THE ISLETS OF LANGERHANS

Edited by
Md. Shahidul Islam

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The Islets of Langerhans

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The Islets of Langerhans

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This book is dedicated to the living memory of
Henrik Kindmark, M.D., Ph.D., (1964–2009)

Preface

When new fellows join my lab, I give them some reading materials so that they can orient themselves in their assignment in a new field. When fellows leave my lab, some after writing their dissertations, I prefer to give them a book as a symbolic present. I was longing for a book that contained something on more or less everything about the islets. At the same time, I wished it contained information as recent as possible. There are a few such books in the market but they are pretty outdated. I started picking islets myself from October 1990, when I joined the Rolf Luft Center, Karolinska Institutet. Over the years my fascination for islet research remained high. Since last year, I felt a stronger urge to do more for these mysterious and hidden mini-organs that are directly or indirectly involved in the pathogenesis of all forms of diabetes that affects ~250 million people in the world. After I launched the *Islet* (landesbioscience.com/journals/islets) and founded the *Islet Society* (isletsociety.org), there was a momentum that could be utilized to create something equally meaningful i.e. this book.

The idea cracked in September 2008. Starting September 19, 2008, I contacted an estimated 90% of the authors who published anything on the islets during 2007–2008 and who could be traced from the internet. I asked them to propose the title of one chapter that they would like to see in this book and to propose the name of potential author(s) who could contribute the chapter. This bottom-up approach tuned the final contents of the book to the need of its potential users. The authors who contributed the chapter are understandably the ones who had time, competence, and interest to write broad and balanced overviews of the backgrounds and advances in their respective areas of research. Together, they spent thousands of hours to do the necessary research to put together their chapters and to include in these their own views, as well as directions for the future. All but three chapters went through time-consuming anonymous peer-review processes. My communications with the authors and referees were smooth and effective. The commitments and the enthusiasm of the authors kept us all steady on the track. The only chapter that was not delivered in time was my own that was completed on July 12, 2009.

In this book one will find topics on a variety of aspects of the islets and the topics are ordered in a logical way. The anatomy, development, evolution, histology, ultra-structure, regulation of hormone secretion, electrophysiology, mathematical modeling, intracellular signaling mechanisms, apoptosis, mitochondrial functions,

islet transplantation, mechanisms of immune destruction, and prospects for regenerative medicine are examples of topics that have been included in this book. But it is by no means complete. For instance, I could not persuade any one to contribute a chapter on islet amyloid polypeptide and amyloids. By the time the book reaches the readers, other exciting new areas may emerge in this fascinating field of research. Readers will benefit maximum if they take the contents of this book as starting points, take everything they read with a pinch of salt, reflect, and do their own research into the respective subject matters. This is what active learning is.

"A man would do nothing, if he waited until he could do it so well that no one would find any fault with what he has done" – Cardinal Newman. There are certainly some mistakes that I am not aware of. Prospective readers may see this book as a beta version and register the bugs at http://isletbook.islets.se, so that they can be fixed in the next (beta) version.

I admire the authors who have put their hearts and minds into their respective chapters. Other potential authors, amongst them, Susan Bonner-Weir, and Michael Dabrowski, to name a few, could not contribute a chapter, but helped out by recommending others who did contribute. I am thankful to the reviewers whose comments were extremely helpful for making decisions and revisions. Thanks to Melania Ruiz who handled the practical aspects so efficiently. Thanks to our near and dear ones who perhaps did not receive enough attention because of our intensive engagement with the writing but were still tolerant and supportive. Finally, I am grateful to the Karolinska Institute, my alma mater, for ensuring the infrastructure that supports creativity. The preface was written on a boat, as it was cruising her way across the beautiful archipelago that symbolizes islets so well.

July 18, 2009
On board Silja Serenade between
Stockholm and Helsinki

Md. Shahidul Islam

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Chapter 1
Microscopic Anatomy of the Human Islet of Langerhans

Peter In't Veld and Miriam Marichal

Abstract Human islets of Langerhans are complex micro-organs responsible for maintaining glucose homeostasis. Islets contain five different endocrine cell types, which react to changes in plasma nutrient levels with the release of a carefully balanced mixture of islet hormones into the portal vein. Each endocrine cell type is characterized by its own typical secretory granule morphology, different peptide hormone content, and specific endocrine, paracrine, and neuronal interactions. During development, a cascade of transcription factors determines the formation of the endocrine pancreas and its constituting islet cell types. Differences in ontogeny between the ventrally derived head section and the dorsally derived head, body, and tail section are responsible for differences in innervation, blood supply, and endocrine composition. Islet cells show a close topographical relationship to the islet vasculature, and are supplied with a five to tenfold higher blood flow than the exocrine compartment. Islet microanatomy is disturbed in patients with type 1 diabetes, with a marked reduction in β-cell content and the presence of inflammatory infiltrates. Histopathological lesions in type 2 diabetes are less pathognomonic with a more limited reduction in β-cell content and occasional deposition of amyloid in the islet interstitial space.

Keywords Pathology · Type 1 diabetes · Type 2 diabetes · Morphology · Anatomy · Insulitis · Amyloid · β-cell · α-cell · δ-cell · PP cell · Autoimmunity · Innervation · Vasculature · Non-endocrine cells

1.1 Introduction

The human pancreas is an unpaired gland of the alimentary tract with mixed exocrine–endocrine function. It is composed of four functionally different, but interrelated components: the exocrine tissue, the ducts, the endocrine cells, and

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the connective tissue. These elements are intimately related through ontogeny, anatomy, histology, and function. Because the scope of this chapter is the microscopic anatomy of the islet of Langerhans, the other components will only briefly be mentioned.

1.2 The Islets of Langerhans

The pancreas has an elongated shape, and somewhat resembles a 17th century pistol with a curved handle and thick barrel. The handle is formed by the head of the gland, which is closely attached to the distal two-thirds of the duodenum, the barrel is formed by the body region, which is overlaid by the posterior wall of the stomach, and by the tapering tail region that ends near the splenic hilus. Macroscopically, the pancreas has a yellowish-pink aspect and a soft to firm consistency depending on the level of fibrosis and fat accumulation in the organ. It has an average weight of 68 g (range 45–120 g) [1] and is composed of small lobules measuring 1–10 mm in diameter. Microscopically, the lobules are formed by a mixture of ductules and well-vascularized epithelial cell clusters that reflect the two main functions of the pancreas: digestion and glucose homeostasis. Exocrine cells (98% of the parenchyma) release a mixture of digestive enzymes and bicarbonate into the duodenum. They are organized into acini that open into intercalated ducts, to which they are connected via centro-acinar cells. The intercalated ducts fuse into intralobular ducts, interlobular ducts, and finally into the main pancreatic ductus of Wirsung, which together with the common bile duct, opens into the duodenum at the papilla of Vater (papilla major). The secondary ductus of Santorini ends in the papilla minor, a few centimeters above the papilla major. Endocrine cells (1–2% of the parenchyma) release nutrient-generated hormones into the portal vein. Clusters of endocrine cells form islets of Langerhans, micro-organs that lie scattered throughout the exocrine parenchyma in between the acini and ductal structures. The islets of Langerhans are of vital importance to the body as they produce insulin, a prime regulator of glucose homeostasis. The name 'islets of Langerhans' was coined by Edouard Laguesse (1861–1927), a histologist working at the University of Lille, who, in a seminal paper in 1893, correctly deduced that they are involved in endocrine secretion. He named them after Paul Langerhans (1849–1888), who was the first to describe these cell clusters in his doctoral thesis in 1869 but who was unable to attribute them with a specific function [2]. The adult human islet of Langerhans has a mean diameter of 140 μm [3]. It is pervaded by a dense network of capillaries [4] and is (partly) surrounded by a thin collagen capsule [5] and glial sheet [6] that separates the endocrine cells from the exocrine component. Islets vary in size and range from small clusters of only a few cells to large aggregates of many thousands of cells. Depending on the exact manner in which an 'islet' is defined, the estimate of islet number in the adult human pancreas varies from several hundred thousand to several million. Total beta mass appears to be highly variable between subjects, ranging from 500 to 1500 mg [7], corresponding to an estimated 10^9 β-cells and 1–2% of

mean pancreatic weight. Adult islets contain four major endocrine cell types: α-cells (also referred to as A-cells), β-cells (also referred to as B-cells), δ-cells (D, formerly also called A1), and PP cells (pancreatic polypeptide cells, formerly also called F or D1 cells). A fifth cell type, the Epsilon or Ghrelin cell has recently been described.

1.3 Embryology and Fetal Development

The pancreas is derived from two primordia in the distal embryonic foregut [8, 9]. At 3–4 weeks of gestation, a dorsal primordium is formed opposite the hepatic diverticulum and a ventral primordium (sometimes bi-lobed) in close apposition to the diverticulum. At 6 weeks of gestation the ventral pancreas rotates, and fuses with the dorsal pancreas around week 7. The ventral primordium gives rise to part of the head region of the gland ('ventral head'), while the dorsal primordium gives rise to the dorsal head, the body, and the tail. This difference in ontogeny is reflected in significant differences in endocrine cell composition, vascularization, and innervation between the ventral and dorsal pancreas. The ventral head is drained of exocrine secretion by the ductus of Santorini and is supplied with blood via the mesenteric artery. The dorsally derived head, body, and tail are drained by the ductus of Wirsung and irrigated by the coeliac artery. The differences in ontogeny are mirrored by differences in islet composition [10, 11].

Pancreas development is controlled by a complex cascade of transcription factors [12]. Pancreatic and duodenal homeobox 1 (Pdx1) induces early (primary) progenitor cells to expand and form duct-like outgrowths into the surrounding mesenchyme. In a second wave of differentiation (secondary transition), cells at the duct tips differentiate into acini, and cells in the duct walls give rise to endocrine cells, a process driven by another key transcription factor Neurogenin3 (Ngn3). Endocrine cells are first detected at 8–9 weeks at the basal side of the ductal epithelium where they grow out to primitive islets. Exocrine acini are observed from 10 to 12 weeks. Growth of the endocrine mass during fetal life follows that of the total gland, with endocrine tissue forming 2–5% of the parenchyma [13]. Growth of β-cell mass in fetal and adult life appears to be partly by neogenesis from endogeneous Ngn3+ progenitor cells [14] and partly by replication of existing β-cells. β-cell replication peaks around 20 weeks of gestation after which replication levels decrease exponentially reaching near zero values a few years after birth [15–17].

During early development the percentage of the various endocrine cell types changes: at 8 weeks approximately 50% of endocrine cells express glucagon, decreasing to 15–20% in the adult. Similarly, the percentage of D-cells decreases from 20 to 25% in neonates to approx 5% in adults [18–21].

1.4 Endocrine Cell Types

Adult human islets contain at least five different endocrine cell types. α and β-cells were both first described in 1907 by Lane [22] on the basis of their histochemical

staining characteristics, while D-cells were first recognized by Bloom in 1931 [23]. Both PP cells [24] and Ghrelin cells [25] were discovered with the aid of immunocytochemistry.

1.4.1 α-Cells

α-cells secrete glucagon, a 29-aminoacid peptide with hyperglycemic action [26]. The peptide is derived from proglucagon (180-aminoacids) through proteolytic cleavage. Other cleavage products that can be derived from the precursor are GLP-1, GLP-2, and glicentin [27, 28]. Glucagon is stored in secretory granules that have a typical morphology with an electrondense core and a grayish peripheral mantle [29]. Glucagon was immunohistochemically localized to the α-cells by Baum et al. [30]. The number of α-cells is estimated at 15–20% [31, 32], although the relative volume taken up by α-cells can vary significantly between islets with some islets containing up to 65% of α-cells [33]. α-cells are most prominent in the dorsally derived part of the pancreas and virtually absent in the ventrally derived part (Table 1.1).

1.4.2 β-Cells

β-cells form the bulk of the pancreatic endocrine cell mass. Depending on the morphometric techniques that were used, the type of samples analyzed, and the extent of the analysis, a relative islet β-cell mass was found between 50 and 80% [31–34]. β-cells secrete insulin, a 51-aminoacid peptide with strong hypoglycemic action. Insulin is essential for cellular nutrient uptake and thus for the survival of the organism. Its isolation and immediate successful clinical application in 1923 by Banting, Best, and Collip was one of the major medical breakthroughs of the 20th century [35, 36]. Like virtually all peptide hormones, insulin is proteolytically derived from a precursor molecule, proinsulin. This biologically inactive precursor is split into

Table 1.1 Cell types in the adult human endocrine pancreas

	Cell type				
	A	B	D	PP	Epsilon
Peptide hormone	Glucagon	Insulin	Somatostatin	Pancreatic polypeptide	Ghrelin
Molecular weight	3500	5800	1500	4200	3400
Number of amino acids	29	51	14	36	28
Volume % (adult)					
Dorsal	15–20	70–80	5–10	<1	1
Ventral	<1	10–20	2	80	1
Total	15–20	70–80	5–10	15–25	1

three parts, an A and a B chain, which remain connected by two sulfur bridges, thus forming the biologically active insulin molecule, and a C chain (Connecting peptide), which is released together with insulin in a 1:1 molar ratio [37]. The β-cell also co-secretes Islet Associated Polypeptide (IAPP, also called amylin), a 37-aminoacid peptide related to calcitonin gene related peptide (CGRP) [38]. Under pathological conditions IAPP molecules may polymerize and form large intraislet amyloid deposits that are characteristic for type 2 diabetes and for insulinoma.

Insulin was first immunohistochemically localized to the β-cell by Lacy [39]. It is stored in cytoplasmic secretory vesicles that have a characteristic morphology with an electrondense core and a clear peripheral mantle (Fig. 1.1). Within the 350 nm granule, insulin (but not proinsulin) is complexed to zinc, forming insulin–zinc hexamers and crystalline granule cores. Depending on the maturation stage of the granule, the mantle may contain unprocessed proinsulin; when the proteolytic enzymes (prohormone convertases PC1-2, carboxypeptidase-H) present in the newly formed secretory granule have not yet resulted in sufficient cleavage of the precursor molecules, the granule core may be absent and typical immature 'gray' granules are found [39]. The biological reason for Zn complexation is not well understood, but its presence is of practical benefit in islet isolation procedures, where zinc-chelating dyes like dithizone [40] are helpful in determining islet yield and purity.

A β-cell is estimated to contain 9–13.000 secretory granules [41, 42]. With an average daily insulin requirement of 40 IU and an average insulin content per granule of 8 fg, it can be estimated that approx 10^{12} secretory granules are released from β-cells each day. Release may occur via a nutrient-regulated pathway or via a constitutive pathway. Nutrient-induced release is initiated via closure of ATP-dependent

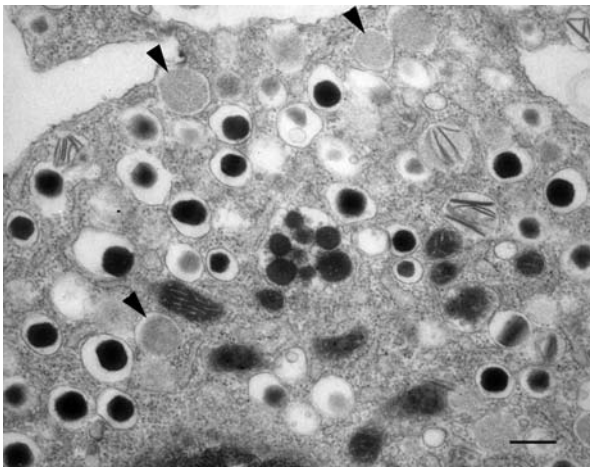

Fig. 1.1 Electron-microscopic image of an islet β-cell with mature dense-cored secretory granules and immature gray granules (*arrowheads*) (bar 300 nm)

potassium-channels, membrane depolization, opening of voltage-dependent calcium channels, and calcium-induced fusion of the secretory granules with the plasma membrane. The process of insulin release is complex and may partly consist of granule fusion with the plasma membrane and partly of temporary opening of small pores between the granule lumen and the extracellular milieu [43].

In addition to (pro)insulin, C-peptide, IAPP, zinc, and proteolytic enzymes, the secretory granule contains calcium, adenine nucleotides, biogenic amines, and a series of additional peptide (pro)hormones including chromogranin A and beta-granin [44, 45]. Several granule (membrane) proteins have been implicated in humoral autoimmunity in type 1 diabetes, like the zinc transporter ZnT8 [46], insulinoma-associated protein 2 (IA-2; ICA-512) [47], and glutamic acid decarboxylase (GAD65) [48].

β-cells in the human pancreas may show marked variation in granulation, cell size, and size of the nuclei (Fig. 1.2). Differences in granulation and cell size may reflect a heterogeneity in glucose responsiveness and biosynthetic activity [49], while differences in nuclear size may reflect polyploidy with nuclear DNA content of up to 8n being relatively common [50]. β-cells in the aging human pancreas display multiple prominent lysosomes with lipid-like content (Fig. 1.3). These strongly autofluorescent organelles resemble the lipofuscin inclusions in aging neurons and linearly increase with age [51].

1.4.3 D-Cells

The D (or δ) cells release somatostatin (formerly called somatotropin release inhibiting factor), first isolated from in the hypothalamus [52]. This peptide hormone is a

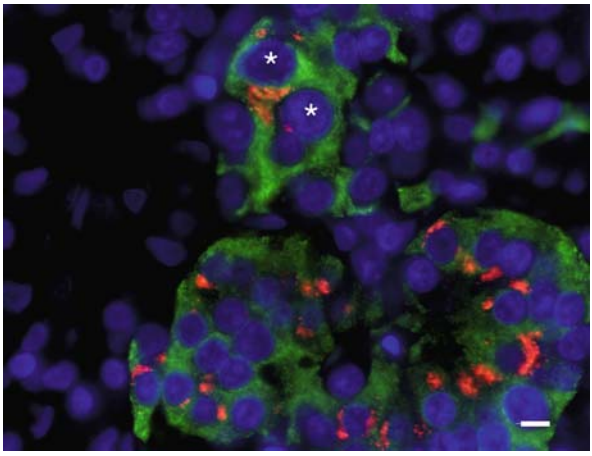

Fig. 1.2 Two-color fluorescent imaging for insulin (*green*) and proinsulin (*red*) of a human islet of Langerhans. Proinsulin has a predominantly perinuclear localization. Note the significant differences in nuclear size between islet β-cells (*asterix*) (Bar 10 μm)

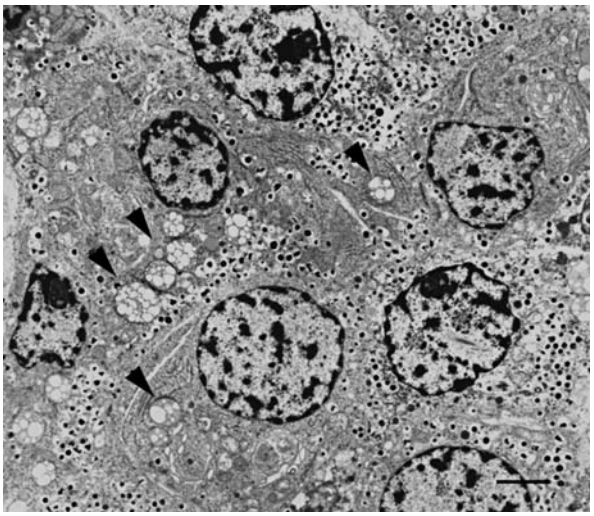

Fig. 1.3 Electron-microscopic image of aging human β-cells with multiple cytoplasmic inclusions (bar 5 μm)

potent inhibitor of glucagon and insulin release and was first immunohistochemically located to the D-cell by Luft et al. [53]. The hormone exists in a 14-aminoacid form and in a 28-aminoacid form [54]. Although all islet cells have neuron-like characteristics, the D-cells resemble small neurons most, as they often form long slender processes with a secretory-granule rich knob-like ending near a capillary suggesting focal and possibly paracrine secretion [55]. D-cells form 5–10% of islet volume (Table 1.1).

1.4.4 PP Cells

The least well studied of the islet hormones is PP, secreted by the PP cell. The peptide has been found immunocytochemically in two morphologically distinct cell types: PP immunoreactive cells (formerly designated as F-cells), characterized by round to angular secretory granules, were found in the ventrally derived head of the pancreas, while cells with small granules, formerly called D_1 cells, were found in the dorsally derived part [56]. In the human pancreas the relative PP cell mass in the ventral pancreas is considerable, constituting up to 80% of the cells (Table 1.1).

1.4.5 Epsilon Cells

The latest cell type that was added is the Epsilon or Ghrelin cell. The hormone ghrelin was first isolated from rat stomach and later localized to a specific cell type in the

adult human islet [25]. Adult islets contain less than 1% epsilon cells. The hormone is thought to be of importance in growth hormone release, metabolic regulation, and energy balance, but its exact role in islet cells has yet to be established.

1.5 Islet Anatomy

Endocrine cells in the pancreas form aggregates of various sizes and microscopic aspect. Larger aggregates, the islets of Langerhans, form small, ellipsoid or spherical structures dispersed throughout the exocrine part. The islet size and number of β-cells increases from birth to adulthood [16]. In fetuses, islets are in close contact with ducts, but they become more separated from the ducts in neonates and adults. In adults, 50% of the islets remain close to the ducts [57]. Size and distribution of islets vary widely from individual to individual, but without recognizable pattern, except that their number seems to increase towards the tail of the pancreas [58, 59]. On light microscopy, the epithelial cells of the islets of Langerhans form trabecular structures, separated by a dense network of anastomosing capillaries [4]. Two architecturally different types of islets are recognized: the diffuse and the compact islet. In the postero-inferior (ventral) head of the pancreas, the islets are of the 'diffuse' type, because the trabeculae seem more loosely arranged than in the islets occurring in the rest of the pancreas and which are known as 'compact islets'. The diffuse islets are very rich in PP cells and are larger than the compact islets. They also contain substantially less A, B, and D cells than the compact islets [60], which are primarily found in the body and tail and have sizes ranging from 50 to 280 μm. Compact islets are well circumscribed and separated by a thin layer of collagen from the surrounding acini. This is less the case in the diffuse islets, which are often irregular. Though occasional islets can measure 1–2 mm in diameter, compact islets larger than 250 μm are generally considered hyperplastic [61].

In humans, the endocrine cells are distributed throughout the islets without apparent organization; this contrasts with murine islets, which show a clear topographical separation of β and α-cell mass. It cannot be excluded that such topographical differences between human and rodent islets are paralleled by differences in endocrine and paracrine islet cell interactions. The cytoarchitecture of the human islet, with its random islet cell distribution, does not support functional islet domains in which the direction of blood flow determines intraislet endocrine signaling [34]. The relative proportion of the various endocrine cell types in the human islets can vary considerably; in one study [33] the percentage of β-cells ranged from 28 to 75%, that of α-cells from 10 to 65% and that of somatostatin cells from 1.2 to 22%. Not all endocrine cells in the pancreas occur in classical islet structures: 15% of all β-cells are found in units with a diameter of <20 μm (1–3 cells) and without associated glucagon, somatostatin, or PP cells [62]. These units, referred to as 'single β-cells' are equally distributed throughout the whole gland and in close association with acini and ductules; they are significantly smaller than β-cells located in larger islets. It has been speculated that these cells are an early stage in the formation of new

islets, although recent studies in rodents using β-cell lineage tracing were unable to confirm this [63].

The different islet cell types can be distinguished with special stains. Nowadays immunohistochemistry is used almost exclusively, but several cell-type-specific histochemical stains are available as well. The best known are Gomori's aldehyde fuchsin for β-cells [64, 65] and Hellman–Hellerström for δ-cells [66]. The Mallory-Azan stain distinguishes between the three major cell types.

1.6 Non-endocrine Islet Cells

Between the islet cell trabeculae, small amounts of connective tissue are present, with blood vessels being most prominent. Other non-epithelial elements present in the islet are nerve fibers, pericytes, macrophages [67], and dendritic cells; the latter express major histocompatibility complex (MHC) class II molecules on their cell surfaces, which may play a role in graft rejection and the initiation of type 1 diabetes.

Pancreatic lymphatics are found in the interlobular septa of the exocrine portion, but are seldom in contact with the islets [68].

1.7 Islet Vasculature

The islet vasculature is critical for adequate glucose homeostasis, not only because of the high oxygen consumption of pancreatic β-cells, but also because of timely responses to changes in plasma glucose concentration and the release of islet hormones into the circulation. Islet perfusion is mediated by neural, hormonal and circulatory signals [69]. The islet capillary network has a density five times higher than the exocrine capillary network [70, 71] and its vasculature is akin to the glomerular system of the kidney: 1 to 3 afferent arterioles provide the islet with oxygenated blood, which leaves through efferent venules; these empty into exocrine capillary networks or collecting venules that in turn empty directly into larger veins. Another similarity to glomeruli is that a variant of nephrin (a podocyte marker) has recently been shown to mark the islet vasculature [72]. The islet endothelium contains 95 nm fenestrations closed by a diaphragm and arranged into sieve plates (Fig. 1.4). Islet capillaries display up to tenfold more fenestrations than exocrine capillaries [73], further illustrating the close interaction between islet cells and the circulation. VEGF-A released from pancreatic β-cells was shown to be a determining factor in inducing islet capillaries and their fenestrated endothelial cells [74]. Islet β-cells are usually bordered by at least one capillary and show polarity in their cytoplasm with the secretory granules at the apical pole towards the blood vessel [75]. Islet capillaries are surrounded by a double basement membrane, each characterized by its own laminin subtypes. One basement membrane is derived from a peri-islet membrane that accompanies the capillary along its winding path throughout the islet; the

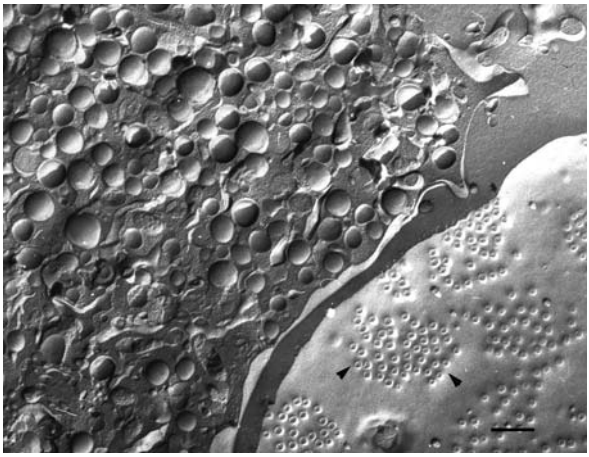

Fig. 1.4 Freeze fracture replica of a rat islet showing a fenestrated capillary with fenestrations arranged into sieve plates (*arrowheads*). Adjacent to the capillary is an endocrine cell with multiple secretory granules in the cytoplasm (bar 300 nm)

endothelial basement membrane constitutes the other. This situation differs from that in rodents where only a single basement membrane was found [76].

1.8 Innervation

Islets have sympathetic, parasympathetic, and sensory innervation; the nerve fibers contain acetylcholine, noradrenaline, and several neuropeptides. The fibers accompany the vasculature and are embedded in non-myelinating Schwann cells. They end blindly in the pericapillary space in close proximity to the islet cells; true synaptic contacts on islet cells have not been described but close nerve–islet cell interactions appear to be mediated by CADM1 (cell adhesion molecule 1) [77]. The ventral and dorsal parts of the pancreas have different innervation, with the dorsal pancreas receiving its sympathetic innervation from the celiac ganglion and the ventral pancreas from the superior mesenteric ganglion. Insulin secretion is stimulated by the parasympathetic system and inhibited by the sympathetic system [78]. It has been postulated that thin peri-islet Schwann cell sheets and sensory afferent neurons may play a role in the initiation of type 1 diabetes [79].

1.9 Islet in Type 1 Diabetes

Patients with recent onset type 1 diabetes (DM1) usually present with a pancreas that is macroscopically normal in appearance and weight. This contrasts with findings in patients with chronic disease in whom the lack of endogenously released insulin

leads to the atrophy of the acinar cells and a decrease in overall pancreatic weight [80, 81].

The characteristic lesion in recent onset DM1 is formed by the presence of inflammatory infiltrates in the islets of Langerhans. In a seminal study in 1965 [80], Willy Gepts described the presence of insulitis in 15/22 young patients with a duration of the disease of <6 months. He observed that the inflammatory lesions were limited to islets in which β-cells were still present and that most remaining islets were pseudoatrophic and contained only non-β-cells (Fig. 1.5), resulting in an overall decrease in β-cell mass to 10% of normal values. He concluded that DM1 was probably the result of a protracted inflammatory disease of autoimmune or viral etiology. Subsequent studies using immunohistochemical staining and precise morphometric methods have confirmed these initial histopathological findings [82], but the use of more sensitive techniques also indicated that residual β-cells are still present many years after clinical onset, especially in older individuals. Our knowledge of the disease processes leading to overt diabetes is still fragmentary due to the fact that only a few dozen cases of very recent onset diabetes could

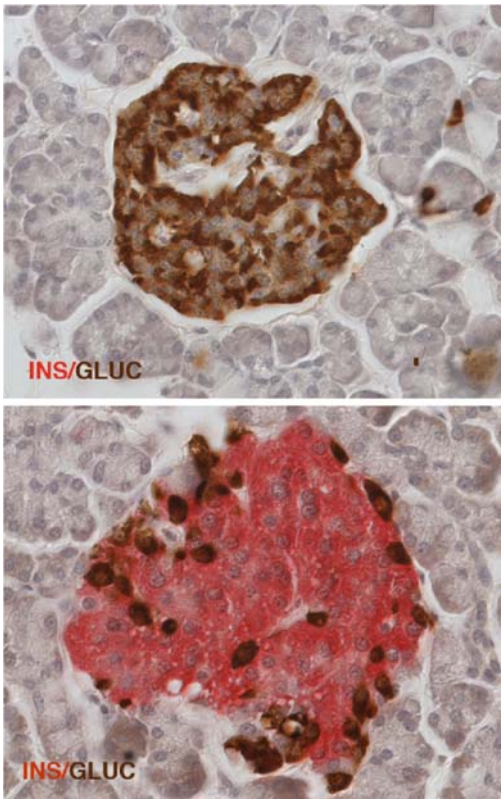

Fig. 1.5 Islets stained for insulin (*red*) and glucagon (*brown*). Islets from chronic type 1 diabetics are pseudoatrophic and consist primarily of α-cells (*top panel*), in contrast to islets from a normal control with both α and β-cells

be studied by autopsy and this often under conditions that precluded extensive molecular and immunological studies [83]. Our current understanding of the disease process indicates that a T-cell-mediated autoimmune reaction against islet β-cells occurs in genetically susceptible individuals and that this process appears to be initiated by environmental triggers [84]. The intensity of the disease process appears to vary between patients and is often more severe in children. At clinical onset, most patients still retain a significant β-cells mass (averaging 10–30% of normal values), but most islets have lost their β-cell component and only contain α, D-, and PP cells; these islets are usually referred to as pseudoatrophic [80, 83]. A small fraction of islets still contain both β-cells and non-β-cells in normal proportions. Such β-cell containing islets may contain an inflammatory infiltrate that predominantly consists of CD8-positive T-cells and macrophages [85, 86]. Neither the mechanism leading to the leucocytic infiltration is known, nor has the antigen toward which the immune response is directed been identified.

Studies of the early phases leading to overt diabetes have indicated that positivity for autoantibodies directed against islet cell antigens often predate the disease by many years. The presence of multiple autoantibodies in combination with a susceptible HLA-DQ genotype was shown to have a predictive value of >70% in relatives of DM1 patients [87]. As the effector phase of the disease appears to be cell mediated, the presence of autoantibodies may function as surrogate markers for islet cell destruction. Histopathological studies in non-diabetic adult organ donors with positivity for multiple autoantibodies and a susceptible HLA-DQ genotype showed that only a minor part (<10%) of the islets presented with insulitis or other histopathological lesions (Fig. 1.6). As such islets also showed high levels of β-cell replication, it cannot be excluded that the clinical outcome of autoimmune attack depends on

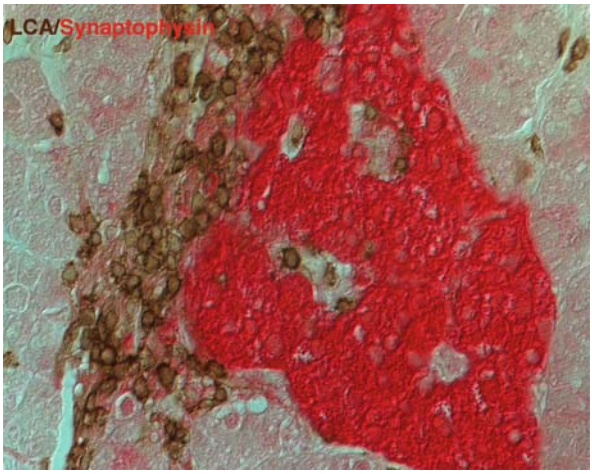

Fig. 1.6 Insulitis in an islet of Langerhans from a non-diabetic autoantibody-positive organ donor. Infiltrating leucocytes are stained with leucocyte common antigen (*brown*) and the islet cells are stained with the pan-endocrine marker synaptophysin (*red*)

the balance between β-cell replication and autoimmune β-cell destruction [88]. Evidence that such regenerative processes may also occur in young patients with recent onset of the disease is found in the early cases described by Gepts, where islet hyperplasia was observed in a 2-year-old child that died 60 days after diagnosis in ketoacidosis. In this patient a single lobe of the gland showed marked hyperplasia of insulin-containing islets in a pancreas that was devoid of β-cells in the remaining part [80]. Additional evidence that β-cell regeneration may play a role in disease progression comes from studies where β-cell apoptosis was found in patients with long-standing DM1 [89], indirectly suggesting that β-cells are still being replenished many years after the onset of the disease. The mechanism underlying β-cell regeneration in the diabetic pancreas is unknown and may either involve neogenesis or replication, although no evidence of β-cell replication was found in recent onset patients who died in ketoacidosis [90].

Although the bulk of the evidence favors an autoimmune etiology of the disease, it is likely that at least some cases of DM1 have a viral origin as the Coxsackie B4 enterovirus could be isolated from a small series of recent-onset DM1 patients characterized by a non-destructive islet inflammation consisting of natural killer cells [91].

1.10 Islets in Type 2 Diabetes

β-cells can adapt to a large number of physiologic stimuli: athletes secrete 2–3 times less insulin than normal individuals in order to reach normoglycemia [92]. Compared to lean non-diabetics, obese subjects can secrete 2–5 times more insulin in response to a glycemic challenge [93]. Pregnancy is another example in which insulin secretion rises drastically in response to physiologic demand [94, 95]. Type 2 DM occurs in predisposed individuals when the adaptive capacity of the endocrine pancreas fails. Several factors can contribute to this failure. DM2 is considered a disease of insulin resistance and insulin deficit, loss of β-cell mass, increased apoptosis, and amyloid deposition. Genetic and environmental factors also play an important role. There is no real histological ‘hallmark’ for type 2 diabetes in the human pancreas. Amyloid deposition comes closest to being such a ‘hallmark’, because the majority of type 2 diabetic subjects show deposition of non-AA amyloid in at least some of their islets. However, not all DM2 subjects show amyloid deposition and islet amyloid can be found in islets of non-diabetics [96–99]. The precursor of amyloid in DM2 is Islet Amyloid Polypeptide (IAPP) or amylin, a 37-amino acid peptide which is present in β-cell secretory granules, and is co-secreted with insulin [100, 101]. Its function is not known. The number of amyloid affected islets is not clearly related to the duration of diabetes in man [98, 102, 103], but may be related to the degree of insulin resistance and islet failure [104]. Affected islets are mostly found in the dorsal head, body, and tail and are rare in the ventral head [105, 106]. Islets located at the periphery of the pancreas exhibit a higher percentage of amyloid deposition than islets in the central regions [105]. The histochemical

staining properties of islet amyloid are the same as for the other forms of amyloid (Congo Red being the most specific stain). Immunohistochemistry for IAPP is another method to demonstrate islet amyloid. It is obvious from a morphologist's point of view that once islets are almost completely invaded by amyloid they can hardly function correctly and this can result in failure to secrete hormones into the blood stream and failure to get sufficient nutrients to the islet cells. However, the number of islets affected in this way is minimal in most diabetics and therefore this does not seem to play a major role in the pathogenesis of DM2 [107]. Most authors do agree that in DM2 the β-cell mass is reduced, but the reduction in β-cell mass early in the disease seems insufficient to cause diabetes in the absence of β-cell dysfunction [108, 109]. When amyloid causes β-cell loss in DM2, this is probably through membrane disruption caused by amyloid fibers. This hypothesis, known as the 'toxic oligomer hypothesis,' is based on findings in neurodegenerative diseases [110]. Since it has been shown that patients with Alzheimer disease are more prone to DM2 than non-Alzheimer patients [111], a link between both diseases is possible.

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Chapter 2
The Comparative Anatomy of Islets

R. Scott Heller

Abstract In the past 20 years, numerous publications on a variety of mammalian and non-mammalian species have appeared in the literature to supplement the excellent comparative work performed in the 70s and 80s by the Falkmer, Epple, and Youson groups. What emerges is that islets are much more complex than once thought and show a lot of similarities in rodents and higher primates. The diversity of lifestyles, metabolic demands, and diets has most likely influenced the great diversity in both structure and cell-type content of islets in lower vertebrate species. In this chapter, I try to provide an overview of the evolution from endocrine cell types in invertebrates to the higher mammals and focus on what has been reported in the literature and some of our own experiences and also include a description of other hormones reported to be found in islets.

Keywords Comparative · Hormones · Islets · Species · Structure

2.1 Introduction

During the past 30 years or so, we have seen emergence of data on islet architecture and cell type expand from just a few species into a broad diversity across many phyla. In three model organisms in which developmental biology studies of the pancreas have been conducted (*Oryzias latipes*, *Xenopus laevis*, and chicken), three buds materialize from the gut tube; two from its ventral side and one from the dorsal [1–3]. In mouse, while initially three buds exist that come from the gut tube (where there is contact between the endoderm and the endothelium), the pancreas only develops from two of these buds, one dorsal and one ventral [4]. This aspect of dorsal and ventral pancreas development of the pancreas has never been examined in species earlier in evolution that teleost fish. The differences in the development

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Table 2.1 Other peptides found in islets

Peptide	Cell type	Species	References
CART	δ	Rat	[75]
		Sheep	[76]
	δ	Ice rat	[54]
CCK	α, β, δ	Spiny dogfish	[77]
	β	Rat	[78]
	β	Ice rat	[54]
CGRP	δ	Rat	[79]
IGF	δ, PP	Lizards	[38]
	α, PP	Frogs, birds	[38]
PYY	PP	Cat, dog, pig	[80]
	PP, α	Mouse, rat	[80]
	α, β, δ	Rat	[81]
		Bullfrog, eel	[33]
	α	Sea bream	[82]
		Brazilian sparrow	[83]
	α, PP	Ice rat	[54]
	PP	Spiny mouse	[84]
Secretin	Unique	Frogs	[36, 85]
TRH	α, β	Rat	[86]
		Rat, guinea pig	[87]

The references cited are mostly based on immunoreactivity.

of the dorsal and ventral pancreas, which later fuse to form one organ in higher verterbrates, also likely explains the different composition of islets in head (ventral derived) or tail (dorsal derived).

The islets of Langerhans have generally been described as round clusters composed mainly of insulin (β-cells) and glucagon (α-cells) and minor populations of somatostatin (δ-cells) and pancreatic polypeptide cells (PP) generally in the mantle or rim of the islets. As the chapter and species evolve you will see there are many exceptions to this generalization. Recent times have shown that in most species during development and the early postnatal period, a unique 5th endocrine cell type, the ϵ-cell, which produces the hormone ghrelin, is found [5–7]. Other endocrine hormones found in the islets are also discussed (Table 2.1). One must remember that almost all of this knowledge has been gained by using immunocytochemical methods based on antisera raised primarily against rodent or human hormones and that differences in the structures of the hormones between different species may be the reason why some hormones are found in some species and not in others. Finally, I have taken a phylogenetic approach to the presentation of the different species discussed (Fig. 2.1).

2.2 Invertebrates

A substantial amount of literature exists on hormones of the pancreatic family in a number of different invertebrates like the silk worm, tobacco hornworm, and

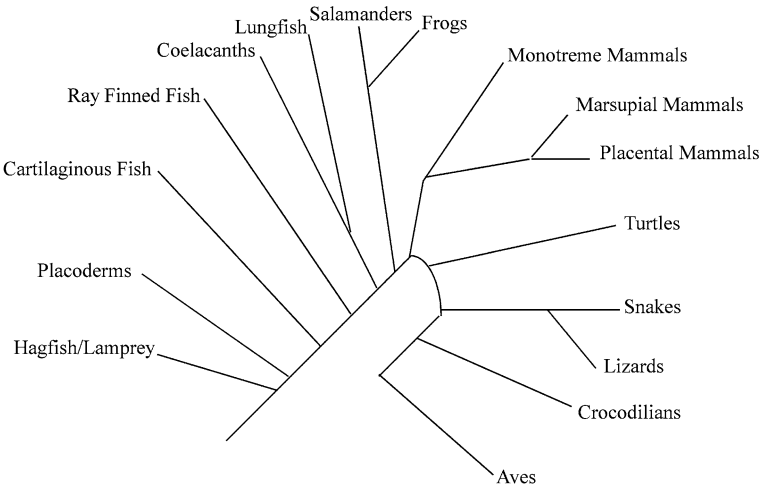

Fig. 2.1 Phylogenetic Tree of Vertebrates. The base of the phylogenetic tree represents the ancestral lineage, and the ends of the branches signify the descendents of that ancestor. When you move from the base to the ends, you are moving from the past to the present. When a lineage divides (speciation), it is demonstrated as branching on a phylogeny. When a speciation episode occurs, a single ancestral lineage gives rise to two or more daughter lineages. The figure was adapted from the Understanding Evolution website from the University of California Museum of Paleontology. http://evolution.berkeley.edu.

dipteran blowfly, in which hormones belonging to the insulin, glucagon, PP (started out as NPY), and somatostatin families have been demonstrated to exist [8]. In addition, a large amount of work on insulin peptides in the phylum mollusca has also been performed [9]. Here I focus on the Drosophilia, where some very important recent molecular studies have been performed that give a great insight into the evolution of the insulin- and glucagon-like peptides.

Pancreatic islets are not found in any invertebrate species but surprisingly many regulatory peptides are found in the midgut of Drosophila, and Ilp3, the equivalent of the Drosophila insulin gene, is found surprisingly in the muscle and not the endocrine cells [10] (Fig. 2.2). The major source of insulin-like peptides (there are seven in Drosophila) is a group of neurons in the pars intercerebralis of the brain [11]. Like insulin from islets, the insulin-like peptides in Drosophila are crucial for the regulation of glucose (actually trehalose, a disaccharide of two glucose molecules) levels in the hemolymph and energy metabolism [12]. Ablation of the insulin producing neurons generates growth deficient and diabetic phenotypes. Interestingly, it has been demonstrated that the insulin producing neurons make direct projections to communicate with the corpora cardiaca (CC) cells located at the heart, which produce glucagon-like peptides. Thus information from insulin-producing cells to communicate with α-cells was established quite early [12].

The insect corpora cardiaca (CC) are clusters of endocrine cells in the ring gland. One of the principal peptides produced is adipokinetic hormone (AKH), which is surprisingly similar to mammalian glucagon, is found in dense core vesicles, is

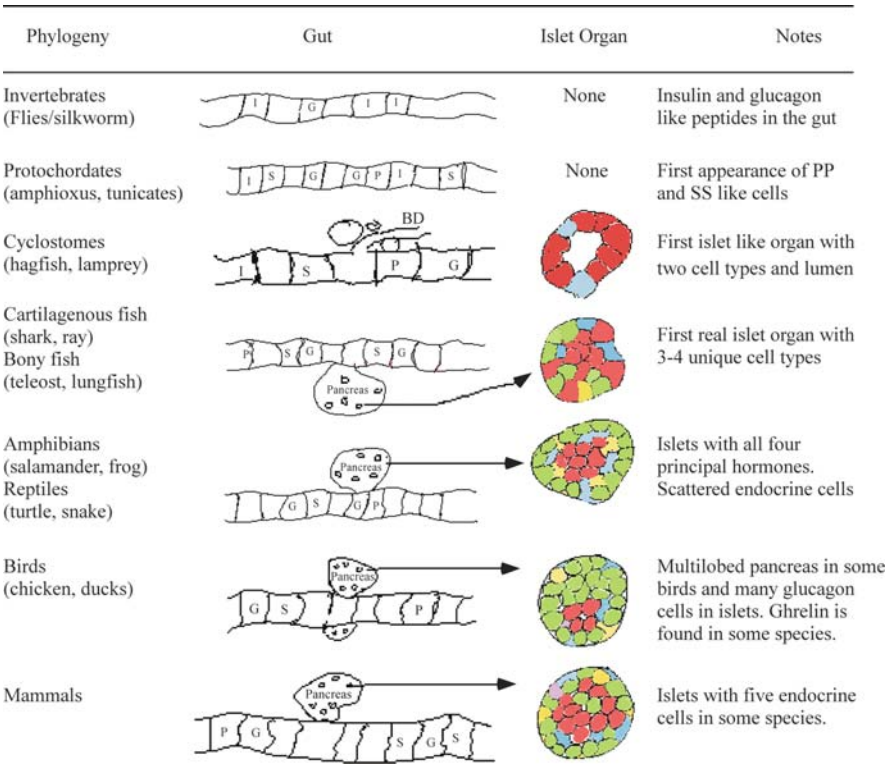

Fig. 2.2 Evolution of the Islet organ from invertebrates to mammals. Considerable species variation occurs in all classes but the scheme is meant to be semi-representative. Family member cell types that still remain in the gut are represented by single letters. I = insulin, G = glucagon peptides, SS = somatostatin peptides, P = PP family peptides. The cyclostomes are the first species where islet like clusters have migrated out of the gut tube into a separate cluster (islet) surrounding the common bile duct. It is with the cartilaginous and bony fish that the first real pancreas is formed with islets containing three and sometimes four hormones. These islets can lie within large islets (Brockmann bodies) or multiple islets within an exocrine pancreas. Reptiles and Amphibia are the first species with islets containing all four of the major hormones. Some species of Aves have multilobed pancreata and the islets tend to contain a lot of glucagon cells and this is the first appearance of ghrelin cells in some species. Mammals have a diverse range of structures but are generally round and contain four or five islet hormones. Insulin (*red*), Glucagon (*green*), Somatostatin (*blue*), Pancreatic Polypeptide (*yellow*), Ghrelin (*purple*). BD = bile duct

synthesized as a preprohormone, and has actions on the insect fat body to increase glycogenolysis and lipolysis, similar actions to mammalian glucagon [13, 14]. Injection of AKH in insects is sufficient to increase glucose in the hemolymph [13, 14]. A recent study demonstrated that ablation of the CC in Drosophila disrupts glucose homeostasis and that overexpression of the AKH gene reverses the effects on hemolymph glucose, thus demonstrating that a glucagon-like peptide is critical to regulation of glucose levels even in invertebrates [15]. In addition,

like in mammalian islets, the CC cells are in direct contact with the vasculature. Interestingly, the CC cells arise during development from delamination from epithelia that give rise to the gut [16]. Kim et al. speculate that CC and neuroendocrine regulatory cells that are important for metabolism may have come from an ancient energy sensing cell and that β-cells may have actually come from ancient α-cells. This is a very interesting and intriguing idea but will require more research to prove the hypothesis.

If we look back to the stem of vertebrate evolution and examine the primitive chordates: urochordates (tunicates) and cephalochordates (Branchiostoma-Amphioxus), we find that peptides related to somatostatin, glucagon, and PP like (primitive NPY) are localized in the tunicate brain, while insulin uniquely moved to the gastrointestinal tract (GI) mucosa [17, 18]. Thus it appears that insulin is the first hormone to have left the nervous system for the gut. Amphioxus is the earliest species for which all four of the main endocrine cell types are found in the GI tract, but not yet organized into an islet organ [19] (Fig. 2.2).

2.2.1 Agnatha-Cyclostomes – First Appearance of an Islet Organ

The Hagfish is a very ancient fish and it has been demonstrated to have an islet organ, which consists of only insulin and somatostatin cells. It is located as a bulge in the intestine near to the exit of the common bile duct (Figs. 2.2 and 2.3). Scattered insulin cells are also found associated with the bile duct as is also found in higher vertebrates [20]. No glucagon, PP or ghrelin (Heller and Christensen, unpublished data) cells have been identified in the structure [19]. The lamprey, a bottom dwelling ocean relative of the hagfish, also has a distinct islet organ, which was described by August Epple as Follicles of Langerhans due to its curious structure and that it was embedded in the submucosa of the intestine and features a duct-like lumen [19]. It is comprised of insulin and somatostatin immunoreactive cells. Interestingly, it appears that many of the somatostatin cells from the gut have now migrated into the islet organ [21]. One very interesting difference between the lamprey and hagfish is that removal of the islet organ in lamprey but not hagfish induced hyperglycaemia [19, 22].

2.2.2 Chondrichthyes (Jawed Fish)

Chondrichthyes is a large class consisting of rays, sharks, and skates. Here we see a large evolution in the islet organ as well as the appearance of some exocrine tissue associated with the islet tissue. Whether the islet organ in these ancient fish is derived from the dorsal or ventral pancreas or both is unknown. The glucagon cells have now migrated out of the GI tract and into the islet organ and are grouped together with insulin and somatostatin (Fig. 2.3) [23]. The first appearance of the PP cells is found and some species such as the elephant fish have abundant PP cells [15].

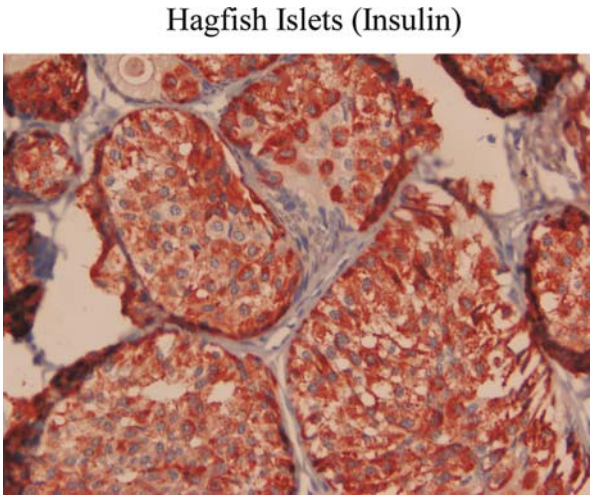

Fig. 2.3 The islets from the hagfish. The section was stained for insulin using the perxoidase staining protocol by Erna Pedersen (Hagedorn Research Institute). Insulin is in *red*. Image is taken at 200*x* magnification

The pancreas of the shark *Scyliorhinus stellaris* has large islets observed around small ducts. In addition, single islet cells or small groups of endocrine cells can also be observed to be incorporated into acini [24]. Ghrelin has not been identified or examined for. Now, for the first time, we see an islet organ with juxtaposed exocrine and endocrine tissue with the four main pancreatic cell types observed in most but not all Chondrichthyes. Interestingly, many glucagon, PP family, and somatostatin cells remain in the gut, a feature that remains with most higher vertebrates as these peptide families play important roles in GI physiology.

2.2.3 Osteichthyes (Lungfish and Teleost Fish)

Lungfish are unique when compared to the vast literature on the teleost fish. The anatomy of the pancreatic region is quite distinctive with a number of scattered encapsulated islets completely surrounded in the dorsal foregut wall. The stomach and intestine wrap around the organ and the spleen is also in close association [25, 26]. For the first time we see islets that are encapsulated by a collagenous-type connective tissue to exclude them from the exocrine tissue [8]. Glucagon, insulin, and somatostatin immunoreactive cells are localized in the islets but few or no PP cells are found [27, 28] (Fig. 2.2).

The teleost are bony fish of the rayfin subclass and have been widely studied. Detailed developmental studies in zebrafish have demonstrated that the dorsal pancreas gives rise to the principal islet body often referred to as the Brockmann body (mainly found in the most advanced teleosts), while the ventral bud leads to

the exocrine pancreas and associated smaller islets, also seen on other fish [29]. Islet structures vary broadly in this class of fish with some members having many islet structures scattered as clumps throughout the abdominal cavity with associated exocrine tissue [30]. Generally, the islet organ is located in the mesentery that connects the stomach, intestine, liver and gallbladder. Teleost fish tend to have islets that very much resemble mammalian islets with the insulin cells in the core surrounded by a mantle of glucagon, somatostatin, and PP cells but not all teleost islets contain all four cell types [31]. Ghrelin cells have been detected in the pancreas of the catfish [32]. Eels have been shown to have numerous peptide YY (PYY) cells in the islets (Table 2.1) [36].

2.2.4 Amphibia

Amphibia, which includes the urodeles (salamanders, newts) and anurans (frogs, toads) vary greatly in their islet structures. In some urodeles, the islets are not encapsulated, appear poorly innervated, and the cell types are more randomly distributed, while in others the islets appear as in most other tetrapods [34]. The literature on newts and salamanders is limited but what has been reported shows that all four of the main cell types are found but are most often in clusters that do not show a distinct distribution, with insulin cells in the core surrounded by the other cell types. It has been reported that the endocrine cells in the mudpuppy appear as groups of cells that are unencapsulated [35]. Ghrelin has not yet been described in urodeles.

Frogs and toads have been more intensely studied and are the first species with five or even six unique cell types in the islets. In addition, Amphibia are the earliest vertebrates to show the classical islet structure of the β-cells in the centre surrounded by the other cell types (Fig. 2.2). While the appearance of the islet structures in frogs is quite close to that of mammals, the cell composition is very different. In some frogs, there are equal numbers of insulin, glucagon, and PP cells and fewer somatostatin cells (Fig. 2.4). Interestingly, like hagfish, frog β-cells appear to lack Zn^{2+}. Also, like mammals, the splenic or tail portion of the pancreas often has larger islets. In addition to the four main cell types, single or small groups of secretin cells have been described in the Red Bellied frog [36]. While, *Xenopus* appear to have ghrelin cells in the islets, bullfrogs have the mRNA but not the immunoreactive peptide [37]. Insulin-like growth factor-1 (IGF-1) has also been observed to colocalize with either PP or glucagon (Table 2.1) [38]. PYY immunoreactive cells have also been described in the bullfrog pancreas [39] (Table 2.1).

2.2.5 Reptilia (Turtles, Crocodiles, Lizards, Snakes)

While these animals were the first to make a complete transition from an aquatic to a terrestrial way of life and represent the animals that evolved into birds and mammals, very little is known about their islets compared to fish and amphibians.

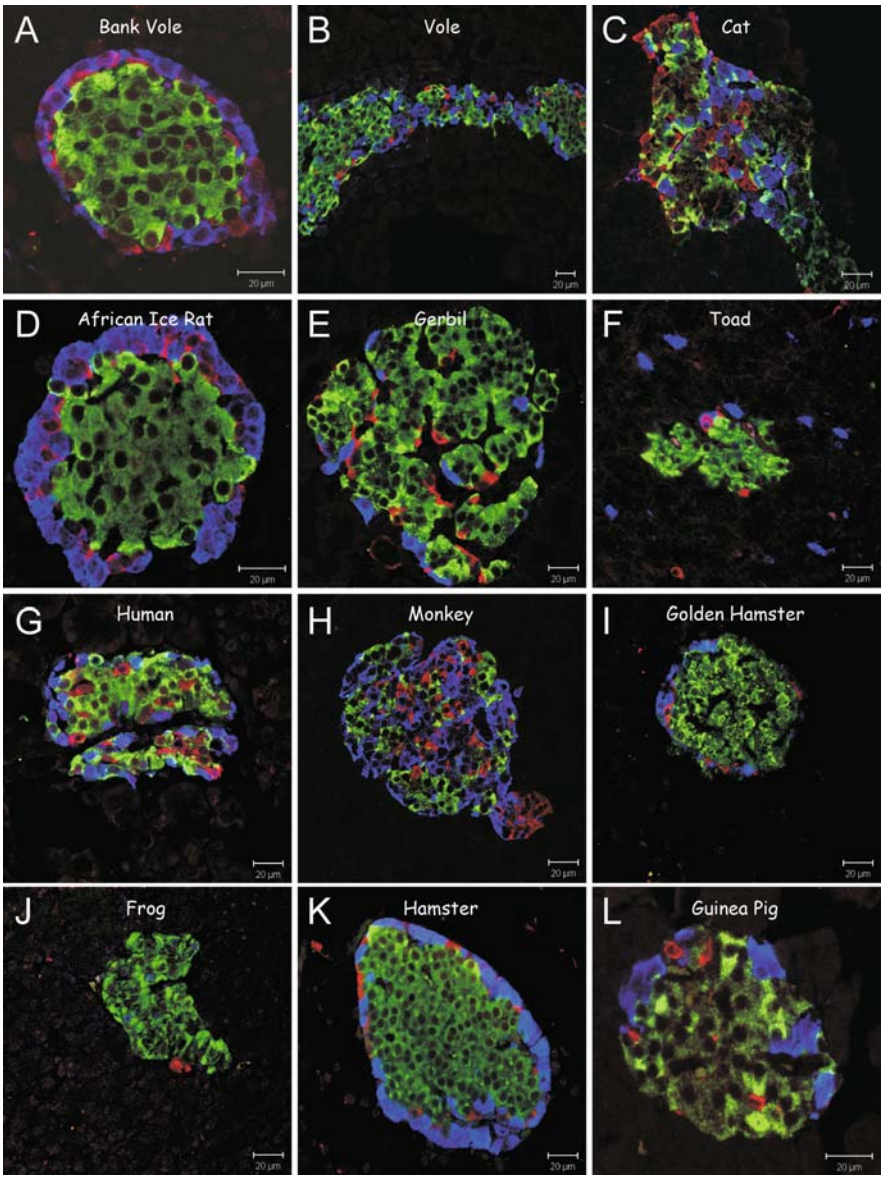

Fig. 2.4 The Comparative anatomy of islets in 12 different species. Bank Vole (**A**), Vole (**B**), Cat (**C**), African Ice Rat (**D**), Gerbil (**E**), *Bufo*(toad) (**F**), Human (**G**), Monkey (**H**), Golden Hamster (**I**), Xenopus (frog) (**J**), Hamster (**K**), Guinea Pig (**L**). Sections were stained for insulin (*green*), somatostatin (*red*) and glucagon (*blue*) and scanned with a Zeiss LSM 510 confocal microscope. Scale bar = 20 μM

Reptiles in general have a distinct pancreas with exocrine tissue and islets. Perhaps not surprisingly, the Crocodilia and Squamata (lizards) are more similar than the turtles. They exhibit a compact pancreas with all four of the established pancreatic hormone cell types. In crocodiles, the insulin cells make up about 50% of the islets while in lizards, the glucagon cells are in abundance with a ratio of 4–5 for every insulin cell. Lizard islets also tend to be large and located in the tail of the pancreas [40, 41]. Interestingly, alligators have been reported to have a large number of somatostatin cells [15]. IGF-1 was shown to colocalize with either somatostatin or PP in lizards (*Lacerta viridis*, *Scincus officinalis*) (Table 2.1) [39] and ghrelin has not been described in these species.

Chelonia (turtles) are the oldest in the class of reptiles and show a large diversity. A recent study in *Melanochelys trijuga* demonstrated that numerous scattered glucagon cells appear in the exocrine pancreas. In addition, small insulin islets of 3–20 cells are found, but when the cells are found together they form islets with β-cells in the centre and α-cells on the periphery and that the α-cells out number the β-cells [42]. In *Chrysemys picta*, it has been described that the duodenal (head) part of the pancreas contains scattered SS and PP cells that are distant from islets made of only glucagon and insulin cells, and that the PP cells are found in an inverse relationship to the glucagon cells. Interestingly, in this species insulin cells are still found in the gut, which is an evolutionary reverse predating the amphibians [43]. Not a lot of literature exists about other hormones in the islets but ghrelin cells have been identified in the pancreas of the red-eared slider turtle [44] and IGF-1 in snakes (*Psamophis leniolatum*, *Coluber ravergieri*) in the glucagon or somatostatin cells [38] (Table 2.1).

The endocrine pancreas in a few snakes have been reported and it appears that in general, glucagon and somatostatin cells are found in the mantle, but also there are scattered somatostatin cells intermingled with the other endocrine cell types. Interestingly, in *Natrix*, there are more α-cells than β-cells but not in *Vipera*, where both appear to have about equal numbers of δ and β-cells, which is also quite different from mammals. Surprisingly, the authors did not find PP or gastrin/CCK family peptides in the islets [45].

2.2.6 Aves

The avian pancreas has evolved as a multilobed and distinct pancreatic organ in the few species that have been studied. Almost all the data comes from chickens, ducks, quail, and pigeons and all of these show quite a lot of similarity and are more closely related to what we observed in the Chelonia compared to the Crocodilia class. Early on it was observed that Aves have what is referred to as A and B islets, which consist of primarily glucagon or insulin cells with somatostatin cells as well as mixed islets [46]. More recently, these observations have been confirmed in the Japanese quail where it was observed that in the β-cell islets, the somatostatin cells were in the periphery while in the α-cell islets they were scattered throughout the islet [47]. Similar data were observed in the domestic duck, where A and mixed

islets were more concentrated in the splenic lobe, and decreased in number in the other lobes [48]. Two exceptions to these species appear to be the Australian eagle and the Houbara bustard, which were reported not to have A and B islets but only islets of the mixed type, and no reciprocal relationship between PP and glucagon was observed [49, 50]. More exceptions may well be observed as more species are studied, but one consistent finding is that birds tend to have large numbers of α-cells.

As we move further up the evolutionary scale, more and more different peptides have been localized in the islets. IGF-1 has been reported to colocalize with either SS or PP [38]. Using specific non-cross-reacting antisera, PYY-specific cells have been observed in the chicken exocrine pancreas with rare cells in islets, which is also similar to what was observed in turtles [51]. Adrenomedullin has been described to be localized with the PP cells in chickens [52]. Ghrelin cells are found in adult domestic chickens (Nils Wierup, personal communication) but nothing is known in other Aves species (Table 2.1).

2.2.7 Mammals

Many more mammals have been investigated than lower vertebrate species and extensive literature is available. There are currently 5,400 species of mammals distributed in about 1,200 genera, 153 families, and 29 orders. This includes species from the Monotremes (echidnas and the platypus), Theriiformes (live-bearing mammals), marsupials, Anagalida (lagomorphs, rodents, and elephant shrews), Grandorder Ferae (carnivorans, pangolins), Grandorder Archonta (bats, primates, colugos, and tree shrews), Grandorder Ungulata, Mirorder Eparctocyona [condylarths, whales, and artiodactyls (even-toed ungulates)], Mirorder Altungulata: perissodactyls (odd-toed ungulates), elephants, manatees, and hyraxes (Fig. 2.1). Many of these have never been examined but I will describe what has been reported.

By the time the mammals evolved, the basic structure of the pancreas, with multiple lobes and encapsulated islets was really set. It is in mammals that we now have strong evidence that the islets are producing much more than the five main islet hormones (insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin) and this includes a wide and diverse group of peptides and proteins, including islet amyloid polypeptide (IAPP), cholecystokinin (CCK), peptide YY (PYY), thyrotropin releasing hormone (TRH), GABA, and cocaine amphetamine regulated transcript (CART) (Table 2.1).

2.2.7.1 Rodents

Forty per cent of all mammals are rodents and this includes mice, rats, chipmunks, squirrels, gophers, hamsters, porcupines, beavers, guinea pigs, gerbils, degus, chinchillas, prairie dogs, and groundhogs. Out of all of these animals, the islet architecture and content has only been examined in mice, rats, hamsters, gerbils, and guinea pigs. In general, rodents such as mice, rats, and hamsters have fairly round

islets with glucagon, somatostatin, and PP cells in the mantle and β-cells in the center [53] (Figs. 2.2 and 2.4). We have recently examined the African Ice Rat (*Otomys sloggetti robertsi*) and observed that these animals have nearly equal numbers of α and β-cells and the islets generally have two layers of glucagon cells surrounding the β-cells [54]. In addition, we have fresh studies on several desert gerbils and have described their islet morphology [55]. In gerbils of the Meriones family, we observed that like rats and mice, the β-cells are in the centre of the islets and are surrounded by a ring of α-, δ- and PP cells. We often observed colocalization of PYY with PP as well and this is seen in a number of mammalian species (Table 2.1). Cocaine amphetamine related transcript (CART) and CCK are also often colocalized with mostly δ-cells and β-cells, respectively (Table 2.1). Hamsters and guinea pigs also tend to have all the glucagon, somatostatin, and PP cells in the mantle, the core being only insulin cells (Fig. 2.4). We have recently examined two species of voles and found that in one the islets showed very similar morphology to other rodents, while the other had larger more elongated islets (Fig. 2.4).

2.2.7.2 Carnivora

Of the approximately 260 species, which includes dogs, foxes, bears, weasels, pandas, elephant seals, and cats, we only really have data on the domestic cat and dog and a few rare animals. The dog β-cells generally occupy the central portion of the islets but are also found as single cells in the exocrine pancreas [53], while the α-cells are generally in the periphery but also found centrally in some islets. The δ-cells are generally mixed in the islets while PP cells appear as single cells or groups [43, 56]. The endocrine pancreas of the Cape fur seal showed very similar morphology to what is observed in other carnivorous species like the dog and cat and this shows a central core of β-cells surrounded by glucagon, somatostatin, and PP cells. Like what we have seen in cats, they also observed scattered endocrine cells in the exocrine pancreas [57]. The red fox, *Vulpes vulpes*, was described to have small islets with insulin in the centre surrounded by glucagon and somatostatin immunoreactive cells. The authors were unable to detect PP cells [58]. Our experience with the examination of the domestic cat shows that these animals have very unusual islets with every shape you can imagine but not round islets, and the endocrine cells can also be arranged in different sorts of clusters mixed together with groups of α-, β- or δ- cells clustered together (Fig. 2.4). Whether this is a common occurrence in other cats is not known.

2.2.7.3 Artiodactyls (Even-Toed Ungulates)

The most widely studied even-toed ungulate is the pig. The minipig has been used in both type 1 and type 2 studies of diabetes [59, 60]. The islets of the minipig have been described to have three types of islets: small with low numbers of β-cells, large islets with β-cells in the core, and large islets with β-cells in the periphery [53]. Interestingly, the left lobe of the pancreas was described to be high in α-cells and devoid of PP cells, while the δ-cells are mostly at the periphery of the

islets or between acinar cells [53]. There has been one description in the literature on the morphology of the camel pancreas. In this paper it was observed that the insulin immunoreactive cells were found in the central and peripheral parts of the islets of Langerhans, as well as some solitary β-cells in the exocrine pancreas outside the islets. Glucagon immunoreactive cells were located in the periphery of the islets and were approximately 23% of the total islet cells while insulin immunoreactive cells were 67% [61]. Little is known about other peptides in these species and the expression of, but not colocalization of CART was recently described in sheep (Table 2.1).

2.2.7.4 Marsupials

The presence of the marsupium (distinctive pouch) is what characterizes this unique class of mammals. A few species have been examined. The fat-tailed dunnart, *Sminthopsis crassicaudata*, was shown to have all four of the major immunoreactive hormones clustered into islets as well as numerous PP cells scattered in the exocrine pancreas [62]. The same group has also looked at the Australian brush tailed possum, *Trichosurus vulpecula* [63]. They found that like the dunnart, the β-cells are in the middle of the islets, with the α-, δ-, and PP cells in the periphery, with numerous PP cells found in the exocrine pancreas. In the possum, *Trichosurus vulpecula*, it was described by another group that insulin cells were found in islets not only centrally but also in the periphery of islets, and in some islets the glucagon cells were the dominant cell type, found both centrally and in the mantle [64]. PP cells were quite rare with usually only 1 or 2 per islet, while somatostatin cells were mainly in the periphery. These data are similar to what was also observed in the opossum, *Didelphis virginiana* [65]. A common feature in marsupials appears to be scattered PP cells in the exocrine pancreas. A recent study of the Tammar wallaby, *Macropus eugenii*, showed that ghrelin cells were found in the developing pancreas up to day 10 but were not present 150 days after birth [66]. These data are the same as found in mice [5, Heller unpublished data].

2.2.7.5 Archonta – Bats, Primates, Tree Shrews

Archonta is the superorder which contains the bats, tree shrews, colugos, and primates (humans). A very interesting study was conducted on the fruit bat, *Rousettus aegyptiacus*[67]. They found that the endocrine tissue makes up about 9% of the pancreas, which is close to double of what is found in all other species studied so far, and this probably relates to the fact that these animals must absorb large amounts of glucose in very short periods of time. The endocrine cells were distributed in islets throughout the gland and also occurred as discrete cells in the exocrine ducts. The four major endocrine cell types were irregularly scattered throughout the islets with insulin (47.4%) cells located throughout the islet and in between the glucagon cells (28.6%). Somatostatin cells made up 7.8% and pancreatic polypeptide (PP) cells 16.2%, which is much higher than normal in other mammals. Interestingly, using pancreatic vascular casts of the common tree shrew (*Tupaia glis*) it was found

that the α- and δ-cells appeared to occupy the core whereas the β-cells were found at the periphery of the islets of Langerhans. This is quite unusual for a higher vertebrate [68].

The primates, which include monkeys, apes, and humans, have been widely studied morphologically. In general, there is a lot of similarity between monkeys and humans, with an intermingling of the major cell types (Fig. 2.4). In monkeys, it is not uncommon to see central groups of glucagon cells and large clusters of insulin cells that occupy specific sides including the mantle of the islet [69]. The somatostatin cells are generally intermixed while the PP and ghrelin cells are in the periphery of the islets [7].

2.3 Conclusion

In conclusion, the islets of Langerhans have evolved from quite simple organs [70] in the ancient fish to very complex organs in higher vertebrates, producing many hormones, neurotransmitters, and other signaling molecules. Many of the variations of the standard map of the islet that we observe are likely to be related to the diet and environment of the animals, while the need to maintain blood glucose and regulate metabolism within a tight physiological range is an evolutionary pressure that is rarely altered. I think that as new immunocytochemical techniques such as whole-mount immunoctyochemistry [71, 72] and optical projection tomography [73, 74] become more widespread in islet research, we should see an expanded knowledge of how these important cellular clusters are localized, shaped, and function in different species and perhaps even reveal greater differences or more similarities than what has been appreciated from 2-dimensional analysis.

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Chapter 3
Approaches for Imaging Islets: Recent Advances and Future Prospects

Ulf Ahlgren and Martin Gotthardt

Abstract The establishment of improved technologies for imaging of the pancreas is a key element in addressing several aspects of diabetes pathogenesis. In this respect, the development of a protocol that allows for non-invasive scoring of human islets, or islet β-cells, is of particular importance. The development of such a technology would have profound impact on both clinical and experimental medicine, ranging from early diagnosis of diabetes to the evaluation of therapeutic regimes. Another important task is the development of modalities for high-resolution imaging of experimental animal models for diabetes. Rodent models for diabetes research have for decades been instrumental to the diabetes research community. The ability to image, and to accurately quantify, key players of diabetogenic processes with molecular specificity will be of great importance for elucidating mechanistic aspects of the disease. This chapter aims to overview current progress within these research areas.

Keywords Nuclear imaging · Optical imaging · Islets · β-cell mass

Abbreviations

BCM	β-cell mass
BLI	Bioluminescence imaging
BLT	Bioluminescence tomography
CLSM	Confocal laser scanning microscopy
CT	Computed tomography
LSM	Laser scanning microscopy
MBT	Multispectral bioluminescence tomography
MPLSM	Multiphoton laser scanning microscopy
MRI	Magnetic resonance imaging
NMRI	Nuclear magnetic resonance imaging

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OCT	Optical coherence tomography
OPT	Optical projection tomography
PET	Positron emission tomography
SPECT	Single photon emission computed tomography
SPIM	Selective plane illumination microscopy
SPIO	Superparamagnetic iron oxide
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TPLSM	Two photon laser scanning microscopy

3.1 Introduction

Diabetes results from an absolute or relative decline and/or function of insulin-producing β-cells in the pancreas. Type 1 diabetes (T1D) results from the autoimmune destruction of β-cells, whereas type 2 diabetes (T2D) is characterised by insufficient insulin secretion to compensate for the increased need of insulin caused by insulin resistance. A common surrogate measure for pancreatic β-cell mass (BCM) is the determination of serum insulin or C-peptide concentration. This procedure provides only a coarse reflection of total BCM and does not provide any spatial information about β-cell distribution in the pancreas itself. Hence, a main challenge for both experimental diabetes research and clinical diabetes care is to obtain spatial and quantitative information about islet or BCM, which is very limited using current technologies. A non-invasive technique to record the spatial and quantitative distribution of islet BCM would have tremendous application in dealing with this critical health-care problem. Such a method would facilitate early clinical diagnosis of β-cell loss during development of T1D. Changes in BCM could be used as an end-point marker for therapeutic regimes aimed at abating continued autoimmune-induced β-cell loss. It would also provide an approach with which to evaluate anti-diabetic drugs with the potential to stimulate β-cell regeneration. It should not be forgotten, however, that the capacity to accurately record the distribution of BCM and different cellular subtypes involved in diabetogenic processes also have significant implications for experimental animal research. By the development of modern mutagenesis techniques, the mouse has become an invaluable tool to help understand the molecular aspects of diabetes pathogenesis and pancreas function. In man and mouse alike, the pancreatic constitution makes it exceedingly difficult to screen for changes in BCM. The β-cell bearing body of the pancreas, the islets of Langerhans, consists of small endocrine clusters ranging from a few cells to ∼4–500 μm in diameter. (For a comparative description of islet anatomy see Chapter 2.) The islets only make up a minute fraction of the overall pancreatic mass (∼1–2%) and are scattered throughout the exocrine parenchyma ($\sim 1 \times 10^6$ in humans and $\sim 2 \times 10^3$ in mice [1]). Furthermore, clear spatial differences exist with regards to islet size distributions within the pancreas, as demonstrated in mouse [2]. In addition, the location of the pancreas, deep in

the abdomen between major organs such as the colon, stomach, spleen and liver as well as major blood vessels, makes it difficult to access. Further, for in vivo imaging, movement due to breathing may influence the output data. Hence, anatomical and physiological constraints put high demands on any technology employed for the enterprise of imaging and calculating pancreatic BCM. Ideally, a methodology for scoring of BCM would depend not only on a contrast agent that is stable with high signal intensity and devoid of toxic effects but also on an imaging modality with paramount spatial resolution. Further, it should be fast, non-invasive (thereby allowing for longitudinal analyses) and, if possible, also permit the simultaneous detection of highly specific contrast agents for different pancreatic cell types/structures. Although an imaging modality combining all these features does not exist, much progress has been made in this area and technologies meeting one ore more of these criteria are available or are being developed. In this chapter we will summarise recent progress in and future prospects for islet imaging modalities pertinent to both clinical and pre-clinical diabetes research. The field of "islet imaging" is rapidly developing, and it should be emphasised that each one of the technologies described herein has only been covered superficially. Therefore, our primary ambition is to introduce technological concepts and their potential for islet imaging.

3.2 Optical Imaging Modalities for Islet Imaging

3.2.1 Laser Scanning Microscopy (LSM)

Benefitting from an abundant pool of available contrast agents and excellent spatial resolution, confocal laser scanning microscopy (CLSM) has for several decades been a useful tool with which to study various aspects of islet architecture ex vivo (see e.g. [3, 4]). Related techniques include two-photon laser scanning microscopy (TPLSM) and multiphoton laser scanning microscopy (MPLSM). In all of these LSM techniques, images are acquired by optical sectioning of the specimen in the focal plane, and the resultant data is reconstructed with a computer, allowing for three-dimensional (3D) reconstructions of fluorescently labelled structures. An important difference between CLSM and TPLSM/MPLSM is that in the latter techniques, the region of probe activation is restricted to the focal plane only and that they utilise longer wavelengths of light. Short-wavelength laser light, as typically used in CLSM, is known to induce tissue damage through the production of free radicals. In this respect, the use of longer wavelengths in TPLSM/MPLSM is advantageous and also allows for deeper penetration into the tissue (down to ~0.5 mm) as well as an increased signal to noise ratio. Although LSM is most often considered as an ex vivo technique, several promising platforms for LSM-based intravital imaging of pancreatic islets have been presented recently. For visualisation of islets in situ, these platforms require prior exteriorisation of the pancreas due to the relatively low penetration depth and short working distances of LSM. In an

experimental setup in which the tail of the pancreas of anaesthetised mice is glued on to a glass slide, Martinic and von Herrath described the potential of LSM for the in vivo study of mediators of the autoimmune attack, transgenically expressing fluorescent proteins, in experimental models of T1D [5]. In a similar approach, Nyman et al. took advantage of a high-speed line scanning confocal microscope to reconstruct the 3D architecture of the islet and time-resolved blood flow pattern throughout the islet vascular bed with high temporal (millisecond) and spatial resolution in vivo [6]. Another interesting approach takes advantage of the eye as a natural body window for LSM imaging. Using this approach, Speier et al. recently showed that islets engrafted to the anterior chamber of the mouse eye retain their morphology and cellular composition and are able to maintain blood glucose homeostasis in the absence of endogenous pancreatic β-cells [7, 8]. This experimental setup strongly facilitates the possibilities for longitudinal analysis of in vivo aspects of islet biology with cellular resolution (see Fig. 3.1). The researchers could hereby, islet graft vascularisation apart, monitor aspects of signal transduction processes involved in β-cell function and death. Although LSM-based platforms are less likely to allow extraction of global (entire gland) information about the pancreatic constitution or disease state, the above developments indicate that LSM-based platforms are likely to play an important role in obtaining mechanistic insights into islet physiology and diabetogenic processes with unprecedented resolution in vivo.

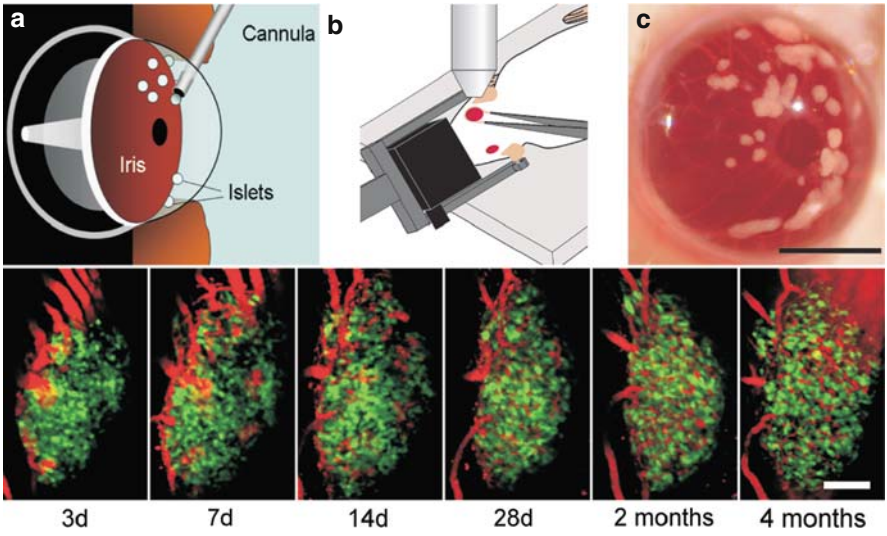

Fig. 3.1 Pancreatic islet transplantation into the anterior chamber of the eye facilitates longitudinal in vivo monitoring of islet cell biology. **a, b** Illustration of islet transplantation procedure. **c** Photograph of islets engrafted on the iris. (*Bottom row*), Non-invasive imaging of RIP–GFP islet engraftment (*green*) and vasucularisation (*red*) at indicated time points. Adapted by permission from Macmillan Publishers Ltd: [Nature Medicine] (Speier et al., 2008a), copyright (2008)

3.2.2 Optical Projection Tomography

A recent invention in optical imaging is the concept of optical projection tomography (OPT) [9]. This imaging technology was originally developed as an ex vivo tool for 3D analysis of embryonic scale specimens and has proven useful for studies of disturbed pancreas development [10, 11]. Similar to computed tomography (CT) and positron emission tomography (PET), OPT is a tomographic technique. This means that in contrast to confocal microscopy, the collected data does not have a direct mapping into 3D space. Instead each piece of data corresponds to the amount of rays, or as in the case for OPT – light, that has been absorbed or emitted along a straight line through the object. When such data is added to similar data obtained from other angles, this contributes to a full description of the imaged object. Hence, OPT data may be visualised by employing the same techniques used for other tomographic methods. By the possibility to operate either in transmission mode or in emission mode, OPT holds the advantage of enabling the visualisation of a wide rage of fluorescent and non-fluorescent contrast agents. Hereby, the abundant pool of highly specific antibodies for molecular determinants relevant to diabetes research becomes available to the investigator. The technology was recently adapted to allow for visualisation and quantification of molecularly labelled structures within the intact adult mouse pancreas [2]. By combined improvements in immunohistochemical processing, tomographic scanning and computational processing, this study demonstrated the ability of OPT to generate accurate 3D representations and quantitative assessments of the BCM distribution, down to the scale of individual islets in the pancreas of normal and T1D diabetic mice (see Fig. 3.2). Due to the optical properties of living tissue, OPT is not likely to become a viable approach for in vivo assessments of pancreatic specimens. However, compared to other methodologies allowing for whole pancreas imaging in rodents, it provides unprecedented resolution and contrast agent specificity. As such, the technique may serve as a powerful tool for phenotypical analyses of BCM distribution in genetically engineered models for diabetes research. At present, adult pancreatic specimens are usually fixed and subjected to relatively lengthy protocols for immunohistochemical labelling and clearing prior to OPT scanning ($\sim$10–12 days). These protocols, however, require little hands on work, and the method is well suited for analyses of BCM distribution in larger populations of experimental animals. We have recently assessed the spatial and quantitative distribution of BCM, down to the level of the individual islets in a time series in a total of 45 T1D prone mice (D. Holmberg & U. Ahlgren, unpublished study). With current setups, the spatial resolution of OPT for assessments of islet distribution in the intact mouse pancreas is $\sim$15–20 μm (the diameter of a rodent β-cell is $\sim$10 μm). Although this figure is sufficient for detection of the majority of islets, it does not allow for resolution at the single cell level. However, by taking advantage of the interactive capability of 3D-rendered OPT data, such data sets can act as templates for directing microbiopsies to individual islets based on their spatial position (origin), shape and volume [12]. By subjecting the isolated biopsies to additional rounds of immunohistochemical labelling and subsequent LSM, this approach allows specific individual islets to be imaged. This

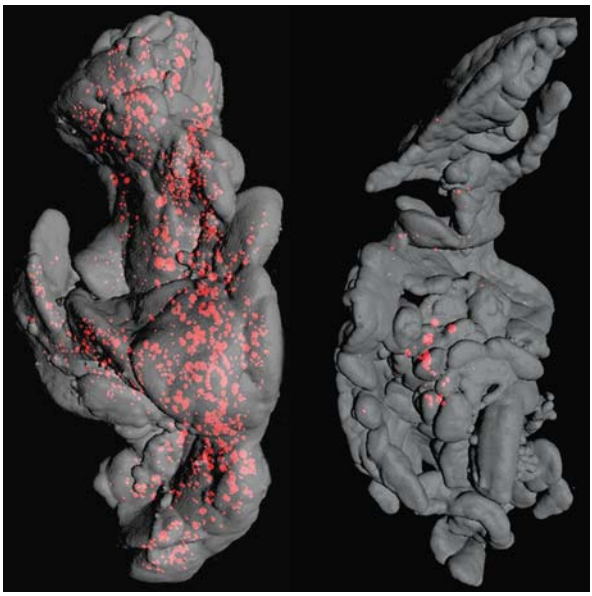

Fig. 3.2 OPT-based isosurface reconstruction of adult mouse pancreas (splenic lobe) from a healthy (*left*) and a NOD type 1 diabetic individual (*right*). The individual islet β-cell volumes (*red*) are reconstructed based on the signal from insulin-specific antibodies, whereas the outline of the pancreatic parenchyma (*grey*) is based on the signal from endogenous tissue fluorescence. The technique facilitates extraction of individual islet β-cell volumes and islet (*x*, *y*, *z*) co-ordinates throughout the volume of the intact pancreas as well as the generation of interactive 3D data sets of the islet distribution. The specimens are ∼1.4 × 0.7 cm. Image by T. Alanentalo and U. Ahlgren

enables high-resolution 3D analyses to be generated, not only of individual islets but also of interacting cell types. Hence, this protocol may become useful in analyses where simultaneous information of both a global (whole pancreas) and a regional (high-resolution) nature is required. Examples include assessment of the 3D infiltration dynamics of different subpopulations of the autoimmune attack in T1D models or the possibility to screen for and analyse rare events or cell niches [12]. Due to the strong autofluorescent properties of pancreatic tissue on the organ scale, OPT has been limited in the simultaneous detection of fluorescent contrast agents and has until recently permitted only quantitative analyses of one wavelength with satisfactory signal to noise ratio. We have taken advantage of the increased channel separation and penetration depth of near-infrared wavelengths, and our preliminary data suggests that this problem could be circumvented (U. Ahlgren, unpublished observation). Hence, the methodology may in this way facilitate the generation of data sets simultaneously displaying detailed 3D and quantitative information of different pancreatic anatomical features or cell types. This development is also likely to facilitate the use of OPT as a tool for ex vivo evaluation of current and perhaps new non-invasive strategies for islet imaging. This would make it possible to perform direct cross-validation analyses of specificity, uptake and read out of virtually

any contrast agent that could be fluorescently labelled, regardless of the detection method for which it was originally designed.

3.2.3 Bioluminescence Imaging

Bioluminescence refers to the enzymatic generation of visible light by living organisms. For bioluminescence imaging (BLI) in experimental research, this usually translates to the catalyses of D-luciferin by transgenic reporters derived from the North American firefly (*Photinus pyralis*) luciferase gene. This process results in the emission of photons that can be detected by a photosensitive detector. Due to the optical properties of living tissue, BLI is primarily an imaging modality for the study of small experimental animals. An optical sensor, usually a charged coupled device (CCD) camera, located at the surface of the specimen detects a continuous current of photons emitted from the site of luciferase gene expression. This detection principle results in the inability of planar [or two-dimensional (2D)] BLI to resolve depth which, in combination with the relatively low resolution, means that the modality is best described as a method for quantification rather than imaging of BCM. For this purpose, however, BLI has proven to be a potent tool for non-invasive longitudinal monitoring of both endogenous and transplanted islets. Using transgenic mice expressing the luciferase gene under the control of the mouse or rat insulin promoter, it has been demonstrated that BCM can be quantified in situ as a function of luciferase gene activity. These studies demonstrate the ability by BLI to correlate changes in luciferase gene activity with changes in BCM and metabolic status, and thus its potential for monitoring endogenous BCM in states of diabetes, insulin resistance and obesity [13–15]. In a similar manner, the technology has been used for longitudinal monitoring of islet graft survival. Islets expressing luciferase under the control of the insulin promoter or by virus-mediated gene transfer (ex vivo) of both human and murine origin could hereby be monitored post-transplantation under the renal capsule or in the liver [16–18]. These studies showed that the signal from the implanted islets directly correlated to the grafted islet mass. Hence, BLI may represent a valuable technique to detect, in transgenic models, early loss of BCM caused by host immune responses even before evident metabolic dysfunction. Another potential application for BLI is its use for embryonic stem cell (ESC) research for the purpose of producing insulin cells for replacement therapies. A limitation for current approaches for differentiating ESCs into insulin-producing cells is the difficulty in following the fate of the transplanted cells post-transplantation. Chan et al. described the potential of BLI to monitor the transcriptional activities of tissue-specific promoters in vivo. ESC or insulin cell-specific promoters (NANOG and Rat insulin 1, respectively) directing luciferase gene expressions were transfected into ESCs, which were then transplanted subcutaneously. In vivo, real-time BLI monitoring of such grafts showed that the undifferentiated ESCs transfected with the ESC-specific construct displayed high levels of luciferase activity as compared to ESCs transfected with insulin cell-specific construct [19]. Hence, this approach may facilitate studies addressing the fate of transplanted insulin-producing cells in vivo.

Although holding great potential as a tool for diabetes research in small animals, it should be noted that BLI is influenced by a number of parameters that have to be carefully characterised to assure accurate measurements of BCM. Since in vivo bioluminescence reflects photons generated by luciferase-mediated oxidation of luciferin, which is dependent on a number of co-factors, it is important that a linear relationship between generated photons and BCM is determined. Further, photon attenuation is non-linear with respect to optical heterogeneity of the tissue and tissue depth. Therefore, differences in overlying structures, the effect of surgical procedures (e.g. wound healing and scar formation in grafting studies) and the imaging angle may influence the quantitative read out [20]. With the recent development of systems for bioluminescence tomography (BLT), i.e. 3D BLI or multiview angle BLI, such impediments may be circumvented. Combining BLT with spatial information on the tissue obtained by CT or MRI, photon scattering and tissue heterogeneity may be corrected for. Another interesting development within the field is multispectral bioluminescent tomography (MBT). Potentially this technology may facilitate quantitative analyses of several bioluminescent tagged cell types during diabetes disease progression in the same experimental animal (for a recent review on BLT and MBT see [21]).

3.3 Nuclear Imaging Modalities for β-Cell Imaging

3.3.1 Magnetic Resonance Imaging

For the purpose of non-invasive islet monitoring, magnetic resonance imaging (MRI) appears as a technology with very high potential due to its capacity for non-invasive longitudinal assessments. MRI, also referred to as nuclear magnetic resonance imaging (NMRI), allows for the generation of tomographic data sets and amongst the non-invasive techniques described in this chapter, it has unprecedented resolution. Further, it is not limited by a penetration depth, at least for human or animal subjects. MRI generates images of the investigated body by using nuclear magnetic resonance (NMR). The patient or animal is positioned in a uniform magnetic field that forces the nuclear spin of hydrogen nuclei (^{1}H), or that of other nuclei, in one of the two possible orientations, i.e. parallel or anti-parallel to the magnetic field. A polarised radio frequency then forces transitions between these orientations, which create a signal that can be detected by the scanner. By applying additional magnetic fields, positional information of the protons in the body can be determined. Gradient magnets allow the magnetic field to be very precisely altered, allowing for the creation of image slices throughout the body in virtually any direction.

In order to resolve the islets of Langerhans from the surrounding exocrine parenchyma, the use of contrast agents must be applied. A contrast agent for this purpose should not only be specific for β-cells but also needs to be stable with high signal intensity and should also be devoid of toxic effects. Hence, the identification

or development of such contrast-enhancing agents is, at present, one of the main challenges for the use of MRI as an imaging modality for successful monitoring of islet mass in humans subjects. Furthermore, MRI is a relatively insensitive technique as compared to, e.g. PET (by a factor of 10^4 to more than 10^6) requiring abundant uptake of the contrast agent into β-cells. Several new methods have been described to tackle the inherent sensitivity limit of MRI and in particular for the application of monitoring transplanted islets, these techniques have demonstrated promising results. Superparamagnetic iron oxide (SPIO) nanoparticles have been used as a potent MRI contrast agent for a number of biomedical applications. In fact, the impact of SPIOs on the MRI signal allows visualisation of cells even smaller than a single image voxel [22]. When co-cultured with islets ex vivo, SPIO particles are internalised by the islets. As first demonstrated by Jirak et al., such islets can be visualised as hypointense (dark contrast) spots when transplanted into the liver of both healthy and diabetic rats [22]. In diabetic animals, the SPIO-labelled islets restored normoglycemia, thus indicating that internalisation of SPIOs does not adversely affect islet function. The same research team also took advantage of this method to monitor the in vivo tolerance of transplanted islets by MRI assessments of SPIO-labelled islets transplanted in to allogenic and syngeneic rat models. Islet rejection could be monitored longitudinally and, in contrast to syngeneic animals, the allogenic rats displayed fewer detectable islets over time [23]. Employing a similar approach, Evgenov et al. reported the real-time detection of human islets transplanted into the liver or beneath the renal capsule of immunodeficient mice [24]. In this study, renal islet grafts were observed longitudinally by MRI for more then 6 months post-transplantation. By modifying the nanoparticles with a near-infrared fluorescent dye, these investigators could show, by ex vivo co-labelling with antibody markers for different endocrine cell populations, that there is no cell specificity for SPIO internalisation by different subpopulations of islet cells. However, β-cells being the predominant cell type, the majority of the cells internalising SPIOs produced insulin. This study further demonstrated that the majority of the near-infrared dye was associated with intracellular endosomal structures, and the authors suggested that fluid-phase endocytosis to be the most likely mechanism for internalisation of the SPIO probe. Although much work remains to be done, the promising results for MRI monitoring of islet grafts in experimental animals makes it tempting to forecast that this imaging modality will eventually also constitute a platform for clinical managing of islet grafts in humans. In support hereof, a recent clinical trial in which SPIO-labelled islets were transplanted into patients allowed the detection of “islet-induced spots” as late as 6 months post-transplantation [25]. This study failed, however, to correlate the MRI read out to the number of transplanted islets. The number of spots was significantly lower than the number of transplanted islets, suggesting that islets could only be visualised when several had been grafted at the same location. Still, the study demonstrates the feasibility and safety of the approach for islet mass scoring.

Apart from SPIOs, other candidate contrast agents have also been explored. One interesting alternative is gadolinium chelates, such as gadolinium 10-(2)-hydroxypropyl)-1, 4, 7, 10-tetraazacyclododecane-1, 4, 7-triacetic acid

(GdHPDO3A). This compound is internalised by pinocytosis [26] and successful in vivo MR imaging of ex vivo GdHPDO3A- labelled islets, grafted under the renal capsule (mouse islets) or into the liver (human islets) of mice, have been reported [27]. In this study by Camussi and co-workers, the effect of GdHPDO3A labelling on human islets was tested by gene array analysis for stress- and toxicity-related gene expression. This experiment could not reveal any significant differences in the expression of genes involved in apoptosis or cell injury as compared to non-labelled islets and in combination with in vitro tests for insulin response suggests that GdHPDO3A labelling does not affect β-cell viability or function. In contrast to SPIOs, GdHPDO3A produces hyperintense (bright contrast) spots in the MR image. Subsequently, when assessing a tissue with low-intrinsic MR signal, such as the liver, this contrast agent may provide a better signal to background ratio. Further, for SPIO-labelled islets the area of contrast surpasses the actual size of an individual islet and the detected hypointense spots may, therefore, also represent a cluster of islets [22, 23]. In contrast, the volume of enhancement equals the cell size for gadolinium-labelled cells [28]. The development of a fully functional system for clinical islet monitoring with islet-level resolution also raises the issue of standardised and automated protocols for islet counting. In the clinical trial of MRI for islet graft scoring by Toso et al. (described above), islets were detected manually with a relatively high degree of variation as a result [25]. Hence, the advent of MRI-assisted islet monitoring, be it endogenous or grafted, the development of computer-aided methodologies able to discriminate islets from background and from non-islet structures (such as vessels) seems to be a vital element. Although not described in attempts to directly address the islets of Langerhans, another interesting approach to circumvent the poor signal to noise ratio associated with metal-ion-based contrast agents (such as SPIOs) for pancreas imaging is the use of perfluoropolyether (PFPE) agents which can be tracked by ^{19}F MRI. Tissues have negligible ^{19}F background signal and therefore the resultant MRI images display only the labelled cells. By combining such images with conventional ^{1}H MRI to provide anatomical context, this approach has been successfully employed to track the homing of ex vivo labelled dendritic T cells to the pancreas in a mouse model for type 1 diabetes [29, 30]. Given that β-cell-specific and non-toxic contrast agents could be identified, ^{19}F MRI may hold potential also for islet monitoring.

As compared to islet graft monitoring, the prospect of imaging native β-cells in vivo put even higher demands on a successful selective contrast agent. Regardless of which imaging modality is to be employed (see also PET/SPECT imaging below), such a contrast agent should meet at least three criteria: (1) it should provide a stable and high signal intensity; (2) it should be highly specific to the β-cell and not be bound or taken up by adjacent tissues to prevent interference from background signal and (3) it should be devoid of toxic effects and allow for monitoring of functional aspects/viability status of β-cells. Hereby, the identification of an MRI contrast agent that can be introduced into the body and bind to the endogenous β-cells with sufficient specificity appears to be a key element that needs to

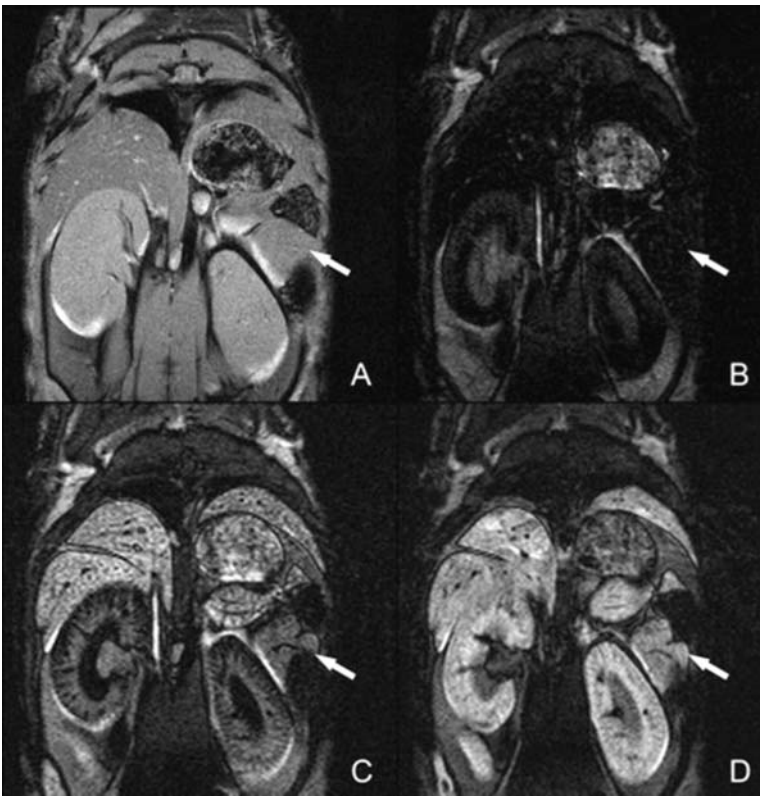

Fig. 3.3 Mn^{2+}-enhanced magnetic resonance (MR) images of mouse pancreas.
A Gradient-echo anatomic reference image. **B** Precontrast inversion recovery image with the pancreas nulled. **C** and **D**, inversion recovery images acquired 5 min (**C**) and 45 min (**D**) after injection of $MnCl_2$. Signal intensity in the pancreas initially increased rapidly and reached plateau ~15 min after injection of $MnCl_2$. *Arrows* indicate location of the pancreas. The figure is used with permission from the *American Journal of Physiology* (Antkowiak et al., 2009)

be resolved and subsequently approaches for MRI-based endogenous islet monitoring appears scarce in the literature. Recently, an approach to in vivo imaging of β-cell response to glucose stimulation has been described [31]. Manganese ions (Mn^{2+}) were used as contrast agent because they are taken up by β-cells as a Ca^{2+}-analogue. Quantitative determination of signal intensity over time reflecting Mn^{2+} influx into β-cells in response to glucose stimulation showed differences of up to 25–40% between healthy and diabetic animals (see Fig. 3.3). These results demonstrate impressively that, despite the limited sensitivity of MRI, the technique can be used successfully for non-invasive in vivo determination of functional β-cell mass.

3.3.2 Imaging with Radioactive Tracer Molecules

Imaging with radiolabelled tracer molecules using SPECT (single photon emission computed tomography) or PET (positron emission tomography) largely relies on the properties of the tracer molecule to specifically target the β-cells with high affinity. The resolution of islets, therefore, does not depend on the spatial resolution of the imaging system but on the "chemical resolution" of the tracer molecule (i.e. the specificity of the tracer molecule for β-cells combined with a high uptake in the target tissue in comparison to the background so that the detected signal will mainly derive from the β-cells). In comparison to MRI, the use of radiotracers is leading to radiation exposure. This radiation exposure largely depends on the radionuclide used for labelling (such as the physical half-life of the radionuclide) as well as other factors (such as the biological half-life of the ligand). In general, the radiation exposure for PET and SPECT can be expected to lie in the range of 5–12 mSv (in comparison, CT scans of the abdomen or thorax will lie in the range of 7–15 mSv). In the following sections, the technical aspects of PET, SPECT and tracer development will be briefly explained and the current state-of-the-art in β-cell imaging with radiotracers will be described.

3.3.2.1 Positron Emission Tomography

For PET imaging, positron-emitting radionuclides are used, either alone or attached to a tracer molecule. The positrons emitted from these tracers hit electrons in their close vicinity, which results in annihilation of the positron/electron pair. This results in simultaneous emission of a pair of high-energetic photons (of 511 keV) at an angle of 180° (coincidence). These photons cause light flashes in a crystal ring, which are detected by adjacent photomultipliers. The source of the photons is located on a straight line between the positions in which the signals have been detected in the crystal ring. This information is used to create 3D images of the tracer distribution. Positron emitters used as tracers or for labelling of tracer molecules include ^{11}C, ^{18}F, ^{15}O or ^{13}N. Complex biomolecules of larger size can be labelled with ^{64}Cu, ^{68}Ga or ^{86}Y which bind to chelators attached to the tracer molecule.

The major advantage of PET is its unmatched sensitivity that allows detection of tracer molecules in picomolar concentrations. Furthermore, quantification is relatively simple and reliable. However, anatomical information is limited (only organs can be visualised that have tracer uptake) and the (physical) spatial resolution of modern clinical PET scanners reaches about 2 mm, while with pre-clinical imaging systems 1 mm is reached. PET scans are often co-registered with CT (and currently also MRI) scans for anatomical correlation.

3.3.2.2 Single Photon Emission Computed Tomography

For SPECT imaging, low-energy photons (circa 140–200 keV) are used for image generation. The gamma rays emitted by radionuclides are detected by special

gamma cameras. In analogy to PET, this technique also uses crystals for detection of photons. Because these photons are not coincident (and therefore information about the 3D location of the source is missing), so-called collimators are used to create an image. Collimators are lead plates with bores that let only photons pass which hit the crystal orthogonally (90°) and eliminate all others. The disadvantage of this technique is the lower detection sensitivity because a part of the emitted radiation is absorbed in the collimators. Alternatively, pinhole collimators (with just one small bore) can be used that result in a higher spatial resolution but have an even lower sensitivity. In clinical imaging systems, the spatial resolution is about 5 mm. In pre-clinical SPECT systems equipped with multipinhole collimators (that compensate for the low sensitivity of single-pinhole collimators as described above), spatial resolutions below 500 μm are reached.

The advantages of SPECT in comparison to PET are the relative low costs and the wider availability. SPECT radionuclides often have longer half-lives, which facilitates labelling and use of tracers (typical SPECT radionuclides are ^{99m}Tc, ^{111}In, ^{123}I). Furthermore, the SPECT signal can easily be quantified if a standard with known activity is included in the scan.

3.3.2.3 Radiotracer Development

For the development of radiotracers, a number of factors have to be taken into consideration. These factors include the size and the physical/chemical properties of the tracer molecule (such as specificity and affinity of binding to a given target, metabolic behaviour, metabolic stability and stability of the label). For imaging of β-cells, a highly diffusible small tracer molecule rapidly binding to a target with high specificity and affinity is optimal. At the same time, the tracer should rapidly be cleared from the background (blood, non-target tissues), preferably via the kidneys. Clearance via the liver may lead to high uptake into the gastrointestinal tract resulting in background activity, possibly blurring the pancreas signal on the images obtained [32]. If the tracer is taken up into the cell, metabolic trapping is one method to obtain high target-to-background ratios. Metabolic trapping means that a tracer is taken up into the cell and is trapped when no metabolic pathway exists for further degradation or externalisation. This can be achieved by the use of a specific label which is then called a “residualising label”. Because the uptake into the target tissue increases over time, the target clearly can be delineated from the background activity. If a tracer is not internalised, a high affinity is required to obtain a good target-to-background ratio. An example for such a tracer would be a radiolabelled antibody binding with high affinity to an antigen on the surface of the target cells. If a potential tracer molecule would lose its specificity for or binding affinity to a target due to necessary modifications for a residualising label, it can be labelled with ^{18}F or ^{11}C. These radionuclides can be incorporated in the tracer molecule without major changes to its structure. However, the short half-life of the radionuclides requires efficient and rapid labelling which is a radiochemical challenge. For specific targeting with radiotracers, the choice of the best approach is dependent

on the target, the potential tracer molecules and the available synthesis/labelling techniques.

3.3.2.4 Radiotracer Imaging of β-Cells

A large variety of potential β-cell tracers have been tested so far. These include mannoheptulose, glibenclamide, tolbutamide, serotonin, L-DOPA, dopamine, nicotinamide, fluorodeoxyglucose, fluorodithizone, glyburide analogs and antibodies (33–36]. To date, most tracers have not been useful for in vivo determination of β-cell mass. Often, the uptake into the pancreas is too low so that the sensitivity of the imaging systems is not sufficient. In other cases, the specificity for the islets (β-cells) was low and uptake into the exocrine pancreas hampered detection of the β-cell-specific signal [34, 35, 37].

In order to develop a method for more specific β-cell imaging, the use of radiolabelled antibodies against pancreatic β-cells has been proposed. It has indeed been shown that uptake of the β-cell-specific antibody IC2 is correlated to the β-cell mass [33]. However (small animal), in vivo imaging has been hampered by the low uptake into the pancreas. A major obstacle for the use of antibodies for imaging is their long circulatory half-life combined with a low diffusion capacity, hindering rapid uptake into the target tissue. For the time being, the use of antibodies for β-cell imaging is limited as the high blood activity decreases the target-to-background ratios [32]. This is especially true if the target is small, consists of solid tissue, and is surrounded by other well-perfused organs and large blood vessels, as is the case for pancreatic β-cells.

More recently, a compound targeting VMAT2 (vesicular monoamine transporter 2) specifically expressed on β-cells has been used for PET imaging of β-cells (dihydrotetrabenazine (DTBZ) labelled with ^{11}C or ^{18}F). In a rat model of spontaneously developing diabetes (BB-DB rat) as well as in non-human primates, a significant decline in pancreatic uptake of DTBZ anticipating the loss of glycemic control could be found in longitudinal PET studies [38, 39]. However, after a major chemical eradication of β-cells, the pancreatic uptake of DTBZ was reduced by only 40–50%, indicating that the uptake of the compound might lack specificity [40]. In clinical studies with optimised dynamic imaging protocols and including several parameters into image analysis (uptake, pancreatic mass, etc.), the maximal decrease of tracer uptake in patients with long-standing T1D with complete loss of β-cell mass never exceeded 50% in comparison to healthy volunteers [41, 42]. Therefore, the currently used tracer molecules might need optimisation in order to improve the specificity for β-cells. Furthermore, optimisation of imaging protocols is ongoing.

Currently, a tracer based on the GLP-1 analog Exendin targeting the GLP-1 receptor is under development (see Fig. 3.4). Currently, it is optimised for imaging in humans. In comparison to DTBZ, this compound has a lower in vivo uptake in the pancreas but seems to be more specific, resulting in reduction of pancreatic uptake by >80% after chemical eradication of β-cells [43, 44].

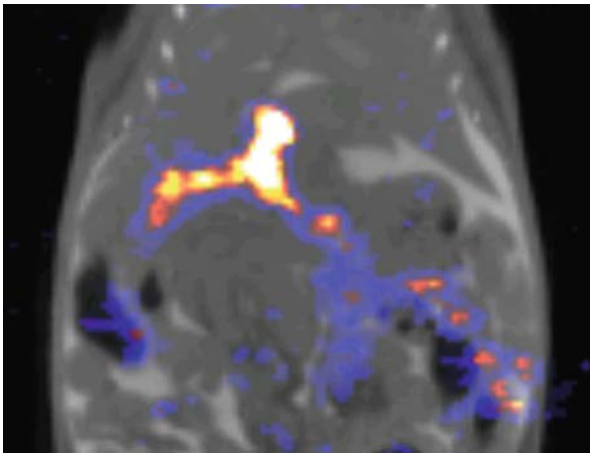

Fig. 3.4 SPECT image of a mouse 4 h after injection with ^{111}In-DTPA-Exendin 3 fused with CT (computed tomography). On the coronal slice, the pancreas can clearly be delineated by high uptake, reaching from the right upper to the left abdomen. The CT for anatomical correlation has been taken after intraperitoneal injection of contrast agent allowing to delineate the abdominal organs such as liver, stomach, spleen and bowel

3.4 Emerging Technologies for Islet Imaging

Several optical techniques have been suggested for future use as islet imaging modalities, in particular for experimental animal research. One such technology is optical coherence tomography (OCT). This interferometric technique amplifies photons backscattered by the tissue [45] and allows deep tissue penetration. Interestingly, recent observations show that islets backscatter light stronger than neighbouring pancreatic tissue, thereby allowing for the generation of high contrast images of islets in vivo or in intravital preparations. In live mice, the application of an extended focus scheme [46] allowed illumination over an axial range of 300 μm with a lateral resolution 2 μm (Villiger et al. accepted for publication in Diabetologia). Hence, although limited by its penetration depth, this imaging technology holds great potential for experimental diabetes research by allowing in vivo imaging of islets with excellent resolution and without prior labelling.

Another interesting optical modality for islet imaging is selective plane illumination microscopy (SPIM) [47]. Technically, this approach has aspects in common with both OPT and confocal microscopy. SPIM functions by focusing excitation light onto a controlled region within the specimen (a 2D optical section) so that a clean image can be formed without much interference from above and below. However, SPIM achieves deep 3D imaging by a concept which is broadly related to OPT – the technique accesses the specimen from more than one angle. In the case of SPIM, the illumination and detection are decoupled so that the z-resolution is not dependent on the objective lens. This allows optical sections to be captured

directly by a CCD. Three-dimensional capture of voxel data simply involves translating the specimen along the optical axis and capturing a sequence of 2D slices. To date, the approach has successfully been applied for time-lapse imaging of live *Drosophila* and zebrafish embryos [47, 48]. However, it also shows potential for imaging larger fixed specimens, similar to the pancreas imaging performed by OPT (J. Sharpe, Personal communication). Hence, if successfully adapted to imaging of intact pancreatic specimens, SPIM would allow multichannel extraction of molecularly labelled pancreatic cell types and structures, throughout the volume of the gland, with cell-level resolution.

3.5 Concluding Remarks

In 1906, in an article about the morphology and physiology of the islets of Langerhans, Lydia Dewitt wrote that: "Probably no organ or tissue of the body has been the subject of more thought or investigation than have the areas of Langerhans, especially during the last years" ([49]. More than a century later, this statement still holds true. Today, as T1D and T2D start to reach epidemic proportions, all efforts to further our understanding of the pathogenesis and natural history of diabetes is vital to improve the possibilities to diagnose and develop preventive and curative measures. No doubt, the development of viable strategies to image the islets of Langerhans and other cell types affected by/or mediating the disease must be considered a cornerstone in these efforts. In general terms, the imaging modalities applied so far could be divided into two groups: optical and "nuclear". The optical techniques are relatively cost-effective and provide a wide range of labelling techniques but are unfortunately confined to the assessment of small animals due to insufficient tissue penetration. However, they provide the best option to study multiple cellular markers or processes in an individual specimen and are therefore well suited to address mechanistic aspects of the disease. At present, nuclear imaging techniques (MRI, PET and SPECT) provide the best option for islet monitoring in human subjects. These technologies are not held back by limitations in their capacity for deep tissue penetration and hold the potential for direct transfer in to clinical practice. Still, several technological hurdles need to be circumvented before these will develop into routine instruments for islet scoring. In this respect, the identification of suitable β-cell-specific ligands represents a significant challenge. Although many promising results have been obtained with these technologies for monitoring transplanted islets, assessments of native β-cells in humans are still limited to autopsy biopsies.

The concept of "islet imaging" often implies the ability to quantify islet or β-cell mass. Across the range of technologies described in this chapter, the spatial resolution for each technology is, generally speaking, inversely correlated to the imaging depth [50]. Further, for the non-invasive nuclear technologies (PET, SPECT and MRI) the one with the highest spatial resolution (MRI) has the lowest sensitivity, whereas the most sensitive (PET) has the poorest spatial resolution (but the highest

chemical resolution). These modality-associated features raise the question as to whether "imaging" or "quantification" is the key issue when attempting to "image islets" in diabetes research or diabetes care. The answer obviously depends on what type of information is required to answer a specific question. Naturally the acquisition of both parameters would be ideal, but it should be noted that the ability to perform both operations simultaneously may not always be required. Hence, imaging technologies that cannot resolve individual islets or cells may well be ideally suited for assessments of BCM as long as a linear correlation with actual BCM can be verified. On the other hand, if spatial information is required, e.g. in attempts to understand spatial dynamics of disease aetiology or to follow the fate of grafted islets, high-resolution imaging modalities may be necessary to provide sufficient information. At present, it appears that combinatorial or complementary approaches may be required to obtain more complete functional and anatomical imaging of the islets of Langerhans.

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Chapter 4
Islet Cell Development

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Abstract Over the last years, there has been great success in driving stem cells toward insulin-expressing cells. However, the protocols developed to date have some limitations, such as low reliability and low insulin production. The most successful protocols used for generation of insulin-producing cells from stem cells mimic in vitro pancreatic organogenesis by directing the stem cells through stages that resemble several pancreatic developmental stages. Islet cell fate is coordinated by a complex network of inductive signals and regulatory transcription factors that, in a combinatorial way, determine pancreatic organ specification, differentiation, growth, and lineage. Together, these signals and factors direct the progression from multipotent progenitor cells to mature pancreatic cells. Later in development and adult life, several of these factors also contribute to maintain the differentiated phenotype of islet cells. A detailed understanding of the processes that operate in the pancreas during embryogenesis will help us to develop a suitable source of cells for diabetes therapy. In this chapter, we will discuss the main transcription factors involved in pancreas specification and β-cell formation.

Keywords Endoderm · Islet cell fate · Pancreas development · Transcription factors · PDX1 · Ptf1 · MafA · Neurogenin3 · Endocrine progenitors · Endocrine cells · β-cell

4.1 Introduction

The adult pancreas is a heterogeneous gland with both exocrine and endocrine compartments. The exocrine pancreas is composed of clusters of secretory cells called acini that produce the digestive enzymes that are released into the duodenum through the pancreatic ducts. The endocrine pancreas is made up of individual

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islets of Langerhans that monitor blood glucose levels and release hormones into the bloodstream to maintain proper glucose homeostasis. Each islet is composed of different cell types: α (glucagon-secreting), β (insulin-secreting), δ (somatostatin-secreting), PP (pancreatic peptide-producing), and ε (ghrelin-secreting) cells.

Diabetes results when insulin production by the pancreatic β-cell is unable to meet the metabolic demand of peripheral tissues such as liver, fat, and muscle. A reduction in β-cell function and mass leads to hyperglycemia (elevated blood sugar) in both type 1 and type 2 diabetes. In type 1 diabetes, β-cells are progressively destroyed by an autoimmune response. In type 2 diabetes, both insulin secretion defects and reduction in β-cell mass are observed [11, 51, 101]. More recently another form of diabetes has been identified. Thus, autoimmunity has been detected in a subset of patients with type 2 diabetes [68]. Decreased β-cells function also underlies early-onset monogenic forms of diabetes called MODY (maturity-onset diabetes of the young) resulting from mutations in transcription factors that regulate β-cells development and differentiation [64]. In conclusion, reduced β-cell number and function leads to a full spectrum of diabetes, prompting intense effort to develop new sources of insulin-producing cells for replacement therapies.

To date, the most promising form of β-cell replacement has been islet transplantation [76, 77]. However, this strategy is far from ideal since it requires a large supply of cadaveric material from which whole islets are isolated, and also the immunosuppresion of the recipient. The limited success of this approach has fostered excitement for alternative stem cell therapy, where precursor cells can be expanded and directed to a β-cell fate, thus providing an unlimited source of material for transplantation.

Although success in generating insulin-producing cells from stem cells has been highly variable and even controversial, recent reports show promising results in the derivation of endoderm cells from embryonic stem cells and subsequent in vitro or in vivo differentiation into insulin-producing cells [14, 15, 34, 35, 44, 65, 79]. This enormous progress has been achieved thanks to the advances in our understanding of signaling pathways and transcription factors that govern the embryonic development of the pancreas and β-cell formation. Thus, to develop a source of cells that can be manipulated and functionally converted into β-like cells, it is crucial to understand how β-cells normally develop during embryogenesis.

There is great progress in understanding the molecular processes that lead to β-cells development; however, there is still much to be learned regarding the transcriptional network and regulatory relationship among inductive signals and transcription factors underlying β-cells differentiation. In this chapter, we will discuss the recent advances in our understanding of how transcriptional networks control early pancreas organogenesis and embryonic endocrine cell determination with special attention to the key transcription factors that play important roles in β-cells formation.

4.2 Overview of Pancreas Development

After gastrulation, the three germ layers, ectoderm, mesoderm, and endoderm, are generated. The gastrointestinal organs, including the pancreas, are derived from

the endodermal germ layer. The commitment to a pancreatic fate occurs through a progressive refinement of potential endoderm as a result of interactions with neighboring tissues. Dorsal endoderm, which will give rise to the dorsal pancreatic bud, contacts the notochord, aorta, and pancreatic mesenchyme. On the other hand, ventral endoderm, which will give rise to the ventral pancreas, lies adjacent to the septum transversum and cardiac mesoderm [41, 46]. Each pancreatic bud receives distinct signals from their surrounding tissues. Several studies have identified some of the morphogenic signaling systems, including FGF, TGFβ, VEGF, retinoic acid, Sonic hedgehog ligands, that are involved in pancreatic endoderm patterning (review in [98]). Signals from the notochord, including FGF and activin ligands, repress Sonic Hedgehog, a ligand of the Hedgehog signaling pathway, in the dorsal pancreatic bud [24, 25, 41]. The ventral bud is formed from cells that escape the inhibitory effect of FGFs and BMPs from the cardiac mesoderm [45]. The differences in the genetic program between the dorsal and the ventral pancreatic buds are also present later in development even though they form similar mature tissues. Thus, several targeted mutations in the mouse leads to phenotypes that are manifest only in the dorsal or the ventral bud.

Around the embryonic day (E) 9.5 in the mouse embryo, epithelial buds invade the surrounding mesenchyme by subsequent branching morphogenesis (Fig. 4.1). Recent studies have shown that multipotent progenitors that give rise to all pancreatic cell types are located at the tips of the branching network [106]. Although some endocrine cells appears at this stage of development, mostly glucagon positive cells, most of the hormone-expressing cells become apparent around E13.5, a period known as second transition (Fig. 4.1). By this time, the gut tube rotates to bring both buds into proximity. As embryogenesis proceeds, organ differentiates and grows while the digestive enzyme-producing acinar cell clusters that empty into the ductal cells and the endocrine cells organize into islet clusters (Fig. 4.1) (see review [38]).

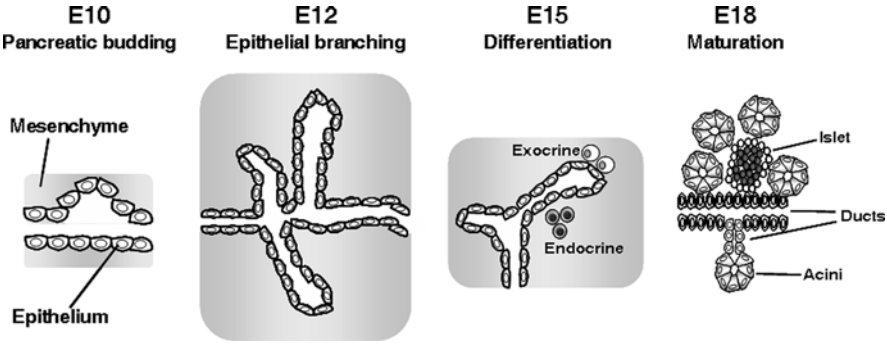

Fig. 4.1 Mouse pancreatic organogenesis. Formation of pancreatic buds begins around 9–10 days of gestation (E10). Starting at E12, the pancreatic ductal epithelium undergoes extensive branching and growth into the surrounding mesenchyme. Pancreatic epithelial cells differentiate into exocrine and endocrine cells (E15). By E18, three pancreatic cell types are found: ductal, acinar, and islet cells

4.3 Transcriptional Control in Pancreas Development

Ultimately, cell differentiation is achieved by the initiation and maintenance of a well-orchestrated gene expression patterns controlled by specific spatial and temporal combinatorial of transcription factors (Fig. 4.2). Dysfunction in β-cell transcriptional regulators might lead to diabetes. Targeted gene disruption in mice has given us enormous information about the role that transcription factors play during pancreas development and β-cell function. In humans, dysfunction of some of the β-cell regulators identified results in maturity-onset diabetes of the young (MODY) [64]. In this review we will emphasize in those transcription factors that have been shown to play crucial role in pancreatic progenitors, islet progenitors, and β-cells differentiation and maturation (Fig. 4.2).

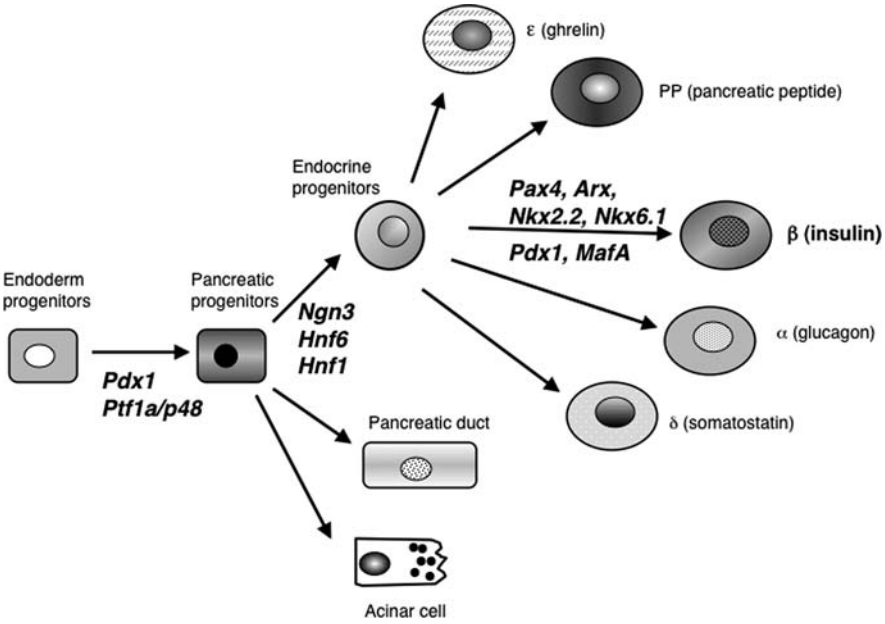

Fig. 4.2 Pancreatic cell differentiation. Upon activation of *Pdx1* and *Ptf1a*, the pancreatic fate is induced from endoderm progenitors. Pancreatic progenitors give rise to ductal, acini, and endocrine progenitors. Endocrine progenitors are then differentiated into different hormone-secreting cells, α, β, δ, PP, and ϵ. Key transcription factors involved in different steps of β-cell formation are indicated

4.3.1 Regulators of Pancreatic Progenitors

4.3.1.1 Pdx1

Among the earliest transcription factors expressed in the pancreatic progenitors is the *pancreatic duodenal homeobox 1* (*Pdx1*). In mice, *Pdx1* is expressed in

all pancreatic progenitors around E8.5 [21, 62]. Using Cre/loxP technologies, Melton and colleagues showed that *Pdx1* expression fate all pancreatic progenitors, indicating that exocrine, endocrine, and ductal cells have a common origin [20]. At midgestation, *Pdx1* expression becomes restricted to a subset of acinar and endocrine cells. In adult, PDX1 is mainly found in insulin-producing cells [21, 49, 57, 62]. Germline inactivation of *Pdx1* in mice arrest pancreas development at bud stage, indicating the essential role of this transcription factor at early stages of pancreas formation [37, 62]. Loss of PDX1 function also results in pancreatic agenesis in human [88]. Using a sophisticated strategy to control gene inactivation, Holland and colleagues have been able to temporally inactivate *Pdx1* [28]. By using this method, the authors showed that PDX1 function is also required at midgestation for the differentiation of both islet and acinar cells. In adult stages, PDX1 plays an important role in β-cell function, including regulation of insulin expression as shown by the defects in glucose homeostasis in heterozygous *Pdx1* mice [10]. Moreover, specific inactivation of *Pdx1* in β-cells in mice leads to diabetes [1].

The transcriptional network linked to *Pdx1* has been extensively studied in the last decade [103]. Analysis of *Pdx1* noncoding sequences revealed three areas of highly conserved sequences, named areas I to IV. A β-galactosidase reporter transgene containing the conserved regions I to III recapitulate the endogenous *Pdx1* developmental expression [17, 87]. These regions contain binding sites for known transcription factors, such as Foxa2, HNF6, PDX1, Ptf1a, and MafA [18, 32, 47, 58, 95, 99], indicating that they can be direct regulators of *Pdx1*. Some other transcription factors are involved in *Pdx1* regulation selectively in the dorsal or ventral pancreas. Thus, for example, mice lacking the homeobox Hlxb9 present selective agenesis of the dorsal pancreas [22, 50]. Similarly, Hex transcription factors have been shown to be required for the proper development of the ventral pancreas [8]. These observation reinforce the idea that the dorsal and ventral buds are governed by different combinatorial transcription factors and that *Pdx1* expression might depend on various sets of transcriptional cues controlling each pancreatic bud.

4.3.1.2 Ptf1a/p48

Another important transcription factor that is expressed in the pancreatic progenitors is the *Ptf1a/p48* basic helix-loop-helix (bHLH). Ptf1a/p48 is detected throughout the pancreatic epithelium and becomes restricted to acinar cell [43]. Originally it was thought that Ptf1a/p48 was exclusively required for exocrine pancreas, given its restricted expression at later stages in pancreas development and the absence of exocrine pancreas when *Ptf1a/p48* is inactivated in mice [43]. However, more recent lineage tracing experiments revealed that *PTF1A/P*48 is expressed in all pancreatic progenitors that give rise to endocrine, exocrine, and ductal cells [40]. Thus, Ptf1a/p48 is important for the specification of early pancreatic progenitors and, as it was mentioned before, in the regulation of *Pdx1* expression.

4.3.2 Establishment of the Endocrine Pancreas

4.3.2.1 Ngn3

The most important of the transcription factors that have been identified as specific for endocrine development is the bHLH transcription factor *Neurogenin3* (*Ngn3*). *Ngn3* expression is first observed at E9.5 in the mouse, and its expression peaks around E15.5, a stage that corresponds to the endocrine differentiation wave [3, 33, 74]. In the adult pancreas, *Ngn3* expression is almost undetectable. Lineage tracing experiments in mice using Cre recombinase under the promoter of *Pdx1*, which is expressed in all pancreas progenitors, showed that *Ngn3*-expressing cells ($Ngn3^+$) function as endocrine precursor cells and give rise to all hormone-secreting pancreatic cells [20]. That observation agrees with the phenotype of *Ngn3* knockout mice, which lack all endocrine cells types [19]. In contrast, overexpression of Ngn3 in the pancreatic progenitors leads to a premature endocrine differentiation [3]. Thus, these studies indicated that the $Ngn3^+$ cells are endocrine progenitors.

The mechanisms by which $Ngn3^+$ cells give rise to different hormone-expressing cells is not clear yet. A more detailed study using inducible expression of *Ngn3* in *Ngn3* – null pancreas at various times throughout development – suggests that the context of different developmental windows dictates lineage allocation. Early *Ngn3* induction in mice forces the induction of predominantly glucagon-producing α-cells, not β-cells, whereas later induction additionally leads to $insulin^+$ and PP^+ cells, and even later induction (E14) contributes to somatostatin-expressing cells while decreased glucagon-expressing cells [36]. The specific signals that influence competence of $Ngn3^+$ progenitors to generate specific endocrine lineages at each stage of development remain to be identified.

Given the importance of Ngn3 in endocrine differentiation, many questions arise about the transcriptional network linked to *Ngn3*. Regarding the upstream network governing *Ngn3* expression, binding sites for several transcriptional activators that are broadly expressed in the endoderm and pancreatic buds including hepatocyte nuclear factor 1β (HNF1β), Foxa2, and HNF6 have been found in the promoter of *Ngn3* [48]. *HNF1β* is expressed in the primitive endoderm and later in the pancreatic epithelium. Its expression eventually become restricted to the ductal cells [6, 52]. Analysis of transgenic mice in which *HNF1β* is inactive revealed the requirement of this transcription factors for pancreas organogenesis [23]. Similarly, *HNF6* knockout mice lack most *Ngn3*-expressing cells and develop very few and disordered islets [31]. In the mouse embryo, HNF1α activates *HNF*6 [67] and mice mutant for HNF6 fail to express *HNF1α* [52], indicating a cross-regulatory of these two transcription factors in the early pancreas. All these studies pointed that positive cells for HNF1α and HNF6 may be the precursors for $Ngn3^+$ endocrine progenitors population [52].

Because of the it crucial role of Ngn3 in establishing endocrine cell fate is very important the identification of its target for a better understanding of endocrine differentiation. Several Ngn3 targets have been identified, including paired box gene 4 (*Pax4*), aristaless-related homeobox (*Arx*), NeuroD/BETA2, insulinoma-associated antigen 1 (*IA-1*), *Nkx2.2*, *Nkx6.1*, and *Ngn3* itself [30, 56, 82, 83, 97]. A specific

endocrine lineage determination likely occurs through a coordinated cascade of these and other transcription factors activation and inactivation in cells derived from common, already committed endocrine progenitor.

4.3.3 Endocrine Lineages Specification

4.3.3.1 Pax/Arx

In the mouse embryo, *Pax4* and *Arx* are expressed in the developing pancreas. As development proceeds, the expression pattern of these two transcription factors becomes mutually exclusive, especially at later stages. *Pax4* is restricted to β- and δ-cells, whereas *Arx* is expressed in ε- and ϵ-cells [13, 84]. After birth, *Pax* expression is almost completely absent in β-cells and *Arx* expression persists in mature α-cells. Inactivation of *Pax4* in the mice leads to islets form of α- and δ-cells exclusively and lack of β- and δ-cells. In these mice, *Arx* expression is upregulated [85]. Similarly, mice mutant for *Arx* present islets composed exclusively of β- and δ-cells and the expression of *Pax4* is upregulated [13]. Thus, in the hierarchical network in endocrine lineage specification, *Pax4* and *Arx* act downstream Ngn3 and repress each other to determine the final proportions of the different endocrine cell types.

4.3.3.2 Nkx Transcription Factors

Three members of the Nkx protein family are expressed in the developing pancreas, Nkx2.2, Nkx6.1, and Nkx6.2, and are involved in endocrine lineage differentiation. *Nkx2.2* is expressed in the pancreatic bud until E13, overlapping with $Ngn3^{+}$ cells. Its expression persists in mature α-, β-, and PP-cells. Mice deficient for *Nkx2.2* display reduced numbers of α- and PP-cells and a complete absence of β-cells. [90]. It seems that in the absence of *Nkx2.2*, β-cells initiate their specification but are not able to fully differentiate into mature insulin-producing cells. This phenotype could be due to downregulation of *Nkx6.1*, which is required at later stages of endocrine development since the phenotype of mice that lacks *Nkx6.1* features a selective reduction of β-cells with a normal complement of other endocrine cell types. *Nkx2.2* expression in the *Nkx6.1* knockout mice is not affected [71]. The pattern of *Nkx6.1* expression is similar to that of *Nkx2.2*. It is broadly expressed until E10.5 but is expressed exclusively in β-cells by the end of gestation. *Nkx2.2*-null mice do not express *Nkx6* [90]. Comparison of single and double knockout mutant mice demonstrated the hierarchical and epistatic relationship between Nkx2.2 and Nkx6.1. Thus, mice with homozygous mutations in both *Nkx6.1* and *Nkx2.2* are identical to those with *Nkx2.2* homozygous single mutant mice [71]. Moreover, the promoter analysis of *Nkx6.1* showed a conserved binding site for Nkx2.2, suggesting that Nkx2.2 likely directly activates *Nkx6.1* transcription [71].

Nkx6.2 has a similar expression pattern that of *Nkx6.1*, beginning at E10.5 [60]. In Nkx6.1-null mice, the overexpression of Nkx6.2 rescues β-cell differentiation, suggesting a high degree of redundancy between these two transcription factors

[61]. Although the *Nkx6.2*-null mice do not have a clear pancreatic phenotype, the *Nkx6.1–Nkx6.2* double knockout mutant mice *2* have a further reduction in the number of insulin-positive cells compared with the single *Nkx6.1* knockout mice [2, 26]. Moreover, the double knockout mice also present reduction in glucagon-positive cells, suggesting an additional role of Nkx6 factors in a cell development.

4.3.4 Maintenance of β-Cell Identity

Once the cells within the islet are differentiated into the hormone-expressing cells, each of these different endocrine cells has to maintain its identity. That is achieved by the constant expression of transcription factors necessary for the maturation and expansion of each cell type. A general regulator for the expansion and organization of all endocrine cells within the islet is Pax6. *Pax6* expression at birth is restricted to the islet. The inactivation of *Pax6* in the mouse embryo reduces the number of endocrine cells, and the few remaining cells appear disorganized and produce low hormone levels [5]. Three transcription factors, NeuroD/BETA2, MafA, and PDX1, represent specific key players for β-cell maturation and identity. These three factors cooperate to synergistically activate the transcription of the *Insulin* promoter in β-cell.

4.3.4.1 NeuroD/BETA2

The bHLH transcription factor BETA2/NeuroD is expressed from E9.5 in scattered pancreatic cells and from E14.5 is expressed in *Ngn3*$^{+}$ cells. After birth, its expression becomes restricted to mature β-cells. Inactivation of NeuroD/BETA2 in mice leads to a decreased in islet cells number, especially in β-cells by undergoing apoptosis [59]. NeuroD/BETA2 is a strong inductor of *Insulin* by directly binding to the E-box present in the insulin promoter [78] and regulates its own transcription [102].

4.3.4.2 MafA

The basic leucine zipper MafA is β-cell-specific transcription factor that bind to the well-characterized insulin promoter [39, 55, 63]. MafA expression starts at E13.5 in the first insulin-producing cells, and its expression continue in the β-cells to the adulthood [55]. Inactivation of MafA in the mouse embryo do not perturb β-cell development, but newborn become glucose intolerant as a consequence of β-cell mass decrease and β-cell apoptosis [104]. Thus, MafA is not required for embryonic pancreas development but is crucial for the maintenance of functional β-cell. This idea becomes apparent in the transcriptional hierarchy in pancreas development, where MafA is located downstream of Pax4 [84]. MafA interacts with Pdx1 and NeuroD to activate the *Insulin* transcription [4, 63].

4.3.4.3 Pdx1

In addition to its role in early pancreas development, Pdx1 is also a key regulator of differentiated cells. As mentioned above, from a broad expression in the pancreatic epithelium, Pdx1 is restricted to β-cells at later stages of pancreas development and persist in the adult life. Its activity is required to activate many β-cells genes, including *Glut2* and *Glucokinase* [39, 55, 63, 75]. Pdx1 and MafA regulate the transcription of each other [69, 70]. This feedback regulation between MafA and Pdx1 contribute to reinforce the regulatory network to maintain the β-cell identity by activating *Insulin* promoter [4].

4.4 Human Islet Cell Development

Most of our understanding of islet cell development comes from developmental studies in mice. It is currently believed that the key transcription factors involved in mouse pancreas development are similar to those in humans. In this regard, the observation that homozygous mutations of PDX1 result in human congenital pancreatic agenesis evidences a clear role for this transcription factor in human pancreas development [88]. Furthermore, heterozygous loss-of-function mutation of PDX1 in human has been associated with maturity-onset diabetes of the young 4 (MODY4) [86]. Dysfunction in other pancreatic transcription factors has also been linked to MODY [64]. MODY1 is a result of mutations in hepatic nuclear factor 4-alpha (*HNF4*α) [100]. Mutations in hepatic nuclear factor 1-alpha result in MODY3. Humans with MODY3 suffer from defective glucose utilization, insulin secretion, and glucose disposal [89]. MODY5 results from mutations in hepatic nuclear factor 1β (*HNF1β*) [29]. Mutations in the *NeuroD1/BETA2* gene cause MODY6 [53].

Other transcription factors that are important for human islet pancreatic development or for differentiated β-cell function exist and are candidate diabetes genes. These include *Pax4* and *Isl1*. Heterozygous mutations in *Pax4* and *Isl1* are associated with late onset diabetes [80, 81]. The role of Ngn3 in human islet cell fate is unclear. A recent human genetic study raises the possibility that human β-cells may develop without Ngn3 function [96]. Three unrelated children have been identified with homozygous missense mutations in the bHLH region of the human *Ngn3* gene. An analysis of biopsy samples of intestinal tissue from these children revealed a near-complete absence of endocrine cells. These children develop diabetes at 8 years of age. However, it remains to be determined whether diabetes was related to the absence of cells since the pancreata of these children have not been examined [96].

The study of human pancreatic development has been mostly restricted to histological analysis of different stages of pancreatic development [9, 66]. Besides the limited access to human tissues, studies in human pancreatic development have been hampered by the absence of appropriate experimental models. However, several reports have described in vitro or in vivo experimental systems using human fetal pancreata. When human embryonic pancreatic tissue was grafted onto immunoincompetent mice, endocrine cell mass and insulin content increased, suggesting

that endocrine cell differentiation took place [91]. Similarly, other groups reported that engraftment of human islet cell clusters resulted in mature, functional islet-like structures [7]. These studies have predominantly relied on fetal pancreata at 18–24 gestational weeks. At this stage, considered a late stage of pancreatic development [72, 92], the human pancreas is already rich in endocrine cells [54] and therefore the analysis of islet differentiation under these conditions might be somewhat limited. However, other studies have shown that when noninsulin positive human embryonic pancreata obtained at an early development stage (7–8 weeks) were transplanted under the kidney capsule of immunoincompetent mice, pancreatic growth and insulin positive cells were observed after 4 months [12]. These same authors have recently developed a new experimental model to study human islet cell fate. In this model, a GFP reporter transgene is transferred into developing human pancreatic cells by lentivirus infection, allowing tracing of the infected cells [73]. The researchers demonstrated that, similarly to what has been described in mice, β-cells from human islets derive from multiple progenitors.

In summary, although rodent and human pancreas development appears to share many similarities, further studies in human pancreas are clearly needed to completely understand how human islet cells are formed. To that end, the generation of new experimental techniques and genetic tools is crucial.

4.5 Lessons from Islet Development for Cell Replacement Therapy in Diabetes

Cell replacement therapy appears as an attractive approach to treat diabetes and thus has become a major goal in the field of diabetes research. Several strategies have been attempted to obtain a renewable source of β-cells for transplantation purposes including the genetic manipulation of cells that are closely related to β-cells. For example, adenovirus-mediated transfer of *Pdx1* to mouse liver cells has been shown to activate the expression of genes that encode mouse insulin and prohormone convertase [16]. Similarly, adenoviral-mediated expression of *Ngn3* in adult human pancreatic duct cells resulted in the generation of cells that express several β-cells markers and were able to synthesize insulin [27]. In addition, transfection of rat intestinal stem cells with genes encoding *Pdx1* and *Isl1*, followed by exposure to betacellulin, induced insulin production. When transplanted to diabetic animal models, these cells led to reduced levels of glucose [42]. More recently, the generation of β-cells from acinar cells in vivo has been reported. In these series of exciting experiments, the researchers delivered via adenoviruses three key pancreatic transcriptional factors (PDX1, Ngn3, and MafA) specifically to acinar cells in adult mice. The forced expression of these three transcriptional factors resulted in the differentiation of acinar cells into β-cells, in what represents a remarkable example of adult cellular reprogramming [105].

Another interesting approach to obtain insulin-producing cells aims to fully recapitulate the processes of pancreatic and β-cell development in embryonic stem cells.

Two recent studies successfully used this idea to obtain insulin-secreting cells from human embryonic stem cells (hESC) [14, 15]. In these studies, the authors generated definitive endoderm, primitive gut tube-like cells, pancreatic endocrine precursors, and endocrine cells from hESC using a differentiation protocol. This protocol is based on the sequential addition of ligands of the signaling pathways that have been shown to be involved in different stages of pancreas development, attempting to recapitulate the signaling cascades that govern β-cell differentiation in the embryo. Thus, under these conditions, the cells express specific combination of transcription factors at different stages, to finally resemble the β-cell signature. That success has been reached thanks to many years of pancreas developmental studies.

Although in the last decade there has been an enormous progress in the elucidation of the basic transcriptional hierarchy in β-cell development, the identification of the signaling pathway that imprint this hierarchy and the contribution of extracellular signals [93, 94] will be essential for the generation of β-like cells from stem cells for diabetes therapy.

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Chapter 5
High Fat Programming of β-Cell Failure

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Abstract High saturated fat intake contributes to insulin resistance, β-cell failure, and type 2 diabetes. Developmental programming refers to a stimulus or insult during critical periods of life which includes fetal and subsequent early neonatal life. Programming alters offspring physiology and metabolism with both immediate and lasting consequences. Maternal nutrition in gestation and lactation shapes offspring development and health. A high saturated fat diet ingested by mothers during gestation and/or lactation is a form of nutritional insult that induces diabetogenic changes in offspring physiology and metabolism. High fat programming is induced by maternal high saturated fat intake during defined periods of gestation and/or lactation and programs the physiology and metabolism of the offspring in early life. This more recently adopted form of developmental programming reflects society in both affluent and developing countries. High fat programming induces adverse changes in β-cell development and function in neonatal and weanling offspring. These changes are characterized by compromised β-cell development and function, evident by altered expression of key factors that maintain the β-cell phenotype. High fat programming is likely to result in β-cell failure and eventual type 2 diabetes.

Keywords Glucolipotoxicity · Nutrition · Pancreas · Type 2 diabetes

5.1 Introduction

Changes in lifestyle, such as consumption of a high-calorie diet and lack of exercise, have increased the global prevalence of obesity and diabetes [1]. Between 60 and 90% of cases of type 2 diabetes appear to be related to obesity [2] with a

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strong correlation between obesity and insulin resistance in both diabetic and non-diabetic subjects [3]. Developmental programming is defined as a stimulus or insult in utero or in early postnatal life (during suckling) that induces long lasting changes in progeny physiology and metabolism. High fat programming (HFP) is induced by maternal high saturated fat intake during defined periods of gestation and/or lactation and programs the physiology and metabolism of the offspring. This contrasts from previous studies focusing on protein deficiency in utero. Instead of mimicking famine as in protein restriction and undernutrition, which still exists in several parts of the world, HFP reflects current society in both affluent and developing nations. In affluent societies, there is an overabundance of food available. With economic improvement and migration in the developing world, there is a transition from the traditional way of life, where the diet comprises whole food and exercise is a regular routine in daily life, to a more Westernized lifestyle – characterized by the consumption of convenient processed and fast foods with high saturated fat content and a sedentary lifestyle due to a greater reliance on transport which reduces the need for exercise. With migration and changing economic status, people often switch diets which may be from whole food or a state of undernutrition to a diet that includes a high content of saturated fat and overnutrition. Disease risk is amplified by a greater mismatch between the prenatally predicted and actual adult environments [4]. As a result, societies in rapid economic transition are particularly vulnerable [5–8]. Long-term consumption of a high fat diet (HFD) concomitant with physical inactivity leads to obesity which is a major risk factor for inducing insulin resistance and β-cell failure and contributes to the increase in incidence of type 2 diabetes.

5.2 Critical Programming Windows

Both the intrauterine and lactational environments represent critical developmental periods that provide a platform for programming. The intrauterine environment influences the health of the fetus. The developing fetus is highly sensitive to its environment and nutrition is an important factor that affects both fetal growth and maturation [9]. The fetus makes adaptations that anticipate the postnatal nutrition which impacts its future metabolic status. A mechanism by which diet influences fetal growth is by altering circulating concentrations of key maternal metabolic hormones, which regulate placental nutrient transport and therefore fetal growth [10]. Lactation is a critical developmental stage for metabolic programming and later disease and for modifying the impact of prenatal challenges [11, 12]. Fluctuations in glycemic and saturated fatty acid levels have adverse effects on progeny. The level of nutrition available during pregnancy and lactation plays a major role in determining offspring metabolic phenotype [13]. The offspring adopts the nutrition it experienced during these critical developmental periods i.e., the diet that it is exposed to during fetal and early postnatal life. A high saturated fat diet is strongly associated with the pathogenesis of β-cell failure, insulin resistance, and type 2 diabetes [14]. In pregnant mothers, maintenance on an HFD results in the exposure of offspring

to an insult during the critical period of fetal life. This, together with the changes in the metabolic profile and state of insulin resistance in the pregnant mother (which occurs in mothers during normal pregnancy), is likely to increase the risk of the offspring to develop metabolic disease.

5.3 HFP Concept

HFP can be described by maternal high saturated fat intake during defined periods of gestation and/or lactation which results in programming of the physiology and metabolism in the offspring in early life [15]. Offspring can be studied at various stages of life: as neonates, weanlings, young adults, and old age. In terms of experimental design, high saturated fat diets are administered to pregnant and/or lactating Wistar rats, thereby exposing progeny to this dietary insult. Specifically, mothers and their progeny were maintained on an HFD throughout gestation only (gestational high fat programming = HFG), throughout lactation only (lactational high fat programming = HFL), or throughout both gestation and lactation (gestational and lactational high fat programming = HFGL; Fig. 5.1a). These offspring are therefore programmed as high fat exposure during these critical developmental periods influences their metabolic status. The HFD used contained 40% energy as fat compared to 10% energy as fat in the standard laboratory diet. Similar protein levels were maintained in both diets to avoid the effects of protein deficiency on β-cell morphology and function.

Furthermore, HFP studies can be extended to include different time period interventions (Fig. 5.1b). In these studies, the nutritional trajectory of pregnant mothers was maintenance on an HFD for either the first, second, or third week of gestation (to mimic human trimesters) and throughout gestation [16]. Neonatal offspring, maintained on the HFD for those specified periods of fetal life, were studied. All of

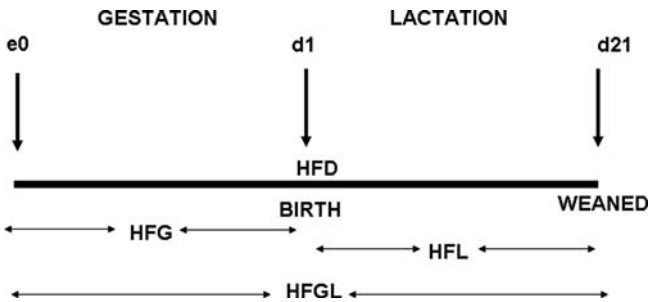

Fig. 5.1a High fat programming: basic experimental design. e, gestational day; d, postnatal day (lactational day), HFD, high fat diet; HFG, gestational high fat diet; HFL, lactational high fat diet; HFGL, gestational and lactational high fat diet; *downward arrows* denote time points; *horizontal arrows* denote period of high fat diet maintenance

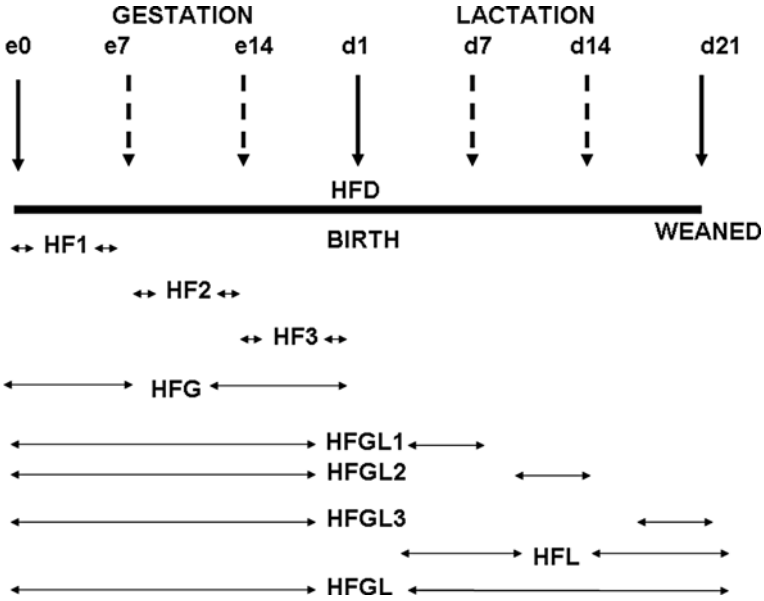

Fig. 5.1b High fat programming: extended experimental design. e, gestational day; d, postnatal day (lactational day). Numerals refer to the specific day of gestation (e) or lactation (d). HFD, high fat diet; HF1, high fat diet for the first week of gestation; HF2, high fat diet for the second week of gestation; HF3, high fat diet for the third week of gestation; HFG, gestational high fat diet; HFGL1, high fat diet throughout gestation and for the first week of lactation; HFGL2, high fat diet throughout gestation and for the second week of lactation; HFGL3, high fat diet throughout gestation and for the third week of lactation; HFL, lactational high fat diet; HFGL, gestational and lactational high fat diet; *downward arrows* denote time points; *horizontal arrows* denote period of high fat diet maintenance

these neonatal groups were also studied at weaning (3 weeks of age) [17]. During lactation, these offspring were maintained on a standard laboratory (low fat) diet.

Further extension of the weanling groups included those maintained on a gestational HFD including either the first (HFGL1), or the second (HFGL2), or the final (HFGL3; Fig. 5.1b) week of lactation [18]. When the neonatal and weanling offspring were not maintained on a HFD, they were instead maintained on a standard laboratory diet.

These studies demonstrated that high fat consumption resulted in increased food intake in pregnant mothers which subsequently increased their body weights and induced hyperglycemia [16]. In terms of HFP, neonates displayed alterations in glycemia and in β-cell development [16], which persisted in weanlings [17]. HFP over different time periods of gestation and lactation also altered expression of key β-cell genes, including GLUT-2, glucokinase (GK), and to a lesser extent, Pdx-1 [18]. A gestational HFD-induced maternal hyperglycemia and the programming effects resulted in neonates with hyperglycemia [16]. Both the mothers and neonates

displayed no changes in insulinemia. Different outcomes in β-cell development and function were evident in both neonatal and weanling offspring from dams maintained on an HFD during specified periods of gestation and/or lactation [16–18]. These alterations appear to be dependent on the specific period of exposure to the HFD.

5.4 β-Cell Regulation

The ability of an organism to maintain its β-cell mass during adulthood is critical for maintaining glucose homeostasis and preventing diabetes [19]. β-cell mass is increased by β-cell neogenesis (differentiation from precursor cells), β-cell proliferation or replication (from existing β-cells), and β-cell hypertrophy (increase in β-cell size), and is reduced by β-cell death, primarily through apoptosis and also via hypotrophy (decreased β-cell size) [19]. The balance between proapoptotic and anti-apoptotic (protective) processes determines the fate of β-cells [20]. ATF3, a stress-inducible proapoptotic gene, represses the expression of IRS2, a prosurvival gene, thus providing a direct link between the stress response and a potent prosurvival pathway [20]. Because ATF3 is induced by a variety of stress signals, it can function as a conduit for stress signals to dampen a potent prosurvival pathway in β-cells [20]. An early loss of β-cell mass might subsequently favor dysfunction of the residual β-cells, possibly due to overstimulation or toxic effects of even mild chronic hyperglycemia and/or hyperlipidemia [21]. Partial recovery of the lost mass may result from post-insult induction of β-cell regeneration and/or neogenesis and eventually with treatment with growth-stimulating pharmacological agents [22]. Hypertrophy and increased insulin responsiveness to glucose and free fatty acids (FFA) also occur in residual β-cells [22]. The adaptive response might be insufficient or temporary because of incomplete differentiation of newly formed β-cells and/or acquired dysfunction of residual β-cells chronically exposed to a metabolically altered environment [22]. An increased frequency of apoptosis due to prolonged overstimulation of residual β-cells, chronic hyperglycemia, and/or hyperlipidemia might accelerate decomposition [22].

An increase in β-cell mass usually takes place over a period of time for the endocrine pancreas to maintain glucose homeostasis when challenged by diabetogenic states such as obesity and insulin resistance. This allows the pancreas to cope, for a defined time, with the maintenance of glucose homeostasis. However, after a threshold is reached, the β-cells become exhausted and hyperglycemia is manifested usually along with an altered insulinemic profile. Initially hyperinsulinemia is exhibited, i.e., the β-cells secrete more insulin in order to restore glucose homeostasis, but eventually, the β-cells fail to secrete sufficient insulin resulting in hypoinsulinemia which often progresses to type 2 diabetes. Hypoinsulinemia is indicative of the more severe type 1 diabetes, which indicates how type 2 diabetes evolves to closely mimic type 1 diabetes, as it progresses to deteriorate to a state

where exogenous insulin is required to counteract the elevated circulating glucose concentrations. Molecules, signaling pathways, and cellular machinery involved in the demise of β-cells under seemingly divergent pathophysiological conditions, i.e., type 1 and 2 diabetes are to a large extent the same [20]. Chronic high saturated fat consumption and persistent hyperglycemia contribute significantly to reducing β-cell mass.

5.5 HFP May Induce β-Cell Failure Via Glucolipotoxicity

Glucotoxicity can be described as the slow and progressively irreversible effects of chronic hyperglycemia on β-cell function [23]. Chronic hyperglycemia decreases β-cell mass by inducing apoptosis [24, 25] and adversely affects insulin secretion [23]. Lipotoxicity, characterized by chronic exposure to elevated FFA concentrations, impairs insulin secretion leading to hyperglycemia and β-cell failure [26]. Hyperglycemia is proposed to be a prerequisite for lipotoxicity to occur [27]. The glucolipotoxicity theory proposes that simultaneous elevation of glucose and lipids results in intracellular accumulation of lipids and lipid metabolites, which are ultimately detrimental to β-cell function and survival [28]. HFP may induce adverse effects on metabolism and physiology by elevating both circulating glucose and fatty acid levels in offspring. This impairs β-cell development and function resulting in loss of glucose homeostasis.

Chronic exposure of islets to elevated levels of nutrients induces β-cell dysfunction and triggers β-cell death. Exposure of isolated rodent islets to hyperglycemia for several days raises basal insulin secretion but impairs insulin secretion in response to stimulatory glucose concentrations [29, 30]. Similarly, exposure of islets to elevated levels of FFA does not impair glucose-stimulated insulin secretion (GSIS) unless the islets are cultured at or above a threshold concentration of glucose (about 8 mmol/l) [23, 31]. This suggests that β-cell failure is likely a consequence of glucolipotoxicity as opposed to either gluco- or lipotoxicity as separate entities [23, 31]. HFP may induce β-cell failure by a glucolipotoxic mechanism due to potential exposure of offspring to maternal hyperglycemic and hyperlipidemic intrauterine environments. In addition, the milk of hyperglycemic mothers may also contain elevated FFA concentrations which may have a glucolipotoxic effect on the suckling offspring. One hypothesis is that β-cells become sensitized to FFA and preferentially metabolize FFA rather than glucose as fuel, which may explain the reduced glucose-stimulated insulin release (GSIR) typically observed following prolonged exposure to FFA [27]. In HFP, it is therefore likely that preferential metabolism of FFA over glucose will further exacerbate hyperglycemia.

Elevated levels of glucose or saturated fatty acids can in their own capacity or synergistically induce β-cell failure. However, the presence of both gluco- and lipotoxicity, i.e., glucolipotoxicity will accelerate the impairment of β-cell function. This simultaneous dual insult will more rapidly increase the β-cell metabolic overload, inhibiting β-cell compensation and thus increase susceptibility to β-cell failure.

In obese type 2 diabetic patients, their hyperglycemic state concomitant with the readily available fat stores for release of FFA, will place them in a glucolipotoxic state. Obese type 2 diabetic individuals who continuously ingest a high intake of harmful saturated fatty acids will further exacerbate this condition.

β-cells initially compensate for the insulin resistance associated with obesity by upregulating the secretion of insulin [1]. During β-cell compensation, β-cells are exposed to metabolic changes associated with obesity, so factors commonly associated with obesity such as insulin resistance, adipokines, FFA, reactive oxygen species, and ER-associated stress are likely inducers of β-cell failure [1]. β-cell failure in type 2 diabetes occurs when islets are unable to sustain β-cell compensation for insulin resistance [32]. The failure is progressive, particularly after hyperglycemia is established, which leads to poorly functioning, dedifferentiated β-cells and loss of β-cell mass from apoptosis [32]. β-cell destruction in various pathophysiological conditions can be viewed as a stress response [20]. The likely mechanisms of early β-cell failure include mitochondrial dysfunction, oxidative stress, ER stress, dysfunctional triglyceride/FFA (TG/FFA) cycling, and glucolipotoxicity [32]. Furthermore, β-cell failure is likely induced by a combination of chronic hyperglycemia, hyperlipidemia, and/or certain cytokines that interfere with the signaling pathways that maintain normal β-cell growth and survival [33]. This results in a reduction in functional β-cell mass in a diabetic state [33]. Other underlying mechanisms in β-cell failure include genetic susceptibility, β-cell metabolic overload and amyloid fibrils [34]. Once hyperglycemia has developed, which occurs in specific instances of HFP dependent on the period of exposure, additional processes linked to glucotoxicity and the diabetic milieu, such as islet inflammation, O-linked glycosylation, and amyloid deposition accelerate β-cell failure, resulting in severe β-cell phenotypic alterations and loss of β-cell mass by apoptosis [32].

Mice fed a diet rich in saturated fat develop overt diabetes characterized by hyperinsulinemia associated with hyperglycemia [35]. In offspring where the HFD was administered only during fetal life (similar to HFG) and during both fetal and neonatal life (similar to HFGL), the β-cell insult was severe evident by sustained hyperglycemia during adulthood [35]. Thus it seems feasible that the programming effects in both HFG and HFGL, and likely also in HFL weanlings, will further exacerbate as they reach adulthood. Obesity often leads to insulin resistance but only a subset of obese insulin-resistant individuals progress to type 2 diabetes [34], which may be due to genetic predisposition, poor dietary control, and physical inactivity. In both animals and humans, the triggering factor is β-cell failure, which involves a decrease in β-cell mass and a deterioration of key β-cell functions like GSIS [34]. The severity of HFP in inducing β-cell failure may be dependent on the stage of programming (gestational and/or lactational), the metabolic status of the mother, and the duration of the insult. It appears that limited exposure to programming effects, such as maintenance on an HFD for only a single gestational week, may have a reduced impact on adversely affecting β-cell function. This however makes the offspring susceptible to the predictive adaptive response whereby they cannot accurately anticipate future nutrition as it differs from the nutrition experienced

in utero which is hypothesized to have adverse health consequences. Maintenance on an HFD throughout both gestation and lactation represents extreme HFP. It is hypothesized that if these progeny are maintained on an HFD, with time β-cell failure will ensue. Initially the β-cell may undergo compensation and adaptation to cope with the maintenance of glucose homeostasis. However, HFP coupled to chronic high fat feeding is likely to increase glucolipotoxicity resulting in eventual β-cell failure.

Chronic hyperglycemia can increase the rate of development of the early diabetic state by affecting the secretion capacity of pancreatic cells, which in turn, increases blood glucose concentrations [36] and ultimately leads to the total incapacity of β-cells to secrete insulin [37, 38]. HFP induces hyperglycemia and hypoinsulinemia, which is characterized by reduced β-cell volume, number, and sizes, with reduced expression of GK. Collectively, these adverse metabolic effects of programming would predispose these progeny to β-cell failure. Also, HFP may increase circulating FFA concentrations due to metabolism of the HFD which releases excess FFA. This may lead to reduced insulin secretion, which induces hyperglycemia resulting in β-cell failure. Apoptosis and oxidative stress are also likely candidates in the demise of β-cell integrity. HFP may follow a glucolipotoxic mechanism to impair β-cell function ultimately leading to the β-cell failure.

5.6 HFP Degrades β-Cell Integrity

A HFD is known to compromise glucose sensing and insulin signaling, evident by reduced expression of insulin, Pdx-1, GLUT-2, and GK after high fat feeding or exposure to FFA [39–42]. The percentage of L-type calcium channels that are considered most important for insulin secretion is reduced in neonatal rats, concomitant with reduced expression of GLUT-2 [43]. Preliminary results show that control neonates display a normal insulin secretory response to glucose stimulation, a function that was absent in HFG neonates – HFG neonates released reduced insulin at stimulatory 13 and 22 mmol/l glucose concentrations concomitant with reduced Pdx-1 and GK immunoreactivity (unpublished data). Chronic hyperglycemia adversely affects insulin secretion [23] and decreases β-cell mass by inducing apoptosis [24, 25]. As HFG neonates display reduced β-cell volume and numbers [5] and both Pdx-1 and GK immunoreactivity is reduced, the functional capacity of the HFG β-cells is reduced. These effects, concomitant with the reduced insulin release from HFG islets at stimulatory glucose concentrations, indicate that programming with an HFD during gestation impairs β-cell function.

An altered metabolic milieu decreases Pdx-1 transcription by mediating a cascade of epigenetic modifications which silences Pdx-1 [44]. In intrauterine growth retarded (IUGR) rats, Pdx-1 expression was permanently reduced in β-cells [44]. Gestational HFP reduced Pdx-1 immunoreactivity in the neonates which could adversely affect insulin gene expression and, in addition, appears to have contributed to the reduction in β-cell volume and number in HFG neonates.

Haploinsufficiency of β-cell specific GK ($GK^{+/-}$) results in mild diabetes with impaired insulin secretion in response to glucose [45]. Wild-type mice fed an HFD showed marked β-cell hyperplasia, whereas $GK^{+/-}$displayed insufficient β-cell hyperplasia despite a similar degree of insulin resistance [46]. Permanent exposure of weanlings to an HFD, i.e., during the entire duration of both gestation and lactation results in the reduction of mRNA expression of GLUT-2 and GK [18]. These HFGL weanlings displayed reduced circulating insulin concentrations suggesting impaired insulin secretion attributed partly to the reduced GK expression both at mRNA and protein level [18]. HFL, HFGL, and HFG weanlings display glucose intolerance in descending order of severity (unpublished data). Thus HFGL weanlings display impaired β-cell function which may predispose them to β-cell failure. Both HFG and HFGL weanlings were normoglycemic and hypoinsulinemic [18] but both groups were glucose intolerant, an effect that was more marked in HFGL weanlings. As the HFG and HFGL weanlings were normoglycemic yet glucose intolerant, it is thus important to assess β-cell function in the absence of hyperglycemia. In contrast, HFL weanlings were hyperglycemic and normoinsulinemic [18] and displayed the greatest severity in glucose intolerance. Glucose intolerance may represent an early event in the impairment of β-cell function and appears to be exacerbated by hyperglycemia.

5.7 HFP: Potential Mechanism of Induction of Type 2 Diabetes

Type 2 diabetes is associated with genetic and environmental factors (Fig. 5.2). Genetic factors include candidate genes (several are currently being characterized including TCF7L2 and KCNJ11) and MODY genes (mostly transcription factors, apart from GK), which result in offspring inheriting the disease from their parents. Environmental factors are more broadly defined as they include nutrition (e.g., malnutrition and overnutrition), developmental programming, level of physical activity, oxidative stress, cytokines, and glucolipotoxicity. High saturated fat diets, sedentary lifestyles, high oxidative stress levels, and cytokines all play a role in the pathogenesis of diabetes via different mechanisms. However, glucolipotoxicity may be strongly associated with HFP-induced β-cell failure.

Developmental programming by feeding pregnant dams an HFD is an environmental insult that induces adverse changes in β-cell development and function in young offspring. HFP can be seen as a more robust environmental insult as high saturated fat intake (in its own capacity) and developmental programming (in its capacity) can strengthen the detrimental environmental influence of these combined insults.

Glucolipotoxicity proposes that simultaneous elevation of circulating glucose and FFA concentrations induces β-cell failure. HFP induces hyperglycemia and metabolism of the HFD may result in increased circulating FFA levels, particularly saturated fatty acids which have adverse effects on β-cells. HFP may therefore, via glucolipotoxic effects, induce β-cell failure.

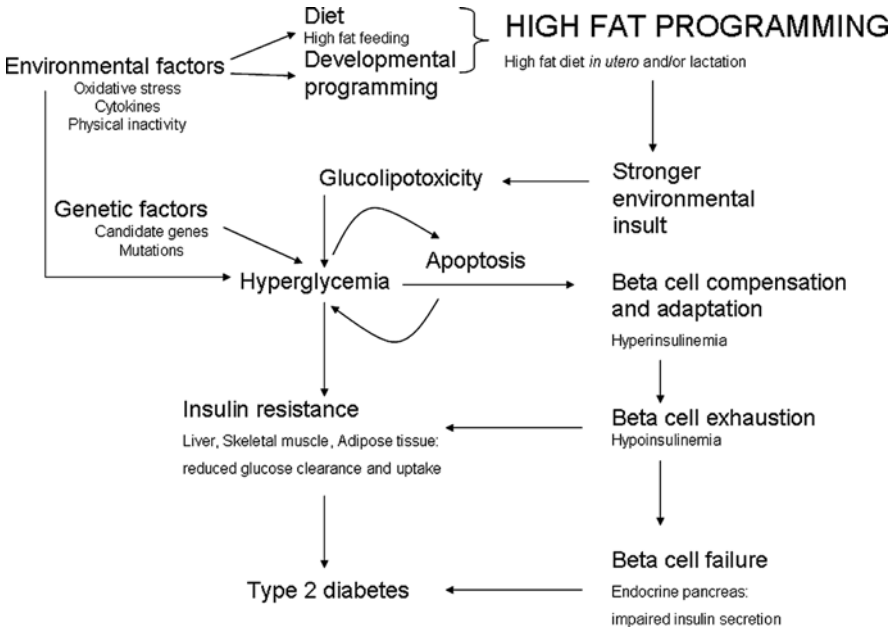

Fig. 5.2 The influence of HFP in the pathogenesis of type 2 diabetes. Apart from environmental and genetic factors, HFP can induce hyperglycemia as a stronger environmental insult. Glucolipotoxicity may be the mechanism whereby HFP induces β-cell failure. Hyperglycemia is the clinical hallmark of type 2 diabetes. Initially β-cells may compensate and adapt to the hyperglycemia (by hypertrophy and hyperplasia) by hypersecretion of insulin. This may still reflect normal glucose homeostasis. Eventually, β-cell exhaustion may set in resulting in reduced insulin secretion characterized by hypoinsulinemia which may lead to impaired glucose tolerance. This will further exacerbate hyperglycemia. In the endocrine pancreas, β-cell exhaustion eventually leads to β-cell failure characterized by impaired insulin secretion which may progress to overt type 2 diabetes. Skeletal muscle, liver and adipose tissue are the main sites of glucose uptake. Hyperglycemia, which is further aggravated by hypoinsulinemia, results in insulin resistance of these peripheral tissues due to their reduced ability to effectively clear glucose. Like β-cell failure, insulin resistance precedes overt type 2 diabetes

Chronic hyperglycemia adversely affects insulin secretion [23] and decreases β-cell mass by inducing apoptosis [24, 25]. Furthermore, chronic hyperglycemia leads to progressive loss of β-cell mass with a prolonged increase in the rate of β-cell apoptosis without a compensatory increase in β-cell growth [47]. Hyperglycemia is central to type 2 diabetes and can be induced by environmental factors, genetic mutations, and by HFP. Apoptosis reduces β-cell mass thereby further aggravating hyperglycemia. With HFP, offspring are compromised at an early age, as normal β-cell development is impaired and they display reduced β-cell function. In the face of hyperglycemia, β-cell compensation and adaptation occurs to restore normoglycemia. This occurs by hyperplasia and hypertrophy of the β-cells, which temporarily maintain glucose homeostasis. However, if hyperglycemia recurs,

β-cell exhaustion may ensue, resulting in subsequent β-cell failure. β-cell failure and insulin resistance are key events that contribute to the pathogenesis of type 2 diabetes. It is likely that HFP accelerates the onset of overt type 2 diabetes by inducing β-cell failure. Further studies are required to elaborate on this potential mechanism of HFP of β-cell failure and to determine the effects of HFP in the possible induction of insulin resistance in skeletal muscle, liver and adipose tissue.

5.8 Perspectives

HFP reduces β-cell integrity by impairing both β-cell development and function, therefore compromising future offspring health by predisposing them to metabolic disease. There may be a possible link between HFP and glucolipotoxicity. Nutrition during critical developmental periods shapes offspring health. The intrauterine milieu and the lactation period have a great influence on the health of the progeny. Dietary intervention to ensure adequate nutrition with the correct macronutrient balance, concomitant with sufficient levels of micronutrients and with the optimum ratios of fatty acids is a strategy to optimize the growth and health of the fetus and neonate. During fetal and early postnatal life, maintenance on an undesirable diet, such as a high saturated fat diet, is likely to induce adverse changes in offspring physiology and metabolism. Specifically HFP has been demonstrated to adversely affect β-cell function thus predisposing offspring to β-cell failure. The key for future research is to clearly elucidate the mechanisms such as glucolipotoxicity, followed by manipulation and correction of these changes in order to maintain a healthy β-cell phenotype that can cope with fluctuating metabolic demands and improve outcomes for β-cell survival.

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Chapter 6
Nutrient Regulation of Insulin Secretion and β-Cell Functional Integrity

Philip Newsholme, Celine Gaudel, and Neville H. McClenaghan

Abstract Pancreatic β-cells are often referred to as "fuel sensors" as they continually monitor and respond to dietary nutrients, under the modulation of additional neurohormonal signals, in order to secrete insulin to best meet the needs of the organism. β-cell nutrient sensing requires metabolic activation, resulting in production of stimulus-secretion coupling signals that promote insulin biosynthesis and release. The primary stimulus for insulin secretion is glucose, and islet β-cells are particularly responsive to this important nutrient secretagogue, It is important to consider individual effects of different classes of nutrient or other physiological or pharmacological agents on metabolism and insulin secretion. However, given that β-cells are continually exposed to a complex milieu of nutrients and other circulating factors, it is important to also acknowledge and examine the interplay between glucose metabolism and that of the two other primary nutrient classes, the amino acids and fatty acids. It is the mixed nutrient sensing and outputs of glucose, amino and fatty acid metabolism that generate the metabolic coupling factors (MCFs) involved in signaling for insulin exocytosis. Primary MCFs in the β-cell include ATP, NADPH, glutamate, long chain acyl-CoA and diacylglycerol and are discussed in detail in this article.

Keywords Pancreatic β-cells · Insulin secretion · Nutrient metabolism · Incretins · Signal transduction · Stimulus-secretion coupling · Gene expression · Desensitization

Abbreviations

ACC	acetyl-CoA carboxylase
CPT-1	Carnitine Palmitoyl Transferase 1
DAG	diacylglycerol
FFA	free fatty acid

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GLP-1	glucagon-like peptide-1
GIP	glucose-dependent insulinotropic polypeptide
GSIS	glucose-stimulated insulin secretion
Gly3P	glycerol-3-phosphate
LC-acyl CoA	long-chain acyl-CoA
MCF	metabolic coupling factors
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C

6.1 Overview of β-Cell Stimulus-Secretion Coupling

Pancreatic β-cells are often referred to as "fuel sensors," continually monitoring and responding to circulating nutrient levels, under the modulation of additional neurohormonal signals, in order to secrete insulin to best meet the needs of the organism. β-cell nutrient sensing involves notable metabolic activation, resulting in production of coupling signals that promote insulin biosynthesis and secretion. The primary stimulus of insulin secretion is glucose, and islet β-cells are particularly responsive to this important nutrient secretagogue, coupling metabolic and other stimuli with the insulin-secretory machinery. In writing this chapter we are fully aware that most of the studies cited have utlilized rat-, mouse- or hamster-derived insulinoma β-cell lines to study function in vitro. This is due to the inherent difficulty in maintaining primary rodent islet β-cell mass and function for more than a few days in vitro and of course the scarcity of human islets for research purposes. There are as yet no suitable human β-cell lines available for unrestricted in vitro studies. However, the major rodent β-cell lines have provided substantial data and insights into cell function in normal or pathogenic situations. The most widely used cell lines include INS 1, MIN 6, RINm5F and BRIN BD11. It is important to state that in vivo intact islet structures (comprising α, β and δ-cells, which secrete glucagon, insulin and somatostatin respectively) are required to maintain appropriate and pulsatile hormone secretion in response to nutrient stimuli.

Elevation in blood glucose concentrations results in rapid rises in intracellular glucose levels as glucose is transported across the β-cell plasma membrane. Glucose uptake and metabolism are two essential steps in the so-called "glucose-stimulated insulin secretion" (GSIS) pathway. GSIS represents the increase in insulin secretion over basal release in response to increased extracellular, and ultimately intracellular, glucose. As illustrated in Fig. 6.1, glucose rapidly enters β-cells, through specific glucose transporters (GLUT-1 in humans; GLUT-2 in rodents), after which it is swiftly phosphorylated by the enzyme glucokinase, which has a high *K*m for glucose. These primary steps, particularly glucokinase, determine the rate of glucose utilization by the β-cell over a range of physiological glucose levels (3–20 mM) and the combination of transport and phosphorylation determines metabolic flux through glycolysis.

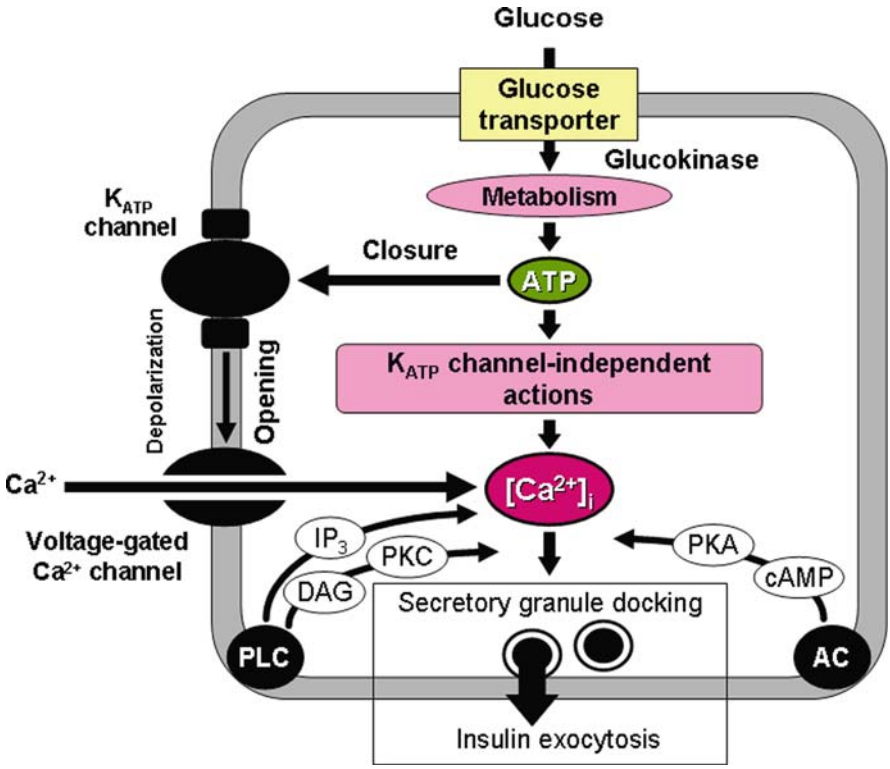

Fig. 6.1 Metabolic stimulus–secretion coupling in the β-cell. Glucose metabolism results in an enhanced cytoplasmic ATP/ADP ratio, which prompts closure of ATP-sensitive K^+ (K_{ATP}) channels in the plasma membrane evoking membrane depolarization, and subsequent opening of voltage-gated Ca^{2+} channels. This culminates in an increase in cellular Ca^{2+} influx – a primary driver of the GSIS mechanism. Ca^{2+} and vesicle docking and fusion events can also be modulated by agents acting through the phospholipase C (PLC)/protein kinase C (PKC) or adenylate cyclase (AC)/protein kinase A (PKA) pathways

Increased β-cell glycolytic flux results in a rapid increase in production of reducing equivalents, an increased activity of shuttle mechanisms (responsible for transferring electrons to the mitochondrial matrix), and TCA cycle activity, leading to increased ATP production in mitochondria. The outcome is an enhanced cytoplasmic ATP to ADP ratio, which prompts closure of ATP-sensitive K^+ (K_{ATP}) channels in the plasma membrane evoking membrane depolarization, and subsequent opening of voltage-gated Ca^{2+} channels (Fig. 6.1). This culminates in an increase in cellular Ca^{2+} influx – a primary driver of the GSIS mechanism [1]. Ca^{2+} and vesicle docking and fusion events can also be modulated by agents acting through phospholipase C (PLC)/protein kinase C (PKC) or adenylate cyclase (AC)/protein kinase A (PKA) pathways as shown in Fig. 6.1.

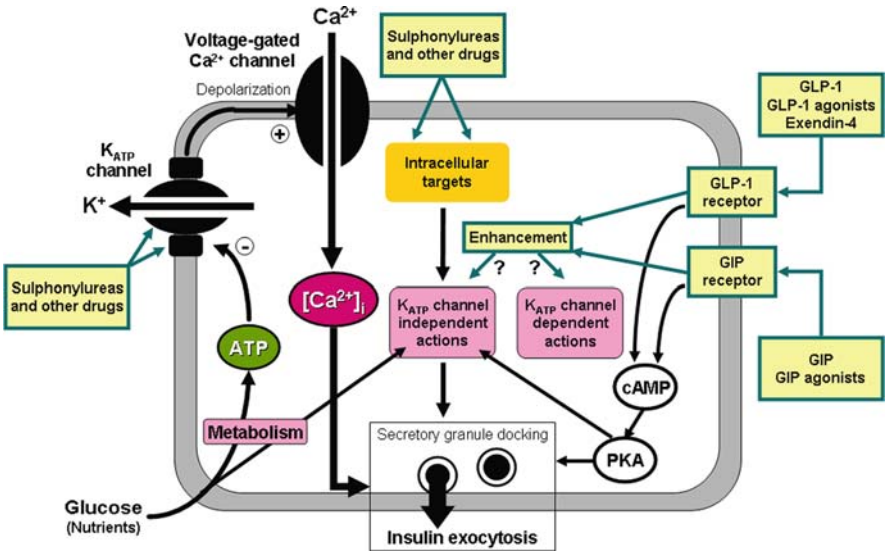

Fig. 6.2 Modulation of insulin secretion by insulinotropic drugs and incretins. In the presence of glucose, insulinotropic drugs (including the sulphonylureas), or neurohormonal signals (including the two incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) and autonomic innervation) can markedly affect insulin secretion via modulation of signal transduction and/or ion channel activity. Sulphonylurea drugs mainly act via promoting closure of the K_{ATP} channel and thus membrane depolarization. GLP-1 and GIP mediate their effects through G-protein-coupled receptors and associated signal transduction pathways which include serine/threonine kinase activation and phosphorylation of proteins associated with the molecular mechanism of exocytosis

Importantly, nutrients (including amino acids and lipids), insulinotropic drugs (including the sulphonylureas), or neurohormonal signals (including incretin hormones and autonomic innervation) can markedly affect glucose-stimulated insulin secretion (Fig. 6.2). Much interest has revolved around acute enhancement of β-cell function by the two incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), currently being hailed as important new therapeutics for type 2 diabetes (for review [2]). These two new classes of therapeutic agent could offer considerable advantages over sulphonylureas and other insulinotropic drugs, as their insulin-secretory action is glucose dependent [2]. The incretin mimetics may also play a role in maintaining β-cell mass in the hostile type 2 diabetes environment [2].

It is convenient to consider individual effects of different classes of nutrient or other physiological or pharmacological agents on metabolism and insulin secretion. However, given that β-cells are continually exposed to a complex milieu of nutrients and other circulating factors, it is important to also acknowledge and examine the interplay between glucose metabolism and that of the two other primary

nutrient classes, the amino acids and fatty acids. Cumulatively, it is the mixed nutrient sensing and outputs of glucose, amino and fatty acid metabolism that generate the metabolic coupling factors (MCFs) involved in signaling for insulin exocytosis [3, 4]. Primary MCFs in the β-cell include ATP, NADPH, glutamate, long-chain acyl-CoA and diacylglycerol and are discussed further below.

6.2 Phases and Pulsatility of Insulin Secretion

Tight regulation of insulin secretion is necessary for glucose homeostasis, where disturbances are associated with glucose intolerance and diabetes. However, glucose-stimulated insulin secretion is under stimulatory and inhibitory control by hormones and neurotransmitters and regular oscillations of circulating insulin in normal subjects can even occur without accompanying changes in plasma glucose – a response related to the so-called "pacemaker" function of the pancreas (for review [5]). Pulsatile insulin secretion from individual islets appears to follow a dominating pancreatic frequency, where a rhythmic variation in islet secretion is synchronized with oscillations in β-cell cytoplasmic Ca^{2+}. In clusters of β-cells exposed to intermediate stimulatory concentrations of glucose, synchronized oscillations can spread to silent cells as the glucose concentration is increased [5]. This demonstrates β-cell recruitment and intracellular coupling in glucose regulation of insulin secretion.

While foetal islets demonstrate a monophasic (first phase) secretory response, in mature adult islets insulin secretion occurs very rapidly after glucose administration, and is reported to occur with precise and biphasic kinetics (Fig. 6.3). Over the years there has been debate as to the underlying mechanisms regulating this biphasic

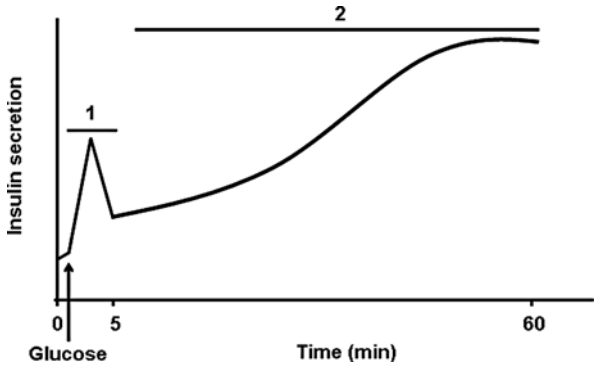

Fig. 6.3 Biphasic insulin secretion. Insulin secretion from islet β-cells occurs very rapidly after glucose administration, and is reported to occur with precise and biphasic kinetics. (i) First phase insulin secretion which is dependent on ATP generation and a rise in intracellular Ca^{2+}. (ii) Second phase insulin secretion which is dependent on mitochondrial metabolism and a rise in intracellular Ca^{2+}

pattern of insulin secretion, which has been proposed to involve at least two signaling pathways, the so-called K_{ATP} channel-dependent pathway, noted above, and another K_{ATP} channel-independent pathway. While both phases would appear to be critically dependent on Ca^{2+} influx, and can be modulated by various agents (including sulphonylureas and incretin hormones; Fig. 6.2), they affect different pools of insulin-secretory granules. It is understood that whereas the K_{ATP}-dependent pathway prompts exocytosis of an "immediately releasable pool" of granules that elicits and represents the first phase response, the K_{ATP} channel-independent pathway, working in synergy with the K_{ATP}-dependent pathway is responsible for the second phase response (see review [1]).

6.3 Primary Metabolic Factors Regulating Glucose-Stimulated Insulin Secretion

Clearly glucose metabolism plays a central role in the regulation of β-cell function, and glucose-derived carbons are understood to be metabolized following three main pathways generating MCFs for activation of insulin exocytosis: (i) glycolysis followed by TCA cycle-dependent glucose oxidation; (ii) anaplerosis; (iii) provision of glycerol-3-phosphate (Gly3P) for glycerolipid/fatty acid (GL/FA) cycling (see review [6]). While the first two of these pathways are linked to GSIS, there is less clarity regarding the latter, though Gly3P is believed to be incorporated into GL and GL/FA cycling which could produce lipid-signaling MCFs for insulin secretion.

Products of glucose metabolism can activate isoforms of PLC, promoting the generation of 1,4,5 inositol-triphosphate (IP_3) and a plasma membrane associated pool of diacylglycerol (DAG), a potent activator of specific isoforms of protein kinase C [4, 6], which can help mediate insulin-secretory granule trafficking and exocytosis [4, 6] (Fig. 6.1). Inositol-triphosphate (IP_3) stimulates Ca^{2+} efflux from the endoplasmic reticulum and increases Ca^{2+} concentration in the cytosol, also favouring activation of the secretory mechanism. Other glucose-derived MCFs such as LC-acylCoA and DAG can also amplify insulin secretion, and Gly3P metabolism through GL/FA cycling can produce nutrient-derived MCFs not dependent on mitochondrial metabolism. Various amino acids and their metabolic products may also impact on GSIS by a combination of enhancement of glucose oxidation, anaplerosis and direct plasma membrane depolarization effects [7–10].

Pancreatic β-cell glucose metabolism also increases arachidonic acid (AA) production, mainly by activation of phospholipase A_2[11]. The AA metabolites, prostaglandins (PGs) and leukotrienes, would appear to provide respective positive and negative modulation of glucose-stimulated insulin secretion [11]. While it would appear that free fatty acids do not stimulate insulin secretion in the absence of glucose, there is a substantial body of evidence that they are essential for GSIS [12]. Recent reports utilizing human islets suggest that non-esterified AA is critical for normal pancreatic β-cell function. Inhibition of the release of endogenous AA by

inhibiting PLA_2 activity resulted in a significant reduction of GSIS perhaps acting via G-protein-coupled receptor(s) [13].

Increases and oscillations in the intracellular Ca^{2+} concentration associated with the mechanism of GSIS can stimulate mitochondrial generation of ROS (via electron transport chain activity), whereas Ca^{2+}, via PKC activation and subsequent phosphorylation/translocation of the cytosolic regulatory subunit $P47^{phox}$, may enhance β-cell NADPH oxidase-dependent generation of ROS [14–16]. The O_2^- and H_2O_2 so produced acutely stimulates [17] but chronically induces inhibitory effects on β-cell metabolic pathways and can promote K_{ATP} channel opening with resulting inhibitory effects on insulin secretion [18].

Pancreatic β-cells have inherently relatively low levels of free radical detoxifying and redox-regulating enzymes, such as, glutathione reductase, glutathione peroxidase, catalase and thioredoxin, rendering them vulnerable to damage and destruction. The consequence of limited scavenging systems is that upon Ca^{2+} stimulation of mitochondrial and NADPH oxidase systems, ROS concentrations in β-cells may increase rapidly, and to high levels. Given this, the β-cell while utilizing necessary and positive aspects of ROS production for insulin production and release [17] is susceptible to the damaging effects of unregulated ROS generation and accumulation. The following sections give an overview of both positive actions of a range of important nutrient regulators of β-cells together with insights into mechanisms underlying nutrient-induced desensitization and toxicity associated with prolonged exposure.

6.4 Investigating Nutrient Regulation of β-Cell Metabolism and Insulin Secretion

Many insights into the mechanisms regulating insulin production and secretion have been gleaned from studies of freshly isolated islets, constituent β-cells and increasingly bioengineered β-cell lines. While early insulin-secreting cell lines, such as RINm5F and HIT-T15, represented rather crude β-cell models, advances in molecular biology and emerging bioengineering technologies offer considerable opportunities to improve and establish more appropriate clonal β-cells (see [19]). Indeed, bioengineered pancreatic β-cells, such as the popular glucose-responsive pancreatic BRIN-BD11 cells [20], have helped facilitate studies of the mechanisms of β-cell metabolism, insulin secretion, cell dysfunction and destruction. Combining the attributes of long-term functional stability of BRIN-BD11 cells with state-of-the-art NMR approaches have enabled the authors to unravel complexities, and provide novel insights into the relationships between glucose, fatty acid and amino acid handling and insulin secretion [7, 19, 21]. The following sections give a brief overview of the complex mechanisms regulating nutrient-stimulated insulin secretion and gene expression by pancreatic β-cells in response to various stimuli, utilizing isolated islet β-cells and other insulin-secreting cells.

6.5 Mechanisms Underlying β-Cell Actions of Glucose

As noted earlier, glucose is a primary physiological β-cell fuel, stimulating insulin secretion as a result of its metabolism and generation of MCFs. As illustrated in Fig. 6.4, after internalization through membrane-associated transporters, glucose is rapidly metabolized to pyruvate, following initial phosphorylation by glucokinase (GK) to glucose 6-phosphate, and subsequent glycolytic reactions. The third reaction in glycolysis is catalysed by phosphofructokinase (PFK), itself a key β-cell metabolic control site, and fluctuations in its activity result in oscillations in glycolytic flux [22–24]. The end product of glycolysis, pyruvate, is metabolized by either pyruvate dehydrogenase (PDH; the glucose oxidation pathway) or pyruvate carboxylase (PC; the anaplerosis/cataplerosis pathway) to acetyl-CoA or oxaloacetate, respectively, resulting in enhanced mitochondrial tricarboxycylic acid (TCA) cycle activity (Fig. 6.4).

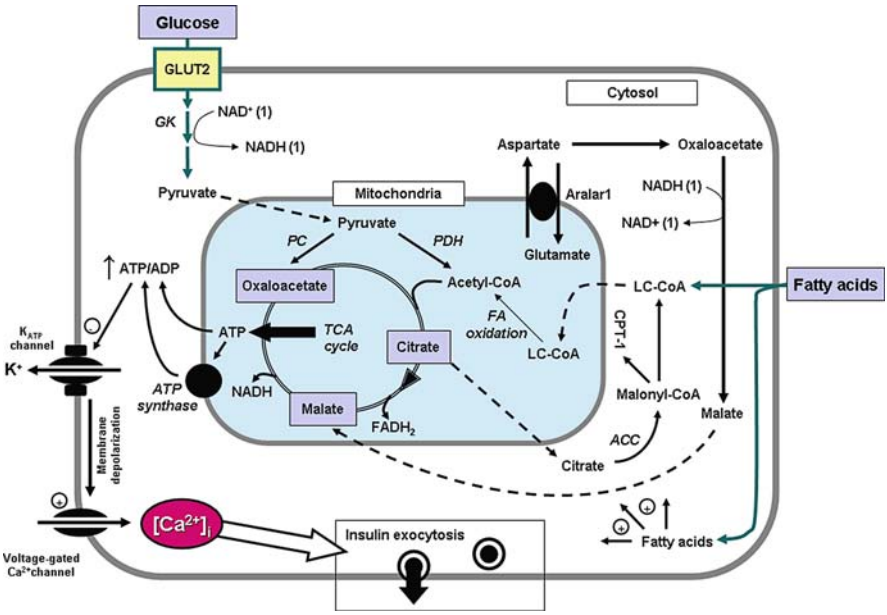

Fig. 6.4 Mechanisms of glucose and fatty acid enhanced mitochondrial activity and ATP generation. The end product of glycolysis – pyruvate – is metabolized by either pyruvate dehydrogenase (PDH; so committing glucose to the oxidation pathway) or pyruvate carboxylase (PC; so committing glucose to the anaplerosis/cataplerosis pathway). The products will be acetyl-CoA or oxaloacetate, respectively, which will contribute to enhanced mitochondrial tricarboxycylic acid (TCA) cycle activity. The malate–aspartate shuttle transfers cytosolic NADH to the mitochondrial matrix, a process which requires aspartate–glutamate exchange across the mitochondrial inner membrane by Aralar1. The generation of ATP and the increase in intracellular Ca^{2+} drives insulin secretion. Fatty acids may potentiate insulin secretion via generation of LC acyl-CoA and stimulation of signal transducing events

Among the enzymes responsible for glucose metabolism, GK, PC and PDH appear to play particularly important regulatory roles in the insulin-secretory pathway (Fig. 6.4). In β-cells, PC activity is high even though the cell does not participate in gluconeogenesis [25], which suggests this enzyme exerts anaplerotic functions. Note that β-cells lack phosphoenolpyruvate carboxykinase (an essential enzyme for gluconeogenesis, converting oxaloacetate to phosphoenol pyruvate) [26]. A recent study has highlighted that siRNA targeted to PC resulted in a reduction of insulin secretion from INS-1 cells [27], consistent with the observation that PC activity may be reduced in type 2 diabetes [28]. Conversely, overexpression of PC in INS-1 cells resulted in increased insulin release [27], again supporting an important role for this enzyme in the maintenance of GSIS. Interestingly, inhibition of PDH by overexpression of PDH kinase 4 in INS-1 cells did not result in a decrease of insulin secretion [27], although it is important not to over-interpret this observation by dismissing an important regulatory role of pyruvate dehydrogenase.

Transfer of electrons from TCA cycle to the mitochondrial electron transport chain is mediated by NADH and $FADH_2$ formation, resulting in ATP generation (Fig. 6.4). The increase in intracellular ATP to ADP ratio leads to the characteristic closure of K_{ATP} channels [29], membrane depolarization, opening of voltage-gated Ca^{2+} channels and rapid rise in intracellular Ca^{2+} concentration, leading to mobilization and ultimately fusion of insulin-containing granules with the plasma membrane and insulin release (Fig. 6.4) [30, 31]. The primary actions of glucose are mediated by potentiation of ATP concentration by enhanced TCA cycle substrate (oxidative and anaplerotic) supply. Generation of other additive factors derived from glucose metabolism might also be promoted by mitochondrial Ca^{2+} elevation [32].

Pyruvate may be converted in the β-cell to both acetyl-CoA and oxaloacetate, as discussed above (Fig. 6.4). A number of possibilities for mitochondrial metabolism of pyruvate exist: (i) generation of CO_2 via TCA cycle activity; (ii) export from the mitochondria as glutamate (due to 2-oxoglutarate conversion to glutamate via transamination or glutamate dehydrogenase activity); (iii) export from the mitochondria as malate to be converted back to pyruvate by $NADP^+$-dependent malic enzyme; and (iv) export from the mitochondria as citrate to be acted on by ATP citrate lyase and subsequently acetyl CoA carboxylase [33] to form malonyl-CoA which is an inhibitor of carnitine palmitoyl transferase-1 and thus an inhibitor of fatty acid oxidation (malonyl-CoA can subsequently be used for fatty acid synthesis via the action of fatty acid synthase).

One of these pathways, the so-called pyruvate-malate cycle, predicts a role for malate in insulin secretion via generation of the stimulus–secretion coupling factor NADPH [34]. Flow of the cycle requires oxaloacetate derived from pyruvate (via PC) to be converted to malate via a reversal of the malate dehydrogenase reaction, consuming NADH and generating NAD^+ in the mitochondrial matrix. Following this, malate is exported to the cytosol, converted to pyruvate via $NADP^+$-dependent malate dehydrogenase, generating NADPH. Glucose stimulation of β-cells or isolated rodent islet cells increases malate levels [35, 36] and while the workings of the malate–pyruvate cycle (recently reviewed [34]) are known, concerns have been raised as to the operation and impact of this cycle under physiologic conditions.

Normal TCA cycle activity ensures the malate → oxaloacetate direction of flux, thus generating NADH in the mitochondrial matrix. In addition, malate → oxaloacetate conversion forms part of the malate–aspartate shuttle, which has a high activity in the β-cell, and is essential for transfer of cytosolic NADH to the mitochondrial matrix (for review [37]). In β-cells, reducing equivalents may be transported to the mitochondrial matrix by either the glycerol–phosphate or the malate–aspartate shuttle [38]. Inhibition of the malate–aspartate shuttle by amino-oxyacetate (which acts on transamination reactions and inhibits cytosolic NADH reoxidation) has been demonstrated to attenuate the secretory response to nutrients, thus highlighting the dominance of this latter shuttle in the β-cell. Most recently Aralar1 (see Fig. 6.4), a mitochondrial aspartate–glutamate carrier which takes part in the malate–aspartate shuttle, has been demonstrated to play an important role in glucose-induced insulin secretion, as its deletion in INS-1 cells leads to a complete loss of malate–aspartate shuttle activity in mitochondria, and to a 25% decrease of insulin release in response to glucose [39].

One key constituent of the malate–aspartate NADH shuttle is the mitochondrial aspartate–glutamate transporter, with its two Ca^{2+}-sensitive isoforms, Citrin and Aralar1, expressed in excitatory tissues [40, 41]. However, Aralar1 is the dominant aspartate–glutamate transporter isoform expressed in β-cells [40], and the function of this transporter in the malate–aspartate shuttle is illustrated in Fig. 6.4. Adenoviral-mediated overexpression of Aralar1 in INS-1E β-cells and rat pancreatic islets enhanced glucose-evoked NAD(P)H generation, electron transport chain activity and mitochondrial ATP formation, and Aralar1 was demonstrated to exert its effect on insulin secretion upstream of the TCA cycle [40]. Indeed, the capacity of the aspartate–glutamate transporter appeared to limit NADH shuttle activity and subsequent mitochondrial metabolism. Thus, it is highly improbable that a malate–pyruvate cycle is active and important to insulin secretion, if the malate–aspartate shuttle is indeed a key component of stimulus–secretion coupling.

An alternative pyruvate-cycling pathway has been proposed, where generation of citrate from condensation of oxaloacetate (OAA) and acetyl-CoA occurs in the TCA cycle, followed by export of citrate from the mitochondria via the citrate–isocitrate carrier, cleavage of citrate by ATP citrate lyase to OAA and acetyl-CoA, and recycling to pyruvate via a cytosolic malate dehydrogenase and $NADP^+$-dependent malic enzyme [34]. In this proposed cycle, OAA to malate formation occurs in the cytosol, similar to the malate–aspartate shuttle. Acetyl-CoA can also serve a substrate for acetyl-CoA carboxylase, leading to formation of long-chain acyl-CoA accumulation in the cytosol via malonyl-CoA and in β-cells, glucose stimulation increases malonyl-CoA levels before insulin release [42], and addition of long-chain acyl-CoA results in a stimulation of insulin secretion [43].

However, the evidence used to refute a role of fatty acid synthesis is not compelling. Suppression of citrate lyase mRNA levels by 92% and citrate lyase protein levels by 75% by adenovirus-mediated siRNA delivery did not affect GSIS in 832/13 β-cells compared with cells treated with a control adenovirus [44]. Also, citrate lyase suppression in primary islet preparations using recombinant adenovirus technology to suppress citrate lyase expression by 65% reported no impact on GSIS

[44]. It is possible to reinterpret these findings if we consider that citrate lyase is expressed at very high levels in β-cells [45], so suppression of protein expression, even by 65–75%, would not be expected to be sufficient to alter the synthesis of key LC-acyl-CoA species.

6.6 Mechanisms Underlying β-Cell Actions of Lipids

Fatty acids appear to freely diffuse into pancreatic β-cells through the plasma membrane [46]. As illustrated in Fig. 6.4, inside β-cells, fatty acids are transformed in long-chain acyl-CoA, by acyl-CoA synthase (ACS), and enter the mitochondria via Carnitine Palmitoyl Transferase 1 (CPT-1), so β-oxidation can occur when glucose levels are low. The resulting acetyl-CoA is subsequently oxidized in the TCA cycle and under these conditions, ATP generation is sufficient for β-cell survival, and to maintain basal levels of insulin secretion (Fig. 6.4). When the extracellular glucose concentration is increased, fatty acid oxidation is inhibited, due to formation of malonyl-CoA by acetyl-CoA carboxylase [33]. Malonyl-CoA under glucose stimulatory conditions is derived from glucose carbon, via formation of citrate. Malonyl-CoA inhibits CPT-1, thus blocking transport of long-chain acyl-CoA into the mitochondria [47] (Fig. 6.4). Accumulation of long-chain acyl-CoA in the cytosol leads to an increase of intracellular Ca^{2+} levels and to changes in acylation state of proteins involved both in regulation of ion channel activity and exocytosis [48, 49]. In addition, long-chain acyl-CoA can also enhance fusion of insulin-secretory vesicles with plasma membrane and insulin release [43].

However, effects of fatty acids on glucose-induced insulin secretion are directly correlated with chain length and the degree of unsaturation, where long-chain fatty acids (such as palmitate or linoleate) *acutely* improve, but *chronically* reduce insulin release in response to glucose stimulation [4]. It is possible that chronic elevated synthesis of triacylglycerol species such as tripalmitin is detrimental to β-cell function due to adverse morphological changes [50] but it is more likely that apoptosis is triggered by lipid-specific signaling pathways and/or endoplasmic reticulum stress-activated pathways, so resulting in β-cell failure and death (reviewed in [4]). A recent study by the authors demonstrated that 24 hours culture of BRIN-BD11 cells with the polyunsaturated fatty acid, arachidonic acid, increased insulin secretion in response to the amino acid L-alanine. On the other hand, 24 hours exposure of BRIN-BD11 cells to saturated fatty acid palmitic acid in culture inhibited L-alanine-induced insulin secretion [51].

A recent advance in the understanding of the mechanism(s) by which non-esterified fatty acids (NEFAs) modulate insulin secretion in vivo was the discovery of high levels of expression of the membrane-bound G-protein-coupled receptor GPR40, a putative NEFA receptor in human and animal islet β-cell preparations [52]. GPR40 mRNA levels positively correlated with the insulinogenic index [52] and while the potential signaling mechanism(s) by which GPR40 regulates insulin secretion are still under investigation, it appears likely they involve changes in intracellular Ca^{2+} mobilization [12, 53, 54].

6.7 Mechanisms Underlying β-Cell Actions of Amino Acids

Under appropriate conditions, amino acids enhance insulin secretion from primary islet cells and β-cell lines [3, 7–10]. In vivo, L-glutamine and L-alanine are quantitatively the most abundant amino acids in blood and extracellular fluids, closely followed by the branched chain amino acids [55]. However, individual amino acids do not evoke insulin-secretory responses in vitro when added at physiological concentrations, rather, combinations of physiological concentrations of amino acids or high concentrations of individual amino acids are much more effective. In vivo, amino acids derived from dietary proteins and those released from intestinal epithelial cells, in combination with glucose, stimulate insulin secretion, thereby leading to protein synthesis and amino acid transport in target tissues such as skeletal muscle [11].

While amino acids can potentially affect a number of aspects of β-cell function, a relatively small number of amino acids promote or synergistically enhance insulin release from pancreatic β-cells [56, 57]. As illustrated in Fig. 6.5, the mechanisms by which amino acids enhance insulin secretion are understood to primarily rely on: (i) direct depolarization of the plasma membrane (e.g., cationic amino acid,

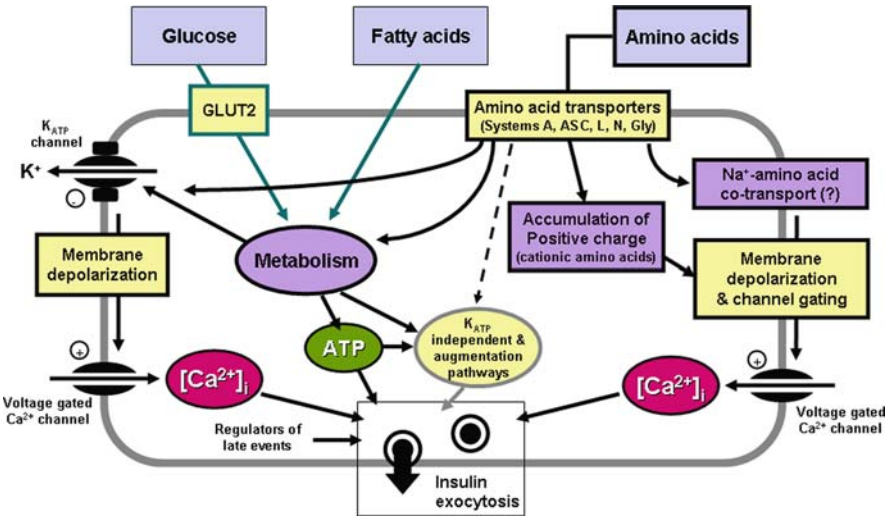

Fig. 6.5 Common mechanisms of nutrient-stimulated insulin secretion. Glucose metabolism is essential for stimulation of insulin secretion. The mechanisms by which amino acids enhance insulin secretion are understood to primarily rely on (i) direct depolarization of the plasma membrane (e.g., cationic amino acid, L-arginine); (ii) metabolism (e.g., alanine, glutamine, leucine); and (iii) co-transport with Na^+ and cell membrane depolarization (e.g., alanine). Notably, rapid partial oxidation may also initially increase both the cellular content of ATP (impacting on K_{ATP} channel closure prompting membrane depolarization) and other stimulus–secretion coupling factors. In the absence of glucose, fatty acids may be metabolized to generate ATP and maintain basal levels of insulin secretion

L-arginine); (ii) metabolism (e.g., glutamine, leucine); and (iii) co-transport with Na^+ and cell membrane depolarization (e.g., L-alanine). Notably, partial oxidation, e.g., L-alanine [7] may also initially increase the cellular content of ATP impacting on K_{ATP} channel closure prompting membrane depolarization, Ca^{2+} influx and insulin exocytosis. Additional mitochondrial signals that affect insulin secretion may also be generated (Fig. 6.6) [58, 59, 60], and in β-cells, the mTOR-signaling pathway acts in synergy with growth factor/insulin signaling to stimulate mitochondrial function and insulin secretion [61]. At present, the mechanism by which amino acids activate the mTOR complex has not been elucidated, but it is interesting to speculate involvement of kinase stimulation or inhibition of a phosphatase utilizing mTOR as a substrate [61–63].

Arginine: This amino acid stimulates insulin release through electrogenic transport into the β-cell via the mCAT2A amino acid transporter (Fig. 6.6), thereby increasing membrane depolarization, rise in intracellular Ca^{2+} through opening of voltage-gated Ca^{2+} channels and insulin secretion [64]. However, in some situations, arginine principally through its metabolism is understood to exert a

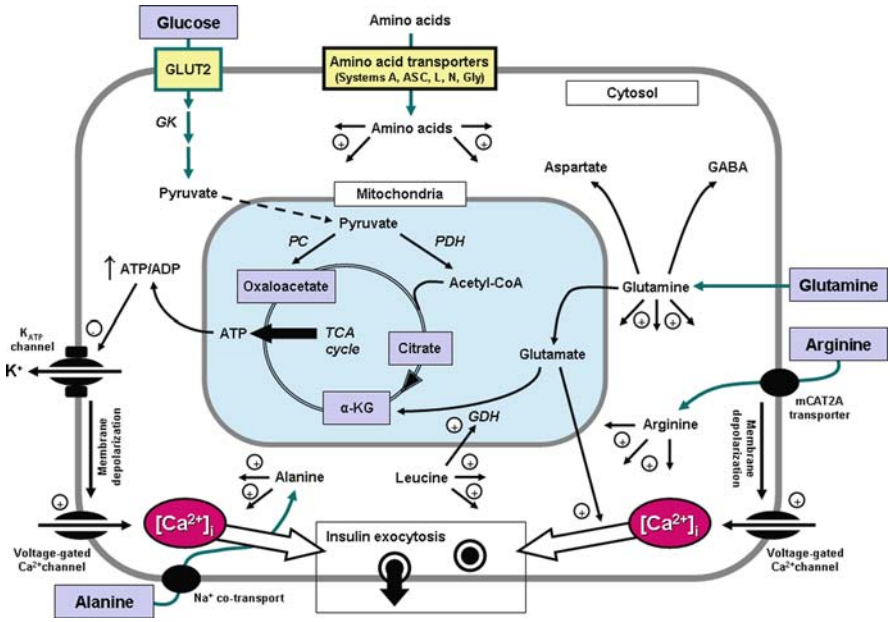

Fig. 6.6 Glucose, alanine, glutamine, leucine and arginine are the major nutrient drivers of insulin secretion. Metabolism of glucose, alanine and glutamine result in enhanced TCA cycle activity and generation of metabolic secretion coupling factors including ATP, Ca^{2+} and glutamate. Leucine may enhance glutamine oxidation via activation of glutamate dehydrogenase (GDH). Arginine may depolarize the plasma membrane by net import of positive charge thus causing opening of voltage-gated Ca^{2+} channels. The key sites of metabolic control in the β-cell are indicated; glucokinase (GK), pyruvate dehydrogenase (PDH), pyruvate carboxylase (PC), glutamate dehydrogenase (GDH)

negative effect on β-cell insulin release. The potentially detrimental effect of arginine metabolism hinges on arginine-derived nitric oxide (NO) through the action of inducible nitric oxide synthase (iNOS). High levels of NO are known to interfere with β-cell mitochondrial function and generation of key stimulus–secretion coupling factors, which could lead to a reduction in cellular insulin output.

Glutamine: Among the amino acids, glutamine is considered one of the most important, playing an essential role in promotion and maintenance of functionality of various organs and cells, including pancreatic β-cells [65]. Both rat islets and BRIN-BD11 cells consume glutamine at high rates [9], but notably while glutamine can potentiate GSIS and interact with other nutrient secretagogues, it does not initiate an insulin-secretory response [57]. In rat islets, glutamine is converted to γ-amino butyric acid (GABA) and aspartate (Fig. 6.6), and in the presence of leucine oxidative metabolism is increased. More recently, a potential glutamine synthetase inhibitor – methionine sulfoximide – was demonstrated to completely abolish GSIS in normal mouse islets [66], a phenomenon reversed by addition of glutamine or a non-metabolizable analogue. However, it is important to note that this inhibitor may block a number of glutamate-utilizing enzymes and so the outcome cannot be interpreted to arise as a result of a specific action on glutamine synthetase.

Glutamate: The ability of glutamate to stimulate insulin secretion and its actions in β-cells has been hotly debated. Intracellular generation of L-glutamate has been proposed to participate in nutrient-induced stimulus–secretion coupling as an additive factor in the amplifying pathway of GSIS [67]. During glucose stimulation, total cellular glutamate levels have been demonstrated to increase in human, mouse and rat islets, as well as clonal β-cells [7, 9, 67, 68], whereas other studies have reported no change [69, 70]. The observation that mitochondrial activation in permeabilized β-cells directly stimulates insulin exocytosis [32] pioneered the identification of glutamate as a putative intracellular messenger [67, 71]. However, in recent years, the role of L-glutamate in direct actions on insulin secretion has been challenged [70, 72]. For example, stimulatory (16.7 mM) glucose did not increase intracellular L-glutamate concentrations in rat islets in one study [70], and while L-glutamine (10 mM) increased the L-glutamate concentration tenfold, this was not accompanied by a stimulation of insulin release. In a separate study, incubation with glucose resulted in a significant increase in L-glutamate concentration in depolarized mouse and rat islets, but L-glutamine while increasing L-glutamate content did not alter insulin secretion [72]. Additionally, in this latter study, BCH-induced activation of GDH lowered L-glutamate levels, but increased insulin secretion. However, it is probable that experimental conditions in which L-glutamine is used as L-glutamate precursor may lead to saturating concentrations of L-glutamate without necessarily activating the K_{ATP}-dependent pathway and associated increase in insulin secretion [68]. It is likely that during enhanced glucose metabolism, the concentration of the key TCA cycle intermediate alpha-ketoglutarate (2-oxoglutarate) is elevated and a proportion of this metabolite is subsequently transaminated to glutamate

[21]. It is the opinion of the authors that the glutamate so formed may indirectly stimulate insulin secretion through additive actions on the malate–aspartate shuttle (as glutamate is a substrate for the mitochondrial membrane aspartate/glutamate carrier 1, thus may increase the capacity of the shuttle, see Fig. 6.4) or by contribution to glutathione synthesis (as glutamate is one of the three amino acids required for glutathione synthesis) and subsequent positive effects on cellular redox state and mitochondrial function (for further detail see [21]). As glutamate is not readily taken up into β-cells it is difficult to design robust experiments considering intracellular actions of this metabolizable nutrient. Indeed, glutamate release from β-cells has recently been reported [73], adding complexity to this story and offering the intriguing possibility of other β-cell actions, perhaps mediated through glutamate receptors which could influence insulin release. This and other aspects of β-cell glutamate signaling and actions are currently under investigation by the authors.

Leucine: Prolonged exposure of rat islets to leucine increases ATP, cytosolic Ca^{2+}, and potentiates glucose-stimulated insulin secretion. In addition, chronic exposure to leucine leads to an increase in both ATP synthase and glucokinase, which can sensitize pancreatic β-cells to glucose-induced insulin secretion [74]. Leucine-induced insulin secretion involves allosteric activation of glutamate dehydrogenase (GDH) leading to an increase in glutamine → glutamate → 2-oxoglutarate flux, elevated mitochondrial metabolism and an increase in ATP production leading to a membrane depolarization (Fig. 6.6). Additionally transamination of leucine to α-ketoisocaproate (KIC) and entry into TCA cycle via acetyl-CoA can contribute to ATP generation by increasing the oxidation rate of the amino acid and thus stimulation of insulin secretion. Moreover, it has been reported that α-keto acids (including KIC) can directly block K_{ATP} channel activity and exert additional K_{ATP} channel-independent effects thereby inducing insulin secretion [75, 76]. Notably, a recent study reported patients with mutations in the regulatory (GTP binding) site of GDH had increased β-cell responsiveness to leucine, presenting with hypoglycaemia after a protein rich meal [75, 77]. In addition, mice harbouring a β-cell-specific GDH deletion exhibit a marked decrease (37%) in glucose-induced insulin secretion, supporting an essential role of GDH in insulin release [78].

Alanine: Effects of L-alanine have been studied in BRIN-BD11 cells and primary rat islet cells, which consume high rates of this amino acid [9]. Moreover, L-alanine is known to potentiate GSIS by enhancing glucose utilization and metabolism [7], and numerous studies have highlighted L-alanine as a potent initiator of insulin release. The authors have utilized BRIN-BD11 cells to study the actions of L-alanine on β-cells demonstrating an influence on GSIS by electrogenic Na^+ transport, and exploited ^{13}C nuclear magnetic resonance technologies to trace L-alanine metabolism, demonstrating generation of glutamate, aspartate and lactate. Additionally, studies using the respiratory poison oligomycin indicate the importance of metabolism and oxidation of alanine for its ability to stimulate insulin secretion [7].

6.8 Overview of Nutrient Regulation of β-Cell Gene Expression

Glucose can impact on insulin secretion and pancreatic β-cell function by regulating gene expression, enabling mammals to adapt metabolic activity to changes in nutrient supply. In pancreatic β-cells, in addition to a fundamental role in the regulation of insulin secretion and pancreatic β-cell function, glucose serves as a principal physiological regulator of insulin gene expression [79]. Glucose is known to control transcription factor recruitment, level of transcription, alternative splicing and stability of insulin mRNA [80]. To cover all aspects of the diverse actions of glucose and other key nutrients on β-cell gene expression is certainly outside the scope of this chapter, but the following gives an overview of some notable aspects of this complex area of study.

In β-cells, three transcriptional factors bind to insulin promoter to regulate insulin gene expression: pancreatic and duodenal homeobox 1 (Pdx-1), neurogenic differentiation 1 (NeuroD1) and V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), acting in synergy and stimulating insulin gene expression in response to increasing plasma glucose [81]. However, consistent with detrimental β-cell actions of prolonged exposure to high glucose concentrations, impairments of Pdx-1 and MafA binding to the insulin promoter have been noted, in turn leading to decreased insulin biosynthesis, content and capacity for secretion. Similarly, prolonged exposure to high fatty acid levels can impair insulin gene expression, this time accompanied by an accumulation of triglycerides in β-cells – particularly palmitate – where the negative effect may be attributable to ceramide formation [82]. Moreover, palmitate is known to induce a decrease in binding activity of transcriptional factors on the insulin promoter, where both Pdx-1 translocation to the nucleus and MafA expression are affected [83].

An important role of amino acids on gene expression has recently been highlighted [84]. In an Affymetrix microarray study utilizing BRIN-BD11 cells, prolonged (24 hours) exposure to alanine and glutamine upregulated β-cell gene expression, particularly genes involved in metabolism, signal transduction and oxidative stress [85, 86]. This upregulation could be due to alanine metabolism, provision of amino acid stimulus–secretion coupling factors and lipid metabolites (such as long-chain acyl-CoAs), and leading to an alteration of cellular redox state [7, 9]. Interestingly, 24 hours exposure of BRIN-BD11 cells to glutamine strongly increased calcineurin catalytic and regulatory subunit mRNA expression [86] and this Ca^{2+}-binding protein has been reported to play a role in the somatostatin-induced inhibition of exocytosis in mouse pancreatic β-cells [87]. Glutamine can also increase Pdx-1 and acetyl-CoA carboxylase mRNA expression. Of the amino acids, alanine and glutamine appear to play particularly important roles in the regulation of gene expression [84, 86] and further study of the precise mechanisms underlying these actions should help understanding of β-cell responses to nutrient supply, metabolism and secretory and functional integrity.

6.9 Nutrient-Induced β-Cell Desensitization, Dysfunction, and Toxicity

Persistently elevated fuel supply such as glucose, amino acids, fatty acids (or a mixture) is known to exert detrimental effects on a number of cells, and can induce insulin resistance in muscle – perhaps as a first line protective adaptation to fuel overload [88]. The β-cell does not protect itself by blocking uptake of excess nutrients and thus is vulnerable to potential excess activation of mitochondrial metabolism, ROS production, elevated intracellular Ca^{2+} and cell injury [6, 16, 89]. While expansion of β-cell mass can offer part of a compensatory response, desensitization may also help reduce the burden on β-cells. Desensitization is commonly observed in eukaryotic cells, is believed to have an underlying role in cell protection [19], and may be defined as a readily induced and reversible state of cellular refractoriness attributed to repeated or prolonged exposure to high concentrations of a stimulus.

While acute exposure to glucose generally promotes increased metabolism and generation of MCFs, as well as changes in insulin gene transcription and translation, chronic exposure to high levels of this sugar has been associated with β-cell deterioration, with glucose desensitization in the first instance progressing to glucotoxicity likely arising from oxidative stress. Likewise, while the acute β-cell actions of fatty acids are usually positive, chronic exposure can exert substantive changes to nutrient metabolism and so-called lipotoxicity, and both the hyperglycaemia and hyperlipidemia of diabetes can alter insulin secretion and β-cell function. However, while experimental glucotoxicity and lipotoxicity can be independently demonstrated, it is clear that these two are interrelated adverse forces on the β-cell [47]. Some characteristics of this so-called "glucolipotoxicity" [47] are: (i) impaired glucose oxidation, resulting in ACC inhibition (due to an increase in cellular AMP levels as ATP generation decreases, subsequent activation of AMP kinase, and phosphorylation of ACC, so inhibiting generation of malonyl-CoA and LC acyl-CoA), (ii) promotion of fatty acid oxidation due to relief of CPT-1 inhibition and (iii) enhanced FFA esterification and lipid accumulation with respect to the excess FFA that are not oxidized. These combined effects lead to a decrease in glucose-induced insulin secretion, impaired insulin gene expression and an increase in β-cell failure and even cell death [4].

Although mechanisms by which chronic exposure to high levels of glucose and/or lipids damage β-cells have been the subject to intense clinical and experimental investigation, much less attention has been directed to other diet-derived factors, including the other major nutrient class, the amino acids. As noted earlier, prolonged exposure to amino acids such as alanine or glutamine may (at least in the first instance) upregulate gene expression of certain metabolic and signal transduction elements, and can also offer enhanced protection against cytokine-induced apoptosis [85]. However, these primary observations also indicated an alteration in

β-cell responsiveness, later studied by the authors in more detail [90]. These latter studies demonstrate for the first time that the desensitization phenomenon previously reported with other pharmacological and physiological agents (see review [19]) may extend to the amino acids, where 18 hours exposure to L-alanine resulted in reversible alterations in metabolic flux (a reduction in flux), Ca^{2+} handling (reduced level of intracellular Ca^{2+}) and insulin secretion (reduction in insulin secretion).

More intriguing evidence for detrimental β-cell actions of amino acids relate to the reported effects of acute and chronic exposure to homocysteine [91, 92]. Interestingly, elevated circulating homocysteine and hyperhomocysteinemia have emerged as important risk factors for cardiovascular disease and other diseases of the metabolic syndrome, including type 2 diabetes. Studies of prolonged effects of alanine and homocysteine in the authors' laboratories represent compelling evidence for the existence of β-cell amino acid desensitization. While these data prompt further study, it is interesting to speculate that nutrient-induced desensitization may be a first line compensatory mechanism to over-nutrition. However, if observations on the "toxic" effects of glucose/lipids also extend to amino acids, this would support the view that prolonged over-nutrition generally results in adverse β-cell events which may contribute to the pathogenesis of diabetes.

6.10 Conclusion

Pancreatic β-cells are well equipped to respond as metabolic fuel sensors, and additionally possess inherent mechanisms to adapt to nutrient overconsumption in order to preserve glucose homeostasis. Glucose signaling is of primary importance in the β-cell, and as discussed both fatty acids and amino acids can interface with central signaling pathways to help regulate insulin secretion. Inherently, the metabolic sensing ability of the β-cell comes at the expense of its protection and islet β-cells are more vulnerable than other cells in the body to excess fuel supply. However, as illustrated in Fig. 6.7, β-cells play a key role in countering nutrient over-consumption through hyperinsulinemia and β-cell expansion as initial attempts to curb the characteristic hyperglycaemia of impaired glucose tolerance (IGT) and type 2 diabetes. Ultimately it is the interplay between nutrient handling by β-cells and other insulin-sensitive cells such as skeletal muscle, adipocytes and liver that dictates whole body nutrient homeostasis (Fig. 6.7). It would seem that β-cell failure due to excess nutrients is dominant in the pathogenesis of type 2 diabetes with a significant underlying genetic or environmental susceptibility defect, contributing to the process [6]. However, the alarming epidemic rise in diabesity only serves to highlight how precious and important β-cells are to the maintenance of whole body metabolism. This also prompts further efforts to understand the complexities of β-cell function, demise and destruction, and indeed novel targets and treatments for diabetes, obesity and the metabolic syndrome.

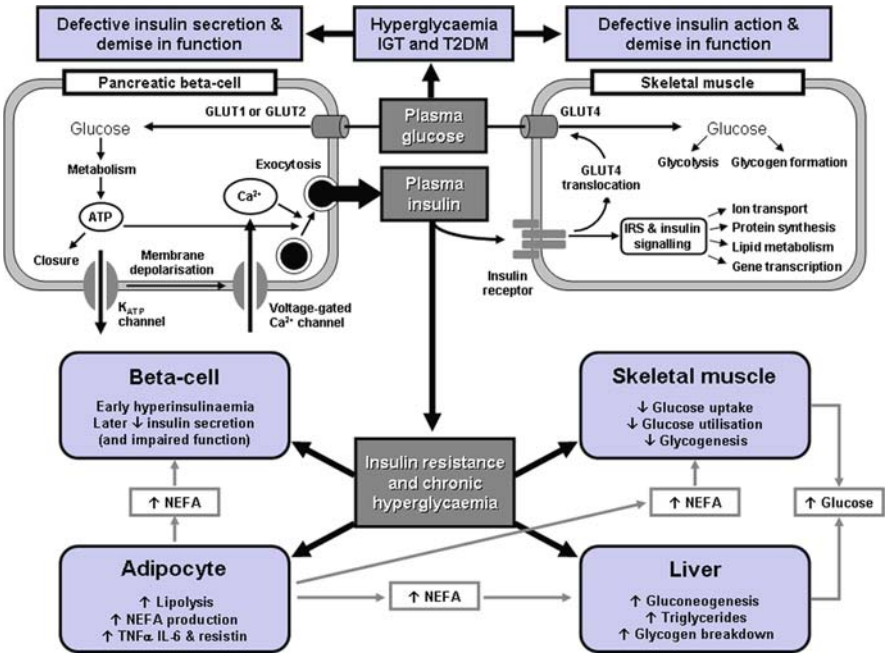

Fig. 6.7 Interplay between β-cells and insulin-sensitive tissues in the pathogenesis of type 2 diabetes. Key interplay between nutrient handling by insulin secreting β-cells and insulin-sensitive cells such as skeletal muscle, adipocytes and liver regulates whole body nutrient homeostasis. Defective insulin secretion (due to excessive nutrient-induced desensitization of the β-cell, see main text) will result in high plasma levels of glucose. Insulin resistance in muscle and adipose tissue will result in reduced glucose uptake. Insulin resistance in the liver will result in enhanced glucose release into the blood, compounding hyperglycaemia. Insulin resistance in the adipose tissue will result in elevated fatty acid release and pro-inflammatory factor release, contributing to insulin resistance due to impairment of insulin-signaling pathways and also reduced insulin secretion from the β-cell due to impairment of regulation of nutrient metabolism

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Chapter 7
Electrophysiology of Islet Cells

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Abstract Stimulus-Secretion Coupling (SSC) of pancreatic islet cells comprises electrical activity. Changes of the membrane potential (V_m) are regulated by metabolism-dependent alterations in ion channel activity.

This coupling is best explored in β-cells. The effect of glucose is directly linked to mitochondrial metabolism as the ATP/ADP ratio determines the open probability of ATP-sensitive K^+ channels (K_{ATP} channels). Nucleotide sensitivity and concentration in the direct vicinity of the channels are controlled by several factors including phospholipids, fatty acids, and kinases, e.g., creatine and adenylate kinase. Closure of K_{ATP} channels leads to depolarization of β-cells via a yet unknown depolarizing current. Ca^{2+} influx during action potentials (APs) results in an increase of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) that triggers exocytosis. APs are elicited by the opening of voltage-dependent Na^+ and/or Ca^{2+} channels and repolarized by voltage- and/or Ca^{2+}-dependent K^+ channels. At a constant stimulatory glucose concentration APs are clustered in bursts that are interrupted by hyperpolarized interburst phases. Bursting electrical activity induces parallel fluctuations in $[Ca^{2+}]_c$ and insulin secretion. Bursts are terminated by I_{Kslow} consisting of currents through Ca^{2+}-dependent K^+ channels and K_{ATP} channels. This review focuses on structure, characteristics, physiological function, and regulation of ion channels in β-cells. Information about pharmacological drugs acting on K_{ATP} channels, K_{ATP} channelopathies, and influence of oxidative stress on K_{ATP} channel function is provided. One focus is the outstanding significance of L-type Ca^{2+} channels for insulin secretion. The role of less well characterized β-cell channels including voltage-dependent Na^+ channels, volume sensitive anion channels (VSACs), transient receptor potential (TRP)-related channels, and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels is discussed. A model of β-cell oscillations provides insight in the interplay of the different channels to induce and maintain electrical activity.

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Regulation of β-cell electrical activity by hormones and the autonomous nervous system is discussed.

α- and δ-cells are also equipped with K_{ATP} channels, voltage-dependent Na^+, K^+, and Ca^{2+} channels. Yet the SSC of these cells is less clear and is not necessarily dependent on K_{ATP} channel closure. Different ion channels of α- and δ-cells are introduced and SSC in α-cells is described in special respect of paracrine effects of insulin and GABA secreted from β-cells.

7.1 β-cells

7.1.1 Ion Channels

7.1.1.1 K_{ATP} Channels

Structure/Assembly – Protein Networks

K_{ATP} channels are hetero-octamers consisting of sulfonylurea receptors (SUR1, SUR2A, and B) and K_{IR} channels, either $K_{IR}6.1$ or $K_{IR}6.2$ [1–5]. β-cell K_{ATP} channels are composed of four $K_{IR}6.2$ subunits that form the pore and four regulatory SUR1 subunits (Fig. 7.1) [2, 6–8].

The $K_{IR}6.2$ Subunit

The $K_{IR}6.2$ subunit is a member of the inward rectifier superfamily. This subunit consists of two membrane spanning helices, M1 and M2, which are linked by a pore-loop containing the pore helix (Fig. 7.1A and [9]). A sub-membrane positioned "slide helix" (Fig. 7.1A) may provide the link between SUR1 and $K_{IR}6.2$, which affects gating of the channel [10]. Four $K_{IR}6.2$ subunits are necessary to establish a pore [9, 11, 12]. Usually only truncated $K_{IR}6.2$ proteins ($K_{IR}6.2\Delta C26$) are able to form a functional K_{ATP} channel in the absence of SUR subunits [13] because of retention of unassembled SUR1 and $K_{IR}6.2$ subunits in the endoplasmic reticulum [14]. However, full length $K_{IR}6.2$ protein can result in functional channels in insect cells using a baculovirus system [15]. Inhibition by ATP is the most prominent characteristic of K_{ATP} channels. ADP can substitute ATP in the absence of Mg^{2+}, however, with a tenfold lower potency. Biochemical studies have shown that the ATP binding site is located on the large C-terminus of $K_{IR}6.2$ [16–19]. Nevertheless, truncation of the $K_{IR}6.2$ N-terminus [20, 21] affects the ATP sensitivity.

The SUR1 Subunit

The SUR1 subunit is a typical ABC protein consisting of two bundles of six transmembrane helices (TMD1 and 2) with two cytosolic nucleotide-binding domains (NBD1 and 2, respectively) [22]. A bundle of five transmembrane helices (TMD0)

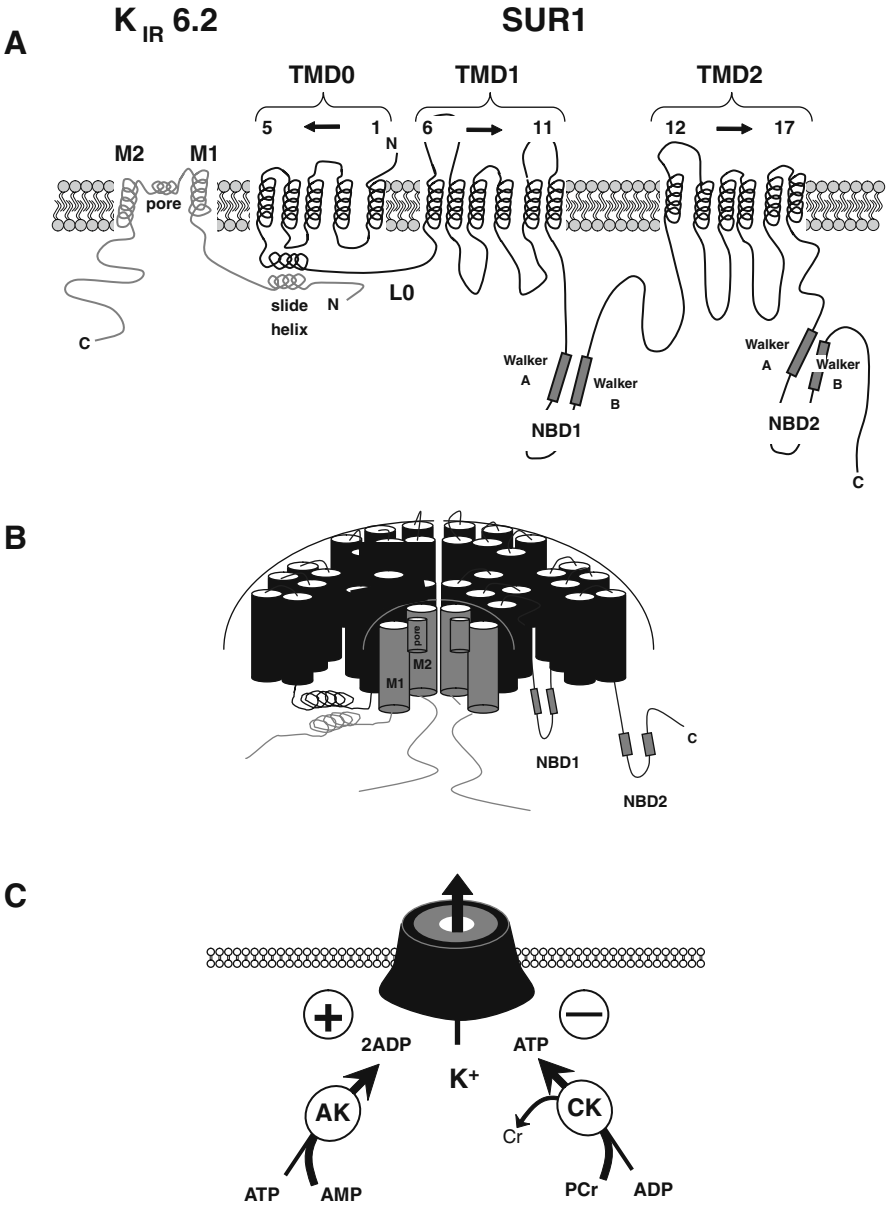

Fig. 7.1 Topology and homology model of the β-cell K_{ATP} channel and regulating enzymes. **A**) The $K_{IR}6.2$ (grey) and SUR1 (black) topologies are illustrated schematically. The amino (N) and carboxyl (C) termini are marked. The Walker A and B consensus motifs are shown in the two nucleotide binding domains (NBD1 and 2), respectively. The three transmembrane domains (TMD0-2) contain 5–6 transmembrane helices each (1–17). **B**) The assembly of four subunits (half of a channel) of the hetero-octamer is shown. The complete channel consists of four $K_{IR}6.2$ pore-forming subunits surrounded by four regulating SUR1 subunits. **C**) A creatine kinase (CK) and an adenylate kinase (AK) regulate the K_{ATP} channel negatively and positively, respectively. Both enzymes determine the nucleotide concentrations in the vicinity of the channel

together with its linker "L0" completes the subunit at the N-terminus (Fig. 7.1A). The TMD0-L0 area interacts with the pore-building $K_{IR}6.2$ subunit to regulate the channel activity [5, 21, 23–26]. Both NBDs of SUR1 contain Walker A and B motifs (Fig. 7.1A) which are directly involved in nucleotide binding and modulation of channel activity [27, 28]. The NBDs are the binding sites for MgADP which activates the channel [28, 29], thus having an important role in the metabolic regulation of the channel [30]. The NBDs also bind ATP. While NBD1 is a high-affinity nucleotide-binding site, NBD2 binds MgATP (or MgADP) with low affinity and has intrinsic ATPase activity [27, 31]. K_{ATP} channel activation occurs by cooperative interaction of MgATP (or ATP) bound at NBD1 and MgADP bound at NBD2 [27, 32]. ATP-induced inhibition of K_{ATP} channels, however, occurs via Mg^{2+}-independent binding to the $K_{IR}6.2$ subunit (see above).

The SURs contain the binding sites not only for the K_{ATP} channel blocking sulfonylureas such as tolbutamide, glibenclamide, or glimepiride but also for other hypoglycemic agents, e.g., the glinides, and K_{ATP} channel openers (for details see Section "Effects of Drugs").

Usually, the assembly of $K_{IR}6.2$ with SUR1 subunits (Fig. 7.1B) is required so that only complete hetero-octamers can yield functional K_{ATP} channels [30]. Unassembled SUR1 and $K_{IR}6.2$ proteins do not leave the endoplasmic reticulum (ER) due to an exposed ER retention/retrieval signal [14]. Therefore, the knock-out of SUR1 (SUR1KO) [33] and $K_{IR}6.2$ ($K_{IR}6.2$KO) [34] abolishes the appearance of K_{ATP} channels in the plasma membrane of β-cells [33, 35].

K_{ATP} channels in pancreatic β-cells are not restricted to the plasma membrane but can also be found in membranes of cellular organelles such as secretory vesicles [36, 37]. However, the protein in organelles may differ from the protein in the plasma membrane. The functional significance of vesicular K_{ATP} channels or the vesicular SUR1 subunit has to be established [37].

Protein Complexes

The K_{ATP} channel of the β-cell plasma membrane cannot be seen as a solitary functioning protein but is part of a protein network that regulates the channel activity. These interactions comprise exocytotic proteins such as syntaxin-1A which is supposed to directly inhibit K_{ATP} channels [38]. Another example is the association with the cAMP sensing protein cAMP-GEFII [39]. It is proposed that the SUR1 subunits of the plasma membrane K_{ATP} channel together with cAMP-GEFII and Rim2, a protein promoting vesicle priming in neurons [40], interact with SUR1 proteins in the granules. This protein complex controls Cl^- influx into the vesicles and may promote granule priming [39]. This is in accordance with the observation that the cAMP-mediated potentiation of insulin secretion is impaired in SUR1KO mice [41].

Very important for the proper regulation of the K_{ATP} channels by mitochondria-derived nucleotides is the link to enzymes that regulate the nucleotide concentrations in a micro-domain in the close vicinity of the channel (Fig. 7.1C). Fine-tuning of the electrical activity of the β-cell according to actual glucose concentrations cannot

be achieved by changing the bulk ATP concentration of the cell. Therefore, a "barrier" is needed to insulate the K_{ATP} channels from the cellular ATP concentration. A "metabolic barrier" may consist of enzymes like the adenylate kinase and the creatine kinase that are tightly associated with the K_{ATP} channels and regulate the actual nucleotide concentrations directly at the channel (see Section "Regulation by Metabolism-Derived Nucleotides and Phosphotransfer").

Electrophysiological Characteristics

The first measurements of single β-cell K_{ATP} channel currents were performed in 1984 by Cook and Hales [42] in inside/out patches where they could show the inhibition by cytosolic ATP. In the same year Ashcroft et al. [43] demonstrated the glucose-induced inhibition of so-called g-channels in metabolically intact β-cells with cell-attached patches. Both publications revealed a conductance of about 50 pS in symmetrical 140 mM K^+ concentration and the inward rectification of the I/V curves. The rectification is due to small blocking cations like Na^+ and not an intrinsic voltage dependency of the channel. In addition, different internal blockers of K_{ATP} channels (ATP, Na^+, Ca^{2+}, Mg^{2+}) interfere with different transitions in channel open and close times, and thus modulate the characteristic intrinsic bursts of channel openings [44–46].

Regulation by Metabolism-Derived Nucleotides and Phosphotransfer

Inhibition by ATP

The predominant characteristic of K_{ATP} channels is the inhibition by ATP derived from glucose metabolism. However, the IC_{50} value for K_{ATP} channel inhibition by ATP is in the range of 5–25 μM [42, 47, 48], whereas the cytosolic ATP concentration ($[ATP]_c$) amounts to 3–5 mM [48, 49]. This means that K_{ATP} channels would be permanently closed if they really sense $[ATP]_c$. Many attempts have been made to explain this paradox: It was speculated that ATP consuming pumps like the Na^+,K^+-ATPase build up an ATP gradient between the sub-membrane space and the cytosol [50] tremendously lowering [ATP] in the vicinity of the channels. Long-chain acyl-CoAs were found to regulate K_{ATP} channels positively [51–54] as well as phosphoinositides, in particular PIP_2 [55–60]. However, these mechanisms are not sufficient to explain the coupling between glucose metabolism and K_{ATP} channel activity.

Phosphotransfer in β-Cells

The breakthrough in understanding the coupling of metabolism to V_m came in 1994 when Dukes and co-workers [61] found that solely ATP derived from reduction equivalents produced in glycolysis is used to regulate K_{ATP} channels. It became clear that the β-cell senses the actual glucose concentration by registering the rate of the glycolytic flux and transfers this signal to the membrane.

Glycolytic reduction equivalents are shuttled into the mitochondria via the malate–aspartate and the glycerol phosphate shuttle systems. Suppression of these shuttle systems completely inhibits glucose-induced insulin secretion again demonstrating that only ATP derived from these glycolytic reduction equivalents is able to influence K_{ATP} channel activity [62]. This concept also explains why pyruvate is not a primary secretagogue in β-cells [63, 64]. Obviously, the metabolism of pyruvate and the reduction equivalents generated in the citric acid cycle are used to produce the bulk $[ATP]_c$ which is needed for the energy demands of the cell, e.g., insulin synthesis or Ca^{2+} sequestration. Reports suggesting that anaplerotic feeding of the citric acid cycle markedly influences K_{ATP} channel activity are often based on experiments with lipophilic pyruvate derivatives, e.g., methyl pyruvate [65] or with α-ketoisocaproate (KIC) [66]. However, since it has been shown that these agents directly inhibit K_{ATP} channels [67–69], the stimulatory effects are rather a consequence of direct interactions than of mitochondrial metabolism.

The discrimination between particular ATP molecules used for either K_{ATP} channel inhibition or energy demands of the cells may be achieved via specialized electron transport chains. Those chains delivering ATP exclusively to the channels may on one side be physically linked to the shuttle systems that transfer glycolytic reduction equivalents to the mitochondrial matrix. On the other side the F_1/F_0-ATPase of these chains may be tightly coupled to a mitochondrial creatine kinase (CK) [70] that directly conveys the energy-rich phosphate from the generated matrix ATP to cytosolic creatine to form phosphocreatine. Phosphocreatine can be channeled through enzyme systems to the plasma membrane where it is reconverted to ATP and creatine by a membrane-associated CK (Fig. 7.1C and [48, 71, 72]). In heart cells, the physical association between CK and K_{ATP} channels has been proven [73]. Recently the model of a "metabolic barrier" (see Section "Structure/ Assembly – Protein Networks") was evolved, whereby ATP producing and consuming enzymes like CK and adenylate kinase (AK) determine the ATP concentration in the direct vicinity of the channels and shield the channels against the bulk cellular ATP [47, 74]. This model is strongly supported by the finding that the ATP sensitivity of K_{ATP} channels is reduced in permeabilized cells (open-cell attached configuration) with efficient enzyme activity compared to excised inside/out patches [47, 48]. For human β-cells the physical association between adenylate kinase and K_{ATP} channels has been shown [74]. The AK activity may even be intrinsic to the SUR1 subunit of the K_{ATP} channel as suggested by Tarasov and co-workers [48]. The "metabolic barrier" model (Fig. 7.1C) can explain why K_{ATP} channels in β-cells are operative despite the high cellular bulk ATP concentration in the millimolar range [49].

Role of K_{ATP} Channels in β-Cell Stimulus-Secretion Coupling

Among all ion channels that are operative in pancreatic β-cells, K_{ATP} channels play a predominant role for regulation of cell activity as they couple nutrient metabolism to membrane depolarization and finally to adequate insulin secretion.

It is known since 1978 that under resting conditions the membrane potential of β-cells is mainly dependent on the K^+ permeability of the plasma membrane [75, 76] which is mediated by K_{ATP} channels. The decrease in the open probability of K_{ATP} channels is closely correlated with rising glucose concentrations [43, 77]. In response to increased ATP synthesis closure of K_{ATP} channels leads to a gradual decrease of K^+ conductance so that a yet unknown depolarizing current prevails which depolarizes the plasma membrane [78–81]. At approximately −50 mV the threshold for opening of voltage-dependent Ca^{2+} channels is reached and action potentials appear. The following increase in $[Ca^{2+}]_c$ constitutes the triggering signal for exocytosis [82]. In response to continuous glucose stimulation, β-cells display a characteristic pattern of electrical activity, the so-called "slow waves." It is now generally accepted that fluctuations in the K_{ATP} current are a key event for generation of the oscillatory activity. For detailed discussion of this point see Section "A Model for β-Cell Oscillations".

In summary, K_{ATP} channels have several indispensable functions for the β-cell, i.e., determination of resting membrane potential, initiation of membrane depolarization in response to nutrient stimulation, mediation of the close coupling between increasing glucose concentration and electrical activity, and finally K_{ATP} channels are a key regulator of membrane potential oscillations.

Effects of Drugs

K_{ATP} Channel Blockers

K_{ATP} channel inhibitors, i.e., sulfonylureas and glinides, are drugs frequently used to enhance insulin secretion in type 2 diabetics. The ability of tolbutamide to depolarize the membrane potential V_m in islet cells was first shown by Dean and Matthews [83], and inhibitory action of tolbutamide on single channel K_{ATP} currents was reported by Trube et al. [84]. As expected from K_{ATP} channel inhibitors, sulfonylureas effectively depolarize β-cells even in the absence of glucose. However, in the complete absence of fuels, they cannot imitate the oscillatory pattern characteristic for physiological nutrient stimulation [85].

Sulfonylureas

Sulfonylureas stabilize the closed state and reduce the duration and frequency of the bursts of K_{ATP} channel openings [86]. Sulfonylureas have no effect on single channel conductance [84]. PIP_2 and acyl-CoA derivatives that induce mechanisms promoting channel opening reduce the maximal sulfonylurea block [87–89], whereas intracellular MgADP enhances the inhibitory potency of sulfonylureas [90]. This contrasts to what is expected from a nucleotide that stimulates channel activity. However, there is evidence that sulfonylureas prevent the activating action of MgADP via SUR1, and thereby the inhibitory effect of the nucleotides (MgADP,

ADP, and ATP) at $K_{IR}6.2$ is unmasked [91, 92]. It has been suggested that sulfonylurea binding to one of the four SUR1 subunits is enough to induce channel closure [93].

High-affinity binding sites for sulfonylureas were first identified in the 1980s [94, 95] long before the cloning of SUR1 in 1995 [22]. They are located within the C-terminal site between transmembrane segment TM 13–16 of SUR1 (Fig. 7.1A). For the sulfonylurea glibenclamide that contains a benzamido moiety, TM 5 and 6 also influence drug binding [96]. High-affinity binding of tolbutamide to SUR1 is completely abolished when serine 1237, which is positioned in the intracellular loop between TM 15 and 16 (Fig. 7.1A), is replaced by tyrosine. Sulfonylureas also bind to pancreatic K_{ATP} channels by a low-affinity binding site which is located on $K_{IR}6.2$ [91].

Glinides

The so-called "glinides" summarize drugs of two structurally different classes: the D-phenylalanine derivative nateglinide and the carbamoylbenzoic acid derivative repaglinide. Nateglinide is the first compound lacking a sulfonylurea and benzamido moiety, respectively, that exhibits a mode of action similar to tolbutamide. K_{ATP} channel inhibition is achieved by high-affinity binding to SUR1 which can be prevented by the S1237Y mutation [97, 98]. In contrast this mutation does not abolish channel inhibition by repaglinide which suggests that repaglinide interacts with different regions located on SUR1 [98, 99] that require functional coupling to $K_{IR}6.2$ for high-affinity binding [100]. Analogous to sulfonylureas, the inhibitory potency of both drugs is enhanced by MgADP [97, 101].

K_{ATP} Channel Openers

The hyperglycemic sulfonamide diazoxide effectively hyperpolarizes the β-cell membrane potential and counteracts glucose-stimulated insulin release by opening of K_{ATP} channels [84, 102, 103]. Diazoxide stimulation requires Mg^{2+} and hydrolyzable ATP and is suggested to act by stabilizing the open state of the channel. In the absence of ATP, ADP is necessary for the enhancement of channel activity by diazoxide [104–106]. The regions that are important for ADP-dependent activation contain the second nucleotide-binding fold and the C-terminal site of SUR1 [107]. It has been suggested that binding sites essential for diazoxide action also include TM 6–11 (Fig. 7.1A) and the first nucleotide-binding fold [108]. The activating potency of diazoxide is modified by PIP_2 consistent with the idea that the potency of K_{ATP} channel openers is not a fixed parameter but depends on the open-state stability of the channel [88].

Diazoxide is the only K_{ATP} channel opener that is successfully used for therapy of hyperinsulinism or inoperable insulinoma (compare Section "Role in Diseases" [109]).

Efforts have been made to use SUR1-specific K_{ATP} channel openers for protection of β-cells from cytokine- or ROS-induced cell damage [110, 111]. However,

up to now it is unclear whether the protective mechanism requires K_{ATP} channel opening to put β-cells at rest or results from a direct depolarizing effect on β-cell mitochondria [112].

Influence of Oxidative Stress

β-cell damage due to the attack of reactive oxygen or nitrogen species (ROS or RNS) is known to contribute to gluco- and lipotoxicity in the development of diabetes. Furthermore, the procedures to isolate functional islets for islet transplantation are complicated by the negative influence of oxidative stress during the isolation process.

Among a variety of deleterious effects, direct and indirect interactions with K_{ATP} channels are an important pathway by which ROS and RNS, respectively, impair β-cell function and inhibit glucose-stimulated insulin secretion [113, 114].

Alterations in electrical activity or K_{ATP} channel current in the presence of hydrogen peroxide (H_2O_2), nitric oxide (NO), or ROS/RNS donors have been described in several studies [113, 115–119]. Oxidative stress can affect the physiologic function of K_{ATP} channels in a dual way: either by direct interference with channel proteins, e.g., due to oxidation of SH-groups [120, 121], or by indirect mechanisms caused by the inhibitory influence of ROS and RNS on mitochondrial function.

In β-cells with intact cell metabolism, H_2O_2 has been shown to drastically increase K_{ATP} current [115, 116]. The rise in K_{ATP} current, concomitant membrane hyperpolarization, and inhibition of insulin secretion are the consequences of a dramatic drop in ATP synthesis which is caused by an H_2O_2-induced breakdown of the mitochondrial membrane potential [113].

The interactions of RNS with K_{ATP} channel activity are more complex: Membrane hyperpolarization and channel opening due to inhibition of mitochondrial ATP production have been described for NO gas in the micromolar concentration range and for several NO donors [117, 118]. However, prolonged exposure to NO or NO donors exerts a biphasic effect: The first drastic increase in K_{ATP} current is followed by channel inhibition which most likely depends on direct interactions of NO with channel proteins [122]. One study reports that sub-micromolar concentrations of the NO donor NOC-7 suppress K_{ATP} channel activity via a cGMP/PKG-dependent pathway, whereas in agreement with earlier investigations, channel activation was achieved by short-term application of higher concentrations [123].

Role in Diseases

Impaired function of K_{ATP} channels can result either in an abnormal increase of insulin secretion or in a pathological reduction of hormone release. The number of mutations on the SUR1 or $K_{IR}6.2$ subunit, respectively, linked to altered K_{ATP} channel activity is steadily increasing. While it is known for decades that decreased K_{ATP} channel activity is a main reason for excessive insulin secretion in patients

with congenital hyperinsulinism, there is now increasing knowledge about channel dysfunctions that cause special forms of neonatal diabetes or type 2 diabetes mellitus.

K_{ATP} Channels and Hyperinsulinism

Congenital hyperinsulinism (HI) usually presents at birth or within the first year of life and is characterized by excessive insulin secretion in the absence of nutrient stimulation. In approximately 50% of all HI patients, loss-of-function mutations located on the SUR1 gene *ABCC8* are causing the disease, whereas mutations in the $K_{IR}6.2$ gene (KCNJ11) are much rarer [124, 125]. In principle there are two mechanisms that account for abnormal β-cell excitability, i.e., decreased expression of K_{ATP} channel protein and a reduced potency of physiological regulators to close the channel. Mutations of SUR1 have been identified that retain "premature" channels in the endoplasmic reticulum, impair K_{ATP} channel trafficking to the plasma membrane, or induce rapid degradation [126–128]. Other HI patients carry mutations which lead to a reduced amplitude of K_{ATP} current or to the loss of MgADP sensitivity [129–131].

K_{ATP} Channels and Diabetes

The first $K_{IR}6.2$ mutations leading to a diabetic phenotype were described by Gloyn et al. in 2004 [132]. Although activating mutations in the *KCNJ11* gene are the most common cause of neonatal diabetes [133], several mutations in the *ABCC8* gene have also been identified [134–137]. Gain-of-function mutations result in an elevated activity of the K_{ATP} channel. The underlying mechanisms include a reduced sensitivity of the channel to ATP, an increased ATPase activity of SUR1, or abnormal channel activation in response to nucleotide diphosphates or long-chain acyl-CoAs [138–143]. It is shown that in some $K_{IR}6.2$ mutations impaired coupling to SUR1 determines the loss of nucleotide inhibition [144].

The severity of the disease is correlated with the extent of ATP insensitivity and ranges from transient or permanent neonatal diabetes to full DEND syndrome, which is characterized by diabetes and neurological defects [132, 133]. Importantly, patients with diabetes due to $K_{IR}6.2$ mutations often benefit from switching insulin therapy to sulfonylureas [145–147]. Short-term investigations suggest that this might also apply to patients with SUR1 mutations [148].

Polymorphisms in the genes encoding $K_{IR}6.2$ or SUR1 are not only associated with neonatal diabetes or DEND syndrome but have also been linked to the development of type 2 diabetes [149–152] or even to secondary failure of sulfonylureas [153]. The $K_{IR}6.2$ mutation E23K that increases open channel probability has been extensively studied with respect to its impact on β-cell function. However, it is still a matter of debate whether the alterations in K_{ATP} channel activity caused by this mutation can explain a diabetic phenotype [138, 143, 154–156].

7.1.1.2 Ca^{2+} Channels

Ca^{2+} influx via voltage-gated Ca^{2+} (Ca_v) channels controls important cellular processes like exocytosis, proliferation, cell viability, gene expression, and cell cycle. In β-cells Ca_v channels play a key role in glucose-induced insulin secretion by mediating Ca^{2+} influx and increasing the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) [157–159]. β-cell Ca_v channel activity and density are decisive for appropriate insulin secretion and up- or down-regulation of both parameters can impair β-cell function.

Structure/Nomenclature and Occurrence in β-Cells

Each Ca_v channel comprises a pore-forming $alpha_1$ subunit and auxiliary beta, gamma, and $alpha_2$/delta subunits. The $alpha_1$ subunit forms the Ca^{2+}-conducting pore and contains the voltage sensor, the selectivity filter for Ca^{2+} and the activation and inactivation gates [159, 160]. The other subunits modulate channel activation, inactivation, and current amplitude and regulate plasma membrane trafficking [161, 162].

Nomenclature

Multiple nomenclatures for Ca_v channels exist according to their biochemical, biophysical, and pharmacological properties and sequence analysis. According to the primary structure of the $alpha_1$ subunits, Ca_v channels are divided into three families of closely related members: Ca_v1, Ca_v2, and Ca_v3. $Ca_v1.1$–1.4 channels are L-type Ca^{2+} channels, $Ca_v2.1$ is a P/Q-type channel, $Ca_v2.2$ belongs to N-type, and $Ca_v2.3$ to R-type channels, whereas $Ca_v3.1$–3.3 are T-type channels. Ca_v1 and Ca_v2 channels have a high threshold for voltage-dependent activation and are named HVA (high voltage-activated) Ca^{2+} currents, while T-type Ca^{2+} currents are referred to as LCA (low voltage-activated currents) because they are stimulated by small depolarizations [163].

Ca^{2+} Channels in β-Cells

It is still a matter of debate which Ca_v channels are present in β-cells and, more important, which are of physiological relevance. The situation is complex because Ca_v channel expression varies between species and often tumor cell lines are used which considerably differ from primary β-cells. Long-lasting changes of $[Ca^{2+}]_c$ as required for stimulation of insulin secretion can only be achieved by L-type Ca^{2+} channels which inactivate slowly but not by the rapidly inactivating T-type Ca^{2+} channels. L-type Ca^{2+} channels are considered to be crucial for β-cell function. In mouse β-cells a large part of the total voltage-dependent Ca^{2+} current is blocked by L-type Ca^{2+} channel inhibitors [164, 165]. Moreover, insulin secretion is almost completely blocked by suppression of L-type Ca^{2+} channel activity [166, 167]. The existence of $Ca_v1.2$ (characterized by the $alpha_{1C}$ subunit) and $Ca_v1.3$ (containing

the alpha$_{1D}$ subunit) has been proven in rodent and human islets at the level of genes, mRNA, and proteins [168–174], although the relative portion of each channel remains controversial and may depend on the species and the methods used. Studies using mice with genetic ablation of $Ca_v1.2$ and $Ca_v1.3$ channels provide additional insights in the specific functions of these channels (see Section "Role in β-cell Stimulus-Secretion Coupling"). Several non-L-type HVA Ca^{2+} channels have been found in human and rodent β-cells and in tumor β-cell lines (for details see reviews: [157–159]).

Electrophysiological Characteristics and Regulation

General Electrophysiological Properties and Influence of Drugs

Ca_v channels are characterized by voltage-dependent activation. Single channel Ca^{2+} currents and whole cell Ca^{2+} currents have been measured in various species, whereas L-type currents are best characterized. Since single channel Ca_v currents are too small to be recorded under physiological conditions, Ba^{2+} has been used as charge carrier because it increases the amplitude of L-type Ca^{2+} channel currents. The single Ca_v channel conductance with extracellular Ba^{2+} is 20–25 pS [175, 176]. From these data, a single channel conductance of 2 pS has been estimated for physiological Ca^{2+} concentrations. Whole-cell Ca_v currents in mouse β-cells are activated at depolarizations to potentials more positive than −50 mV, have a maximum current at −20 mV, and reverse at ∼+50 mV [165, 177]. In human and rat β-cells additionally T-type Ca^{2+} currents have been detected [178, 179]. β-cell L-type Ca^{2+} currents inactivate in a Ca^{2+}-dependent manner during sustained depolarization [165], and thus inactivation is clearly reduced with Ba^{2+} as charge carrier. A smaller voltage-dependent component of inactivation has also been described [180]. To estimate the physiological significance of the different Ca_v channel components for insulin secretion, the correlation between the pharmacological block of channel activity and the inhibition of insulin secretion is decisive. Most important in this context is the sensitivity of L-type Ca^{2+} channels to dihydropyridines and D-600 [165, 177].

Regulation by Kinases and G Proteins

The amount of open Ca_v channels is determined by the membrane potential but fine-tuning must occur by other mechanisms. In several excitable cells including muscles and neurons protein kinases are important modulators of Ca_v channel activity. A variety of protein kinases are present in β-cells. However, in primary β-cells the regulation of Ca_v channel activity by the cAMP-dependent protein kinase A, the cGMP-dependent protein kinase G, and tyrosine kinases seems to play only a minor role [158]. Protein kinase C is assumed to be necessary to sustain a tonic phosphorylation of β-cell Ca_v channels [181]. Ca_v channels in primary β-cells are highly phosphorylated under basal conditions excluding further phosphorylation as a mechanism to alter Ca_v channel activity. Accordingly, protein kinase activation

can have a large effect on exocytosis, determined as changes in cell capacitance, without any change in Ca_v channel activity [182].

It is generally accepted that activation of inhibitory G proteins decreases insulin secretion. It has been shown that the effects of alpha-adrenergic agonists, somatostatin, and galanin are sensitive to pertussis toxin [183–185]. However, it is highly disputed whether Ca_V channel activity in β-cells is regulated by G_i proteins. Once again the discrepancies may be brought about by the use of tumor cell lines. In insulin-secreting cell lines, catecholamines, galanin, and somatostatin seem indeed to inhibit Ca_v channels [183, 184, 186, 187]. However, for primary β-cells the concept that G_i-stimulating agents inhibit insulin secretion via reduction of Ca_v channel activity has not been confirmed, and other mechanisms of action have been proposed [188–193].

Excitosomes

Ca_v channels are not equally distributed in the β-cell plasma membrane but are clustered and co-localized with the exocytotic vesicles [168, 194, 195]. The physical neighborhood of Ca_v channels and secretory granules allows a steep local rise of $[Ca^{2+}]_c$ (>15 μM) which is necessary for fast exocytosis with only marginal enhancement of bulk $[Ca^{2+}]_c$ [168]. Moreover, the formation of excitosomes has been shown for β-cells, i.e., the Ca_v channels form a complex with proteins of the exocytotic machinery like syntaxin 1A, SNAP-25, and synaptotagmin [174, 196]. These complexes may fix the channels in the optimal position but also affect channel activity. SNAP-25 possesses distinct inhibitory and activating domains that modulate Ca_v1 channel activity [197]. These protein networks are suggested to serve as a fine-tuning mechanism of β-cell Ca_v1 channel function. Disruption of the integrity of the complexes impairs channel function [174, 196].

Role in β-Cell Stimulus-Secretion Coupling

Ca^{2+} Influx and Electrical Activity

Despite the diversity of Ca_v channels expressed in β-cells and species differences, it is unequivocally accepted that L-type Ca^{2+} channels play the paramount role for insulin secretion. In human β-cells glucose-induced action potentials and insulin secretion are completely suppressed by blockage of L-type Ca^{2+} channels [167]. In contrast, depolarization-evoked vesicle exocytosis, measured as changes in cell capacitance, is only marginally influenced by L-type channel blockage. Depolarization-induced exocytosis is markedly suppressed by the P/Q-type blocker omega-agatoxin which does not significantly decrease glucose-induced insulin secretion [167]. This emphasizes the necessity to properly discriminate between both processes, insulin secretion and membrane fusion of exocytotic vesicles. A recent paper confirms the outstanding significance of L-type Ca^{2+} channels in INS-1 cells [198]. The authors show that glucose-induced insulin secretion and $[Ca^{2+}]_c$ is

reduced to basal values by inhibiting L-type Ca^{2+} channels but not markedly influenced by suppressing R-type $Ca_v2.3$ activity. This is just the opposite in δ-cells (see Section 7.3).

Induction of electrical activity by glucose is a prerequisite for insulin secretion. Glucose evokes electrical activity in β-cells that is characterized by bursts of action potentials and hyperpolarized interburst phases (see Section "Regulation of V_m by K_{ATP} Channels" and Fig. 7.2). In mouse β-cells the action potentials are provoked solely by Ca^{2+} currents, in rat and human β-cells Na^+ channels contribute to electrical activity [165, 167, 199–201]. The influx of Ca^{2+} from the extracellular space is crucial for glucose-induced insulin secretion; release from the ER can only modulate it [202]. Influx ensures high Ca^{2+} concentrations beneath the membrane in the micro-domains with complexes containing channels, exocytotic proteins, and vesicles.

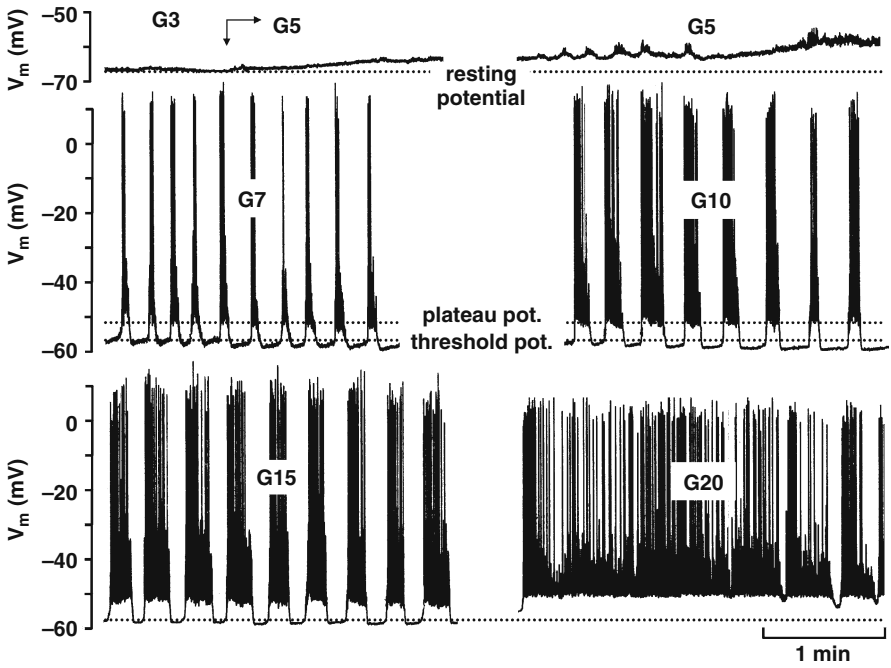

Fig. 7.2 Microelectrode (ME) measurements of membrane potential (V_m) of β-cells in intact islets. The glucose concentration is varied from 3 to 20 mM (G3 to G20). In the upper left panel V_m started to depolarize when the glucose concentration was switched from 3 to 5 mM (arrows). In the upper right panel the cell penetrated by the ME has not yet reached the threshold potential while a neighbouring cell is already electrically active. Current through gap junctions from the neighboring cell elicits voltage deflections in the cell impaled by the ME. Note that the burst time increases with increasing glucose concentrations while the interburst time is shortened

Studies with Knock-Out Mice

Several studies with Ca^{2+} channel knock-out mice gave new insights but until today the problem of the contribution of different channel types to β-cell function is not definitely solved.

Schulla et al. [172] constructed mice with a β-cell specific knock-out of the L-type $Ca_v1.2$ channel. About 45% of the Ca^{2+} channel current was removed by this maneuver, but the remaining current was insensitive to the L-type channel blocker isradipine suggesting that $Ca_v1.2$ carries the L-type current in β-cells. $Ca_v1.2$KO mice were glucose intolerant compared to WT mice. The first phase of insulin secretion and the rapid component of exocytosis were significantly reduced. The authors take their results as disruption of the $Ca_v1.2$ channel/granule complexes. However, no information is given about effects of the $Ca_v1.2$ knock-out on the second phase of insulin secretion (>15 min). Moreover, it is remarkable that complete loss of $Ca_v1.2$ channel activity influences electrical activity and $[Ca^{2+}]_c$ only marginally.

The knock-out of another important L-type Ca^{2+} channel present in β-cells was also investigated. Barg and co-workers [168] did not detect a significant effect of the $Ca_v1.3$ knock-out on β-cell Ca^{2+} currents in accordance with the observation of Platzer et al. [203] that fasting insulin and glucose serum concentrations are equal in $Ca_v1.3$KO mice and WT animals. Moreover, no change in these parameters was obtained after a glucose challenge. In contrast, Namkung and co-workers [171] found a severe impairment of glucose tolerance and reduced serum insulin concentrations in $Ca_v1.3$ channel knock-out mice compared to their littermates. The situation is even more complex because the $Ca_v1.3$ knock-out seems to be counteracted by up-regulation of the $Ca_v1.2$ gene [171].

Two papers describe reduced insulin tolerance in mice lacking R-type $Ca_v2.3$ channels [204, 205]. One of these papers shows that the effect is accompanied by reduced glucose-induced insulin secretion [204]. However, further studies are needed to clarify the role of $Ca_v2.3$ channels for insulin secretion because the effect of the knock-out on glucose tolerance was marked in male but not in female animals and was lost in aged animals [204]. Jing et al. [206] suggest a role of $Ca_v2.3$ channels in vesicle recruitment because in their study the $Ca_v2.3$ knock-out primarily affects second phase of insulin secretion.

7.1.1.3 K_v and K_{Ca} Channels

Pancreatic β-cells express a variety of K^+ channels regulated by voltage (K_v channels) and/or by the intracellular Ca^{2+} concentration (K_{Ca} channels). While the primary function of K_v channels, i.e., the repolarization of action potentials, is well accepted for years, the importance of K_{Ca} channels is less clear. Recent studies show that K_{Ca} channels play a role for the regulation of the characteristic membrane potential oscillations but are also involved in determining the glucose responsiveness of pancreatic β-cells.

Characteristics of K_v and K_{Ca} Channels in β-Cells

K_v Channels

K_v channels belong to the six transmembrane family of K^+ channels of which 12 subfamilies have been described up to now. In primary β-cells K_v channels of five subfamilies (K_v1, 2, 3, 6, and 9) have been detected [207–212]. The members of K_v1, 2, and 3 form functional channels as homo- or hetero-tetramers, whereas K_v6 and 9 are silent subunits that have been shown to modulate K_v2 and 3 channel currents by co-assembly in heterologous expression systems [213, 214]. In clonal and primary β-cells K_v currents consist of at least two components: one 4-aminopyridine-insensitive current without inactivation (delayed rectifier current, K_{DR}) that can be blocked by TEA^+ in the low millimolar concentration range and one inactivating current (A-current) that is inhibited by 4-aminopyridine [215–219]. The inactivating component is maximal at +30 mV, and its contribution to whole-cell K_v current gets progressively smaller with increasing depolarization [220]. K_v channels underlying the A-currents require membrane depolarization more positive than −40 mV for activation, whereas delayed rectifier currents are active at V_m above −20 mV [221]. Consequently, in β-cells K_v channels are not operative at resting membrane potential [222].

A-type currents in clonal or primary β-cells can be mediated by K_v1.4, 3.3, and 3.4 or K_v4.x. [211]. Among K_{DR} channels, K_v2.1 seems to play a predominant role for β-cells where it is clustered with Ca_v1.2 and SNARE proteins in cholesterol-rich lipid rafts [223, 224].

K_v currents can be modulated by hormones and neurotransmitters. GIP has been shown to diminish the A-currents in pancreatic β-cells in a PKA-dependent manner [225]. GLP-1 receptor activation reduces K_v currents [226], thereby antagonizing membrane repolarization – a mechanism that may contribute to the stimulatory effect of GLP-1 on insulin secretion. Other modulators of K_v channels are nonesterified fatty acids. PhospholipaseA_2-beta-mediated hydrolysis of membrane phospholipids has been shown to reduce peak K_v current [227] and islet-PLA_2-beta over-expressing β-cells display reduced K_v2.1 currents with alterations in electrical activity and increased insulin secretion [228].

K_{Ca} Channels

K_{Ca} channels can be divided into three groups with respect to their single channel conductance: large conductance BK channels (K_{Ca}1.1, maxi-K), intermediate conductance SK4 channels (K_{Ca}3.1, IK1), and small conductance K_{Ca} channels (SK1, 2, and 3).

The existence of BK channels in pancreatic β-cells and insulin-secreting cell lines has been verified by several groups [167, 218, 229–231]. BK channels are hetero-octamers of four alpha subunits forming the channel pore and four beta subunits with regulatory functions. BK channels have a single channel conductance of ∼150–300 pS and are sensitive to low concentrations of TEA^+, charybdotoxin, and iberiotoxin. They are active at nanomolar Ca^{2+} concentrations, and Ca^{2+} sensitivity

is increased with membrane depolarization. BK channel inhibition has been shown to increase the duration of action potentials in the MIN6 cell line when $K_v2.1$ channels are blocked [218], whereas others report no effect of iberiotoxin on the shape of action potentials in rodent islet cells [231]. One study shows an elevated amplitude of action potentials in a subset of human β-cells in response to BK channel inhibition [167]. Interestingly, genetic ablation of functional BK channels impairs glucose tolerance (Düfer, Krippeit-Drews, Ruth, Drews, unpublished observation).

The intermediate conductance K_{Ca} channel has been cloned from human pancreas in 1997 [232], and SK4 mRNA and protein, respectively, are expressed in murine islets [233, 234]. SK4 channel opening is largely independent of V_m [235, 236] but strictly regulated by $[Ca^{2+}]_c$ [235, 237]. Single channel currents with SK4 channel characteristics have been observed in clonal and primary β-cells [233, 238]. Genetic ablation of SK4 (SK4KO) channels increases the duration and frequency of Ca^{2+} action potentials, and pharmacologic channel inhibition alters the oscillatory pattern of $[Ca^{2+}]_c$ in WT β-cells [233]. In addition, glucose responsiveness of V_m and of $[Ca^{2+}]_c$ is shifted to lower glucose concentrations in SK4KO β-cells. Compared to their littermates, SK4KO animals exhibit an improved glucose tolerance but no change in insulin sensitivity.

In mouse islets mRNA of the small conductance K_{Ca} channel SK1 has been observed and for SK2 and 3 protein co-localization with insulin has been verified in dissociated islet cells [234]. Up to now there is no investigation characterizing single channel currents of SK1-3 in β-cells, but it has been shown that SK1-3 channel inhibitors influence membrane potential and Ca^{2+} oscillations [234, 239].

Contribution of K_{Ca} Channels to K_{slow} Currents

In 1999, a K^+ current activating with increasing Ca^{2+} influx during burst phases of glucose-stimulated β-cells was detected [200]. The current, termed K_{slow} due to its delayed and slow onset, strongly depends on $[Ca^{2+}]_c$. It can be modulated by Ca^{2+} influx via L-type Ca^{2+} channels and by Ca^{2+} release of the endoplasmic reticulum [240, 241]. Further analysis suggested that approximately 50% of K_{slow} could be ascribed to K_{ATP} current [242]. Another significant component are SK channels. For murine β-cells it has been shown that knock-out or pharmacological inhibition of SK4 channels significantly reduced K_{slow} [233]. Although K_{slow} currents are not sensitive to apamin, a blocker of the small conductance SK channel [200, 241], there is one study suggesting involvement of SK3 channels in generation of K_{slow} [239].

Significance of K_v and K_{Ca} Channels for β-Cell Electrical Activity

Role of K_v and K_{Ca} Channels for Action Potentials

The primary function of K_v channels in β-cells is action potential repolarization [177, 220, 222]. Increasing K^+ outward current repolarizes V_m and terminates Ca^{2+} action potentials prior to Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels.

Inhibition of K_v channels with TEA^+ or several spider toxins extends action potential duration. Consequently, blockade of K_v channels is a potent tool to augment insulin release [219, 224, 243]. As activation of K_v channels requires membrane depolarization, targeting K_v channels affects insulin secretion only in the presence of elevated glucose concentrations or other depolarizing stimuli [211, 222].

At least in rodent β-cells, the most important K_v channel underlying the K_{DR} current is $K_v2.1$ [209]. Inhibition or knock-out of this channel reduces K_{DR} currents by >80%, broadens single action potentials and increases insulin secretion [218, 244, 245]. In human β-cells ~50% of K_{DR} currents are sensitive to the $K_v2.1$ blockers stromatoxin and hanatoxin, respectively [167, 221]. Experiments with K_v1 channel antagonists show that $K_v1.1$, 1.2, and 1.3 channels do not markedly contribute to regulation of insulin secretion in primary β-cells, whereas an adenoviral approach with dominant-negative $K_v1.4$ suggests involvement of this channel in generation of K_{DR} currents [216, 221].

Action potentials and insulin secretion can also be modulated by K_{Ca} channels: Inhibition of SK4 channels with TRAM-34 or genetic channel ablation leads to action potential broadening, increases the frequency of glucose-induced Ca^{2+} action potentials, and elevates Ca^{2+} influx [233]. Interestingly, inhibition of SK4 channels not only affects glucose-stimulated β-cell activity but also shifts the threshold for glucose responsiveness of V_m, $[Ca^{2+}]_c$, and insulin secretion to lower glucose concentrations [233].

Blockade of small conductance SK channels has also been shown to increase the frequency of action potentials and to increase glucose-stimulated insulin release [239]. It is suggested that Ca^{2+}-activated K^+ channels of the BK type play a significant role for action potential repolarization in human β-cells and in clonal MIN6 cells [167, 218].

Role of K_{Ca} Channels in Oscillations of V_m

For decades it was discussed whether K_{Ca} channels participate in the regulation of the characteristic membrane potential oscillations of β-cells [222, 231, 246–248]. At present, it is generally accepted that periodic activation of K_{ATP} channels is a key event that determines oscillations in V_m [249–250] (compare Section "A Model for β-Cell Oscillations" and see Fig. 7.3). Early studies investigating the effect of elevated Ca^{2+} influx on membrane potential already suggested that activation of K_{Ca} current could modulate the length of the hyperpolarized interburst intervals [251]. As blockage of BK channels does not influence membrane potential oscillations [222, 231, 243], these channels are not considered to play a role for regulation of the burst pattern. However, with the detection of a Ca^{2+}-dependent, sulfonylurea-insensitive component of K_{slow} it became obvious that activation of K_{Ca} channels plays an important role for induction of the electrically silent interburst phases [200, 241, 242]. Although the precise nature of the underlying ion channels remains to be identified (compare Section "Characteristics of K_v and K_{Ca} Channels in β-Cells"), the sensitivity of K_{slow} to SK channel blockers and the ability of these drugs to alter oscillations in V_m and $[Ca^{2+}]_c$, respectively, clearly point to an involvement of small

and intermediate conductance K_{Ca} channels in the regulation of membrane potential oscillations [233, 239].

7.1.1.4 Other Ion Channels

Na^+ Channels

Plant [199] was the first to report the existence of voltage-dependent Na^+ channels in the pancreatic β-cell of the mouse. Strangely, in mouse β-cells Na^+ channels are fully inactivated at the resting potential [199] and seem to have no physiological function. This is different in β-cells of dogs [252] and humans [253]. In these species glucose-induced electrical activity consists largely of Na^+ action potentials (Na^+ APs) inhibitable by tetrodotoxin (TTX). Na^+ influx depolarizes the cell membrane to voltages where L-type Ca^{2+} channels open. In human β-cells Na^+ APs play a major role at a V_m negative to −45 mV and disappear due to Na^+ channel inactivation at a V_m positive to −40 mV, e.g., at glucose concentrations higher than 10 mM [201]. More recent work confirms the role of Na^+ APs in human β-cells [167]. Half-maximal inactivation of the Na^+ channel was found at ∼−45 mV, and TTX is more potent to inhibit glucose-induced insulin secretion at low than at high glucose concentrations. Quantitative RT-PCR identified $Na_v1.6$ and $Na_v1.7$ channels to be expressed in equal amounts in human β-cells [167].

Volume-Sensitive Anion Channels (VSACs)

In 1994, Britsch and co-workers [254] published that osmotic cell swelling markedly increased glucose-induced electrical activity. They ascribed the underlying depolarization to activation of a volume-sensitive anion current (VSAC). This current was later confirmed and electrophysiologically characterized [255–257]. The existence of this current is well established; however, its role for the physiological function of β-cells – besides cell volume regulation – is not fully understood, although it has been extensively studied by Best and co-workers [258–268]. Since E_{Cl} is about −30 mV [255, 257], the VSAC will provide a depolarizing current at most physiological potentials. Inhibition of K_{ATP} channels by cell metabolism or antidiabetic drugs leads to depolarization of β-cells, but the underlying current for the depolarization is unknown. Whether VSAC is this "unknown current" or contributes to it is still conflicting [256, 260]. More recently, it has been shown that glucose activates the VSAC by incorporating the channel protein in the plasma membrane of INS-1E cells [269]. However, this effect was elicited by 20 mM glucose and could be mimicked by the non-metabolizable 3-O-methylglucose and may therefore be caused by cell swelling.

Transient Receptor Potential (TRP)-Related Channels

On the search for the unknown depolarizing current, TRP channels were also regarded as potential candidates; however, at the resting β-cell, no activation

mechanism for these channels is described so far [270]. Members of all three subfamilies of TRP channels (C-form for canonical, M-form for melastatin, V-form for vanilloid) have been described for either primary β-cells or insulin-secreting cell lines [270].

TRPC1 and TRPC4 transcripts have been found in islets and β-cell lines [271–273]. These channels are nonselective cation channels which are activated by either $G_{q/11}$ protein or IP_3 or by Ca^{2+} release from intracellular stores [270, 272–274] and may therefore be counted among the store-operated Ca^{2+} channels [275]. Worley and co-workers [276] presented evidence that β-cells also possess store-operated nonselective monovalent cation channels. These channels may be TRP channels [273] and were suggested to be TRPM4 [277] or TRPM5 channels [278], but do not obviously represent the acetylcholine-induced Na^+ current which is independent of Ca^{2+} stores [279]. Another signaling pathway in which TRP channels are involved is the action of incretins such as GLP1 [280, 281], though the exact nature of the channel(s) involved remains undefined. Nevertheless, these TRP channels are candidates to account for the GLP1-induced depolarization which is independent from K_{ATP} channel inhibition [282]. Steroidal compounds often have rapid effects on membrane surface receptors. Wagner and co-workers [283] have recently shown that pregnenolone sulfate activates TRPM3 channels, thereby increasing $[Ca^{2+}]_c$ and insulin secretion. Thus, the cross talk between steroidal and insulin-signaling endocrine systems is enabled.

TRP channels may also be involved in β-cell destruction during the development of diabetes as TRPM2 channels were identified to be activated by H_2O_2 [273, 284]. Since TRPM2 channels are unspecific cation channels [270], these channels can account for the excessive unspecific Ca^{2+} influx in response to H_2O_2 in β-cells [113]. Moreover, the H_2O_2-induced ATP depletion may release Ca^{2+} from intracellular stores [113] and in turn open release-activated Ca^{2+} channels or another group of unspecific cation channels belonging to TRPC4 [270]. Thus, TRP channels may be involved in Ca^{2+} overload of β-cells in response to oxidative stress which is causative for subsequent cell death.

A channel of the vanilloid subfamily, TRPV1, was found to be expressed in primary β-cells and in pancreatic neurons [285] which may link regulation of food intake and pancreatic endocrine function.

Hyperpolarization-Activated Cyclic Nucleotide-Gated (HCN) Channels

HCN channels are pacemaker channels of oscillations in a variety of cells [286–291]. Since β-cells are oscillating, it is tempting to speculate that HCN channels are involved in the pattern of electrical activity. In addition, it has been shown that cAMP has a depolarizing effect on β-cells [292] which may contribute to the depolarizing effect of GLP-1 (see Section 7.1.2.2). To our knowledge there is only one publication dealing with HCN channels in β-cells [293]. It was ascertained by PCR that HCN2 is the predominant channel in MIN6 cells and mouse islets, whereas HCN3 and 4 are most abundant in rat islets. Forskolin and dbcAMP regulate β-cell HCN currents positively while they are inhibited by specific

small interfering (si)RNA against HCN2 or by established HCN blockers such as Cs^+, ZD7288, cilobradine, and zatebradine. However, the authors were unable to demonstrate an effect of HCN channels on acute insulin secretion or membrane potential behavior [293]. Therefore, the function of these channels remains to be established.

7.1.2 Cell Membrane Potential (V_m)

V_m of β-cells is unique due to its regulation by glucose. It links signals derived from glucose metabolism to insulin secretion by determining $[Ca^{2+}]_c$.

7.1.2.1 Regulation by Glucose

Glucose enters β-cells mainly via the high K_m Glut-2 transporter [294]. As this transporter is not rate limiting for glucose uptake, β-cell cytosolic glucose concentration is rapidly adapted upon changes in blood glucose concentration. Glucose induces insulin secretion by activating a triggering pathway (closure of K_{ATP} channels, depolarization of V_m, and increase in $[Ca^{2+}]_c$) and an amplifying pathway (sensitization of the exocytotic machinery for $[Ca^{2+}]_c$) that is independent of changes in K_{ATP} channel activity and V_m [295, 296]. The triggering Ca^{2+} signal is essential. All physiological or pharmacological maneuvers lowering or enhancing $[Ca^{2+}]_c$ impair or improve insulin secretion. The triggering pathway is superior to the amplifying pathway. As long as the triggering signal $[Ca^{2+}]_c$ is slight, amplifying signals are without effect. Thus, low glucose can stimulate amplifying signals but they are silent without an adequate increase of the triggering Ca^{2+} signal. In this case an augmentation of $[Ca^{2+}]_c$, regardless by which means (metabolism-derived or metabolism-independent signal) unmasks the amplifying pathway. The amplifying mechanism strongly depends on metabolism, however, the signal(s) responsible for this phenomenon are not yet identified.

Regulation of V_m by K_{ATP} Channels

In the presence of functional K_{ATP} channels, the actual plasma glucose concentration determines the activity of K_{ATP} channels. At a subthreshold glucose concentration, V_m is silent (∼−70 mV) and is mainly determined by the K_{ATP} current [297]. With increasing glucose concentration, glucose metabolism and thus ATP formation rise and more and more K_{ATP} channels close until the K_{ATP} current is reduced to a level at which the unknown depolarizing current exceeds the hyperpolarizing current through K_{ATP} channels. V_m depolarizes to the threshold for the opening of voltage-dependent ion channels (Ca_v and Na_v channels, depending on the species) and action potentials start from a plateau potential (see Fig. 7.2). At a supra-threshold glucose concentration V_m starts to oscillate. The knowledge about the nature of these oscillations mainly derived from mouse β-cells. The depolarized burst phases with action potentials and the silent hyperpolarized interburst phases

are glucose dependent. With increasing glucose concentration, burst phases are prolonged and interburst phases are shortened until continuous activity is reached at glucose concentrations above ~25 mM (Fig. 7.2). Each action potential is terminated by deactivation of Ca^{2+} channels which is achieved by opening of K_v and K_{Ca} channels (see Section "Significance of K_v and K_{Ca} Channels for β-Cell Electrical Activity" and [220]), a maneuver that repolarizes V_m to the plateau potential from which the next action potential starts. However, the question remains which mechanisms drive the unique glucose-induced oscillations of V_m with bursts of action potentials and silent interburst phases.

A Model for β-Cell Oscillations

$[Ca^{2+}]_c$ plays a pivotal role in insulin secretion. It has been suggested that the glucose-induced increase in $[Ca^{2+}]_c$ augments the mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) with subsequent activation of Ca^{2+}-dependent dehydrogenases and ATP production [298]. However, this positive feedback mechanism is not compatible with oscillations that require a negative feedback process. The following model suggests that the positive feedback mechanism that is induced upon a glucose rise converts into a negative feedback mechanism during sustained glucose elevation (see Fig. 7.3 and [299]). During phase 1, glucose increases and stimulates the β-cell. The metabolism of the sugar leads to the production of reduction equivalents which enter the respiratory chains. This hyperpolarizes the mitochondrial membrane potential $\Delta\Psi$. The resulting H^+ gradient is used by the F_1/F_0-ATPase and leads to ATP production (and phosphocreatine production, see "Regulation by Metabolism-Derived Nucleotides and Phosphotransfer"), closure of K_{ATP} channels, depolarization of V_m, increase of $[Ca^{2+}]_c$, and finally insulin secretion. During phase 2 (see Fig. 7.3), glucose is steadily increased which keeps up insulin secretion, however, the β-cell now undergoes oscillatory activity. The increase in $[Ca^{2+}]_c$ depolarizes $\Delta\Psi$ which diminishes ATP production and leads to reopening of some K_{ATP} channels. In addition, elevated $[Ca^{2+}]_c$ activates K_{Ca} channels. As a consequence of both processes, V_m hyperpolarizes which lowers $[Ca^{2+}]_c$. Subsequently, K_{Ca} channel activity decreases, whereas $\Delta\Psi$ hyperpolarizes. The enhanced ATP formation leads to closure of K_{ATP} channels and finally $[Ca^{2+}]_c$ increases. With this rise in $[Ca^{2+}]_c$ the next cycle starts. This model assumes that during sustained glucose elevation, an increase in $[Ca^{2+}]_c$ does not enhance but diminishes ATP production. This hypothesis is meanwhile supported by many observations: (1) Stimulation of Ca^{2+} influx reduces the ATP/ADP ratio [300], (2) Ca^{2+} influx depolarizes $\Delta\Psi$ [250], (3) K_{ATP} channel activity oscillates and these oscillations are driven by $[Ca^{2+}]_c$ oscillations [249, 301], (4) $\Delta\Psi$ oscillates in dependence on the Ca^{2+} fluctuations [250, 302], and (5) $[Ca^{2+}]_c$ drives NADH oscillations [303]. This model implicates that burst phases of V_m are terminated by activation of K_{slow} composed of K_{ATP} and Ca^{2+}-dependent K^+ currents. K_{slow} counterbalances the depolarizing current and finally hyperpolarizes the plasma membrane below the threshold for L-type Ca^{2+} channel opening [200, 233, 242, 249, 250, 301]. This model is excellently supported by mathematical simulations of β-cell bursting [304–306].

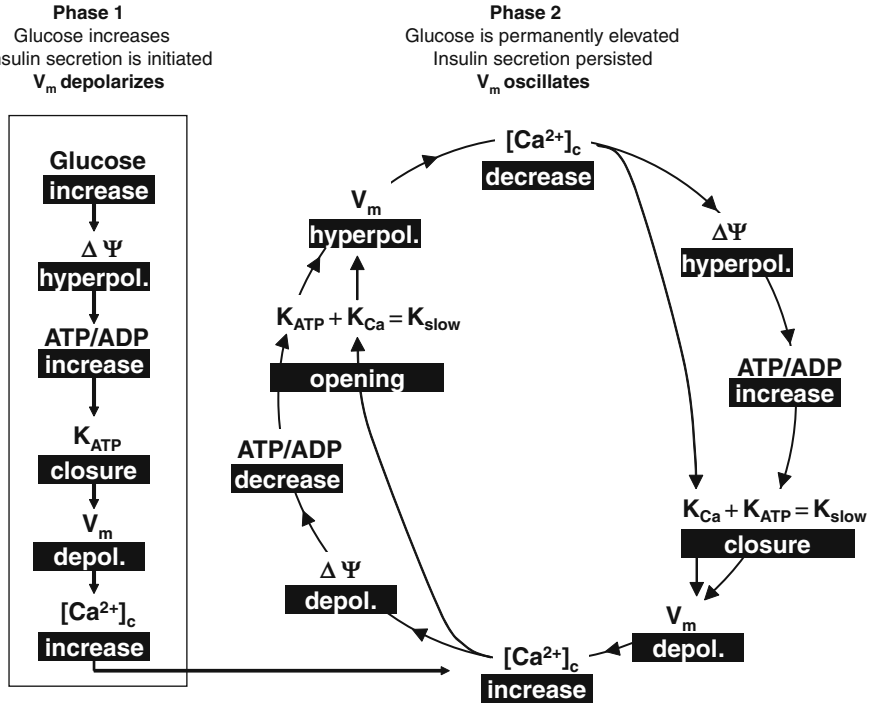

Fig. 7.3 Model for V_m oscillation in WT β-cells. Phase 1 describes the consensus model of β-cell activation by glucose. Phase 2 indicates that Ca^{2+} influx increases the K_{slow} current (for details see text) which counterbalances the depolarization. During the hyperpolarized phase $[Ca^{2+}]_c$ is lowered and the cell depolarizes again. Thus, V_m oscillates at a constant stimulatory glucose concentration

β-cell oscillatory activity is considered to be a prerequisite for pulsatile insulin secretion. Interestingly, oscillations of the membrane potential persist in β-cells without functional K_{ATP} channels (SUR1KO) [35]. This demonstrates that mechanisms exist that can substitute for K_{ATP} channels to hyperpolarize V_m and sustain oscillations (see Section "Regulation of V_m Independent of K_{ATP} Channels" for further details).

Regulation of V_m Independent of K_{ATP} Channels

It is meanwhile well accepted that glucose can mediate insulin secretion by a K_{ATP} channel-independent pathway [307, 308]. Interestingly, V_m of β-cells lacking functional K_{ATP} channels is also regulated by glucose. As expected, SUR1KO β-cells display action potentials even at very low glucose concentration but surprisingly still exhibit an oscillatory pattern of electrical activity with burst and interburst phases [35]. Action potential frequency, percentage of time with action potentials, and interburst length change in response to an alteration of the glucose concentration.

Compatibly, glucose depolarizes V_m of β-cells from $K_{IR}6.2$ knock-out mice [309]. Since oscillations require a hyperpolarizing current, these results suggest that other hyperpolarizing mechanisms besides K_{ATP} channels are regulated either directly by glucose or by signals deriving from the glucose metabolism. Additional hyperpolarizing mechanisms may be up-regulated as a result of K_{ATP} channel loss. As mentioned above, K_{slow} currents are good candidates that may contribute to β-cell hyperpolarization [200]. The K_{Ca} component of the K_{slow} current may gain importance in β-cells lacking K_{ATP} channels. Another possibility proposed recently is the activation of the Na^+,K^+-ATPase by glucose metabolism and insulin. The stimulation of the pump induces a hyperpolarizing current sufficient to maintain oscillatory electrical activity when the membrane resistance is high due to the lack of K_{ATP} channel conductance [310].

7.1.2.2 Regulation by Hormones and Neurotransmitters

Glucose-stimulated insulin secretion is modulated by a variety of hormones and neurotransmitters which affects V_m of β-cells besides other steps of the stimulus-secretion coupling.

GLP-1

GLP-1 that is produced in the neuroendocrine L-cells of the intestine is the most important representative of the incretin hormones, a group of intestinal hormones that increase insulin secretion in the presence of glucose. Since several years the genetically engineered GLP-1 analogue exenatide is used in the treatment of type 2 diabetes mellitus. It has been suggested that GLP-1 depolarizes V_m by closing K_{ATP} channels [311–313]. However, this mode of action of GLP-1 is inconsistent with other findings. Some studies propose that the insulinotropic effect of GLP-1 is mediated by its effects on unspecific cation currents [314, 315], others attribute it to L-type Ca^{2+} currents [282, 312, 316] or Ca^{2+} mobilization from intracellular stores [317].

Noradrenaline and Galanin

The autonomic nervous system has important modulating effects on insulin secretion by adapting hormone release to food intake or increased physical or psychic stress. The sympathetic neurotransmitter (nor)adrenaline and the co-transmitter galanin suppress insulin secretion [190], while the parasympathetic neurotransmitter acetylcholine enhances hormone secretion [318]. Noradrenaline and galanin act on several steps in β-cell stimulus-secretion coupling including the membrane potential. After binding to $alpha_2$ and specific galanin receptors, respectively, noradrenaline and galanin hyperpolarize V_m via G_i protein-coupled processes

[185, 190, 319], however, the underlying mechanisms are still unclear. For insulin-secreting tumor cell lines, it has been proposed that the sympathetic neurotransmitters activate K_{ATP} channels and that this mechanism hyperpolarizes the β-cells [320, 321]. However, this mode of action was never confirmed with primary β-cells. In 1991 Rorsman and co-workers described the activation of a sulfonylurea-insensitive low-conductance K^+ current by clonidine [189]. It was concluded that adrenaline shares this target because it acts via the same receptors. This assumption is supported by the findings that noradrenaline and galanin are able to hyperpolarize mouse β-cells in the absence of K_{ATP} channels [35, 322]. Inhibition of L-type Ca^{2+} channel current by galanin or catecholamines was solely described for insulin-secreting tumor cell lines [183, 187] but not approved in primary β-cells [192].

Somatostatin

Somatostatin is released from δ-cells of the islets of Langerhans and inhibits insulin secretion by a paracrine effect. Like noradrenaline and galanin it hyperpolarizes V_m [185]. The mode of action is not identified but for primary β-cells a similar mechanism is suggested as for adrenaline and galanin [189].

Acetylcholine

The parasympathetic neurotransmitter acetylcholine has complex effects on β-cells that result under physiological conditions in an augmentation of insulin secretion. The effect of the transmitter on β-cells is mediated by M_3 receptors. Membrane depolarization is one mechanism contributing to the insulinotropic effect of acetylcholine. The depolarization is caused by activation of a Na^+ current and the subsequent stimulation of Ca^{2+} influx. The Na^+ current is not voltage-dependent and not regulated by store depletion. Surprisingly, the activation of the current occurs independent of G proteins. It is suggested that distinct Na^+ channels are directly coupled to muscarinic receptors in β-cells via an unknown transduction mechanism [279, 318, 323]. It has been shown that Ca^{2+} store depletion triggers Ca^{2+} or unspecific cation influx in β-cells [272, 324]. Therefore, another possibility for an acetylcholine-induced depolarization is emptying of Ca^{2+} stores by IP_3 with subsequent induction of store-dependent Ca^{2+} influx. However, to our knowledge it has only been shown for insulin-secreting cell lines but not for primary β-cells that acetylcholine stimulates this pathway [325].

Insulin

It is attractive to assume that insulin influences its own secretion by a feedback mechanism. However, the concept that insulin has an autocrine effect is controversial. Numerous papers on this topic demonstrate negative feedback, positive feedback, or no effect of insulin on β-cell function (for review see [326]). The K_{ATP} channel has been identified as a target for insulin. Khan and co-workers [327]

show that insulin activates K_{ATP} channels leading to hyperpolarization of V_m which would suppress insulin secretion. It is suggested that this effect of insulin on K_{ATP} channels is mediated by PI3 kinase/PI(3,4,5)P_3 signaling that alters the ATP sensitivity of K_{ATP} channels [327, 328]. Insulin hyperpolarizes V_m in SUR1KO mouse β-cells showing that the negative feedback of insulin on V_m is present in the absence of K_{ATP} channels. Düfer and co-workers [310] provide evidence that this negative feedback is due to activation of the Na^+,K^+-ATPase by insulin. This mechanism may gain importance in cells with a high membrane resistance where small current changes can induce large effects on V_m.

7.2 α-Cells

7.2.1 Ion Channels

Most studies addressing the expression and function of ion channels in pancreatic α-cells have been performed with rodent islet preparations. In α-cells there have been identified at least four different types of K^+ channels, four types of voltage-gated Ca^{2+} channels, a Na^+ channel, and the $GABA_A$ receptor Cl^- channel [208, 329–332]. Recent studies also prove evidence for a regulatory function of HCN channels [333] and ionotropic glutamate receptors [334].

K_{ATP} Channels

K_{ATP} currents have been observed in clonal glucagon-secreting alphaTC cells [335, 336] and in rodent α-cells [330, 337, 338], and co-localization of $K_{IR}6.2$ or SUR1 mRNA, respectively, with glucagon has been shown in intact islets [337]. Up to now a direct proof for K_{ATP} channel activity in human α-cells is still missing.

In accordance with the characteristics of K_{ATP} channel regulation in β-cells, the sensitivity of K_{ATP} channels toward ATP inhibition is much higher in excised patches (K_i ~17 μM) compared to intact α-cells (K_i ~940 μM) [337, 339]. With regard to nucleotide sensitivity there seem to exist species differences: A reduction of the ATP sensitivity by PIP_2 was reported for rat [337] but not for murine α-cells [338], and the K_i value for ATP in intact murine α-cells is about sixfold higher [338] compared to rats. ATP sensitivity of α-cell K_{ATP} channels has been shown to be reduced by insulin [340, 341], and it has been suggested that the mediator inducing channel opening is not insulin but Zn^{2+} [342].

Other K^+ Channels

Besides ATP-regulated K^+ channels, α-cells are also equipped with voltage-activated K^+ channels. In human α-cells $K_v3.1$ and $K_v6.1$ have been identified on mRNA level [208], and $K_v4.3$ was detected in mouse α-cells [331].

Two groups of currents, a TEA^+-resistant but 4-aminopyridine-sensitive transient K^+ current (A-current) [331, 338, 343] and a TEA^+-sensitive delayed rectifier K^+ current (K_{DR}), have been detected in mouse α-cells [338, 343]. The A-current might, at least in part, be attributable to $K_v4.3$ channels [331]. In addition a G protein-coupled K^+ current composed of $K_{IR}3.2c$ and $K_{IR}3.4$ that is activated by GTP via the somatostatin receptor has been described by Yoshimoto et al. [344].

Ca^{2+} Channels

Ca^{2+}-dependent action potentials in α-cells have been described first by Rorsman and Hellman [345] in FACS-purified cells of guinea pigs.

Currents through L-type Ca^{2+} channels were reported in α-cells of several species. Channel opening starts at membrane depolarization above −50 mV, and the current through these channels mediates about 50–60% of the Ca^{2+} influx induced by membrane depolarization in rat and mouse α-cells [330, 346]. L-type Ca^{2+} currents are suggested to account for most of the Ca^{2+} increase required for glucagon secretion in response to adrenaline or forskolin stimulation [329]. Comparative experiments with knock-out animals suggest that L-type Ca^{2+} current in α-cells is mediated by $Ca_V1.2$ and 1.3 [346].

N-type Ca^{2+} channels seem to play a role for regulation of exocytosis under resting conditions in rat α-cells [329] and for glucose-induced glucagon secretion (see Section 7.2.2 and [347]). In mouse α-cells about 25% of the depolarization-evoked Ca^{2+} current could be ascribed to omega conotoxin GVIA-sensitive N-type Ca^{2+} channels [330]. However, expression of N-type Ca^{2+} channel mRNA ($Ca_V2.2$) was not found in murine α-cells [346].

R-type Ca^{2+} channels that are blockable by the $Ca_V2.3$ channel inhibitor SNX 482 account for ∼30% of Ca^{2+} influx in murine α-cells [346] but seem not to play any role for glucose-regulated glucagon secretion in rat α-cells [347].

Low voltage-activated T-type Ca^{2+} currents have been measured in mouse and guinea pig α-cells [331, 338, 345], whereas one study failed to detect these channels in murine α-cells [346]. As these channels activate at relatively negative membrane potential of ∼−60 mV, it is suggested that they are involved in the initiation of Ca^{2+} action potentials [339].

Na^+ Channels

The Na^+ channels expressed in α-cells are inhibited by tetrodotoxin and activate at potentials more positive than −30 mV. Maximum peak current is achieved between −10 and 0 mV. Inactivation of Na^+ channels occurs with $V_{1/2}$ of ∼−50 mV [331]. This clearly contrasts to mouse β-cells where $V_{1/2}$ is ∼−100 mV and no Na^+ current could be evoked by depolarizations starting from the resting membrane potential (compare Section 7.1.1.4 "Na^+ Channels"). The importance of Na^+ channels in α-cells is underlined by the fact that tetrodotoxin strongly inhibits glucagon secretion [331].

$GABA_A$ Cl^- Channels

The existence of Cl^- currents activated by GABA in α-cells was primarily described by Rorsman et al. [332] for cells isolated from guinea pigs. $GABA_A$ receptor mRNA and protein expression have been identified in clonal and primary α-cells [348–350]. In patch-clamped α-cells application of GABA terminates action potentials. The GABA-activated current as well as GABA-induced inhibition of glucagon release are sensitive to the $GABA_A$ receptor antagonist bicuculline [332, 351]. Translocation of $GABA_A$ receptors and Cl^- currents have been shown to be potentiated by insulin [350].

HCN Channels

There is one report [333] showing mRNA and protein expression of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in alphaTC6 cells and rat α-cells. Blockade of HCN channels resulted in elevation of $[Ca^{2+}]_c$ and increased glucagon secretion in clonal and primary α-cells.

Ionotropic Glutamate Receptors

Cabrera et al. [334] demonstrate that human α-cells express glutamate receptors of the AMPA/kainate type which are Na^+ permeable nonselective cation channels. Stimulation of these receptors results in activation of an NBQX-sensitive inward current and in glucagon secretion. The authors suggest that glutamate release from α-cells provides an autocrine positive feedback mechanism where activation of AMPA and kainate receptors triggers membrane depolarization and promotes opening of voltage-gated Ca^{2+} channels.

7.2.2 Regulation of Electrical Activity

As expected from an electrically excitable cell the degree of membrane depolarization and the extent of glucagon release are closely coupled in α-cells. In the absence of glucose, α-cells are electrically active and display Na^+- and Ca^{2+}-dependent action potentials [345, 352]. In contrast to β-cells where Ca^{2+} action potentials are induced when V_m is depolarized above −50 mV, action potentials in α-cells start at a more hyperpolarized membrane potential of ∼−70 to −60 mV [330, 343, 353]. It is suggested that in mouse α-cells electrical activity is initiated by opening of T-type Ca^{2+} channels. Further depolarization leads to opening of Na^+ and L-type Ca^{2+} channels and activation of K_{DR} channels and A-currents induces action potential repolarization. In rat α-cells there is no proof for the existence of T-type Ca^{2+} channels, but it is suggested that due to the low K^+ conductance V_m is sufficiently depolarized for Na^+ and Ca^{2+} channel activation [339].

Regarding the influence of nutrients, hormones, or drugs acting on ion channels, one must clearly discriminate between studies made with single cells and those with

α-cells within intact islets. Studies performed with intact islets more precisely reflect the situation in vivo. However, such investigations have the drawback that direct effects of nutrients or drugs on ion channels cannot be discriminated from indirect mechanisms mediated by paracrine regulators.

α-cells of intact islets are spontaneously active and increasing glucose results in membrane hyperpolarization [354, 355]. Reports about glucose-dependent regulation of electrical activity in single isolated α-cells are inconsistent. Varying glucose between 5 and 20 mM has no effect on action potential frequency in guinea pig α-cells [345]. In FACS-purified α-cells of rats and in single mouse α-cells increasing glucose above 10 mM results in increased membrane depolarization with reduced action potential amplitude [353, 356]. In contrast, the same groups also report for both species membrane hyperpolarization below the threshold for action potentials in response to high glucose [330, 337].

Recently, it has been shown that in isolated α-cells glucose-mediated K_{ATP} channel closure induces a sequence of events similar to the stimulus-secretion cascade of β-cells: Elevating glucose decreases K_{ATP} current which triggers Ca^{2+} influx and exocytosis [347, 356]. As the α-cell ATP/ADP ratio is higher than in β-cells, K_{ATP} current is much lower which allows spontaneous electrical activity even in the absence of glucose [347]. Interestingly, in contrast to β-cells the potency of glucose to inhibit K_{ATP} current seems to be very low. One study describes that inhibition of K^+ conductance by 20 mM glucose amounts to only 1/3 of tolbutamide inhibition [347], whereas another investigation completely failed to detect any inhibitory effect of 15 mM glucose on K_{ATP} current [357].

Regardless of what happens on the single-cell level there is much evidence that the primary mechanisms governing glucagon secretion are mediated by paracrine signaling pathways. Insulin and GABA which are secreted from neighboring β-cells and somatostatin from δ-cells hyperpolarize the α-cell via activation of K_{ATP} channels, $GABA_A$ Cl^- channels, and G protein-coupled K^+ channels, respectively (compare Section 7.2.1). The importance of a glucose-mediated direct inhibition of glucagon secretion is still in debate. This pathway suggests that with high glucose concentrations membrane depolarization via closure of K_{ATP} channels might exceed the stimulatory range and lead to reduction of exocytosis via inactivation of Na^+ and N-type Ca^{2+} channels [358].

7.3 δ-Cells

Less than 10% of the islet cells are δ-cells producing somatostatin [359]. Since somatostatin is known to inhibit insulin and glucagon secretion [185, 360–362], it is thought to act as a paracrine regulator of β- and α-cells.

δ-cells [37, 343, 363–365] and derived tumor cells [366] are equipped with K_{ATP} channels and respond to an increase in glucose concentration with depolarization [367]. δ-cells were supposed to have a similar glucose-induced stimulus-secretion coupling than β-cells [343], although they are already stimulated at lower glucose

concentrations (~3 mM) [368] possibly because of a lower density of K_{ATP} channels [369]. In contrast, Zhang and co-workers [370] have shown that the β-cell-specific stimulus-secretion coupling is not necessarily valid for δ-cells. They approved that at low glucose concentrations V_m and $[Ca^{2+}]_c$ are at least partly dependent on K_{ATP} channel activity and Ca^{2+} influx through L-type Ca^{2+} channels but that neither exocytosis nor somatostatin secretion are influenced by L-type Ca^{2+} channel blockers. They show that, especially in high glucose concentrations, somatostatin secretion is completely independent on K_{ATP} channel activity, but influenced by inhibitors of R-type Ca^{2+} channel ($Ca_v2.3$) blockers. In addition they illustrated that exocytosis and secretion crucially depend on Ca^{2+}-induced Ca^{2+} release (CICR) through ryanodine receptors (RyR3 type). It is suggested that K_{ATP} channel closure initially depolarizes δ-cells in response to rising glucose concentrations, but that R-type rather than L-type Ca^{2+} channels and CICR are responsible for somatostatin secretion. Accordingly, somatostatin release at high glucose concentrations is tolbutamide insensitive and even exists in SUR1KO mice [370]. Due to the limited number of studies the exact nature of stimulus-secretion coupling in δ-cells remains elusive.

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Chapter 8
ATP-Sensitive Potassium Channels in Health and Disease

Rebecca Clark and Peter Proks

Abstract The ATP-sensitive potassium (K_{ATP}) channel plays a crucial role in insulin secretion and thus glucose homeostasis. K_{ATP} channel activity in the pancreatic β-cell is finely balanced; increased activity prevents insulin secretion, whereas reduced activity stimulates insulin release. The β-cell metabolism tightly regulates K_{ATP} channel gating, and if this coupling is perturbed, two distinct disease states can result. Diabetes occurs when the K_{ATP} channel fails to close in response to increased metabolism, whereas congenital hyperinsulinism results when K_{ATP} channels remain closed even at very low blood glucose levels. In general there is a good correlation between the magnitude of K_{ATP} current and disease severity. Mutations that cause a complete loss of K_{ATP} channels in the β-cell plasma membrane produce a severe form of congenital hyperinsulinism, whereas mutations that partially impair channel function produce a milder phenotype. Similarly mutations that greatly reduce the ATP sensitivity of the K_{ATP} channel lead to a severe form of neonatal diabetes with associated neurological complications, whilst mutations that cause smaller shifts in ATP sensitivity cause neonatal diabetes alone. This chapter reviews our current understanding of the pancreatic β-cell K_{ATP} channel and highlights recent structural, functional and clinical advances.

Keywords ATP-sensitive potassium channel · Neonatal diabetes · Congenital hyperinsulinism · Insulin secretion · Pancreatic β-cell

Abbreviations

ABC	ATP-binding cassette
ADP	adenosine diphosphate
ATP	adenosine triphosphate
CHI	congenital hyperinsulinism

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CL3	3rd cytosolic loop in the sulphonylurea receptor connecting TMD0 to TMD1
DEND	developmental delay, epilepsy and neonatal diabetes
GCK	glycolytic enzyme glucokinase
GIP	gastrointestinal peptide
GIRK	G protein-coupled inwardly-rectifying potassium channel
GLP-1	glucagon-like peptide
GLUD1	mitochondrial glutamate dehydrogenase
HbA1C	Glycosylated (or glycated) haemoglobin
i-DEND	intermediate DEND syndrome
K_{ATP}	ATP-sensitive potassium
MRP	multidrug-resistant protein
NBD	nucleotide-binding domain
NDM	neonatal diabetes mellitus
PNDM	permanent neonatal diabetes mellitus
SCHAD	short-chain L-3-hydroxyacyl-CoA dehydrogenase
SUR	sulphonylurea receptor
TMD	transmembrane domain
TNDM	transient neonatal diabetes mellitus

8.1 Introduction

Insulin, as the only hormone able to lower the blood glucose concentration, is of great importance in glucose homeostasis. Insulin is released from the β-cells of the pancreatic islets of Langerhans in response to changes in nutrient, hormone and transmitter levels [1]. Electrical activity of the β-cell is central to the secretion of insulin. The extent of insulin release and electrical activity are directly correlated: in the absence of β-cell electrical activity no insulin is secreted [2].

The ATP-sensitive potassium (K_{ATP}) channel is a key component of stimulus–secretion coupling in the pancreatic β-cell. The resting membrane potential in β-cells is principally determined by the activity of the K_{ATP} channel [1] (a small depolarizing inward current of unknown origin is also present, but it must be extremely small, as it has proved difficult to measure). The K_{ATP} channel is responsible for the initiation of electrical activity and regulates its extent at suprathreshold glucose concentrations [3, 4]. The electrical resistance of the β-cell membrane is also determined by the K_{ATP} channel, which is low when K_{ATP} channels are open and high when they are closed. Therefore, when K_{ATP} channels are closed and membrane resistance is high, small changes in the K_{ATP} current can lead to membrane depolarization, electrical activity and insulin secretion [5].

Given the critical role of the K_{ATP} channel in insulin secretion and glucose homeostasis, it is not surprising that mutations in Kir6.2 and SUR1 can lead to diseases of both hypo- and hyperglycaemia [6–8]. This chapter focuses on the role of the β-cell

K_{ATP} channels in health and disease, taking into account recent genetic, clinical, structural and functional advances.

8.2 Role of K_{ATP} Channels in the Pancreas and Other Tissues

K_{ATP} channels act as metabolic sensors, coupling the metabolism of a cell to its membrane potential and electrical excitability. They are expressed in many tissues including the pancreas, skeletal and smooth muscle and the brain [9]. They link cell metabolism to electrical activity by sensing changes in adenine nucleotide concentrations and regulating membrane K^+ fluxes [10]. A decrease in metabolism opens K_{ATP} channels, causing K^+ efflux, membrane hyperpolarization and reduced electrical activity. An increase in metabolism closes K_{ATP} channels and prevents K^+ efflux, which triggers membrane depolarization. The resulting electrical activity stimulates responses such as the release of neurotransmitter at brain synapses, insulin exocytosis or muscle contraction [2].

The physiological role of the K_{ATP} channel has been best characterized in the pancreatic β-cell. The pancreatic K_{ATP} channel was discovered 25 years ago by Cook and Hales [11]; its closure by glucose metabolism was first demonstrated by Ashcroft et al. [3]. The link between glucose metabolism and insulin release in the β-cell is illustrated in Fig. 8.1. At substimulatory glucose concentrations, the β-cell K_{ATP} channel is open. Hence the cell membrane is hyperpolarized and voltage-gated calcium channels are closed [1]. Insulin secretion is therefore prevented. In response to an increase in the blood glucose concentration, insulin release from the β-cell is initiated. Glucose is transported into pancreatic β-cells and metabolized,

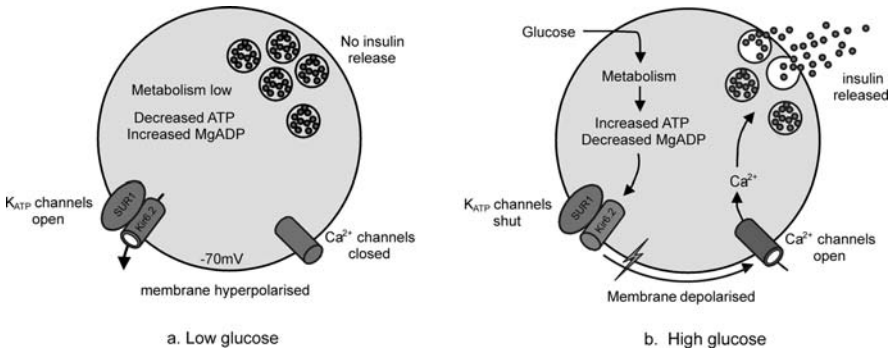

Fig. 8.1 Stimulus–secretion coupling in pancreatic β-cells. (**a**) When extracellular glucose, and thus β-cell metabolism is low, K_{ATP} channels are open. As a result, the cell membrane is hyperpolarized. This keeps voltage-gated Ca^{2+} channels closed, so that Ca^{2+} influx remains low and no insulin is released. (**b**) When extracellular glucose concentration rises, glucose is taken up by the β-cell and metabolized. Metabolism generates ATP at the expense of MgADP, thereby closing K_{ATP} channels. This causes membrane depolarization, opening of voltage-gated Ca^{2+} channels, Ca^{2+} influx and insulin secretion

thereby increasing the ATP:ADP ratio. This closes the K_{ATP} channel, producing a membrane depolarization that opens voltage-gated calcium channels: the influx of calcium into the β-cell triggers insulin exocytosis [12]. K_{ATP} channel activity in the β-cell is finely balanced – increased activity leads to reduced insulin secretion, whereas reduced K_{ATP} channel activity decreases insulin release. Thus, loss-of-function mutations in K_{ATP} channel genes cause over-secretion of insulin and result in hyperinsulinaemia. Conversely, gain-of-function mutations result in under-secretion of insulin, hyperglycaemia and a condition known as neonatal diabetes [6–8]. Similarly, impaired metabolic regulation of K_{ATP} channels, resulting from mutations in genes that influence β-cell metabolism, can cause both hyperinsulinaemia and diabetes.

K_{ATP} channels are also expressed in pancreatic α-cells where they have been proposed to play a role in glucagon secretion [13]. Unlike insulin secretion from β-cells, glucagon secretion exhibits dual dependency on K_{ATP} channel activity: intermediate K_{ATP} channel currents stimulate glucagon release, while both high and low activity have an inhibitory effect [14]. Since the resting activity of K_{ATP} channels in healthy α-cells is low, this would imply that inhibition of K_{ATP} channels due to rise in glucose concentration would inhibit glucagon release. It has been hypothesized that diabetic α-cells have increased resting activity of K_{ATP} channels, above the value optimal for glucagon release, so an increase in glucose metabolism would result in stimulation of glucagon secretion [15]. Consequently, glucose has opposite effects on glucagon secretion in normal and diabetic α-cells.

Göpel et al. have demonstrated the presence of K_{ATP} channels in pancreatic δ-cells [16]. Stimulus–secretion coupling in pancreatic δ-cells is expected to work in the same way as in pancreatic β-cells, with glucose stimulation leading to closure of K_{ATP} channels and the resulting membrane depolarization triggering somatostatin release [17].

The K_{ATP} channel further contributes to glucose homeostasis by controlling glucose uptake in skeletal muscle [18] and GLP-1 secretion from L-cells in the gut [19]. In the hypothalamus it is involved in the counter-regulatory response to glucose [20] and modulates neurotransmitter release in the hippocampus and substantia nigra [21–27]. The K_{ATP} channel is also thought to play important roles in altered metabolic states of tissues, for example hyperglycaemia, cardiac stress, ischemia and hypoxia [28–33].

8.3 Molecular Structure and Functional Properties of the β-Cell K_{ATP} Channel

The K_{ATP} channel is a hetero-octameric complex [34, 35] comprising four Kir6.x subunits and four sulphonylurea receptor (SUR) subunits (Fig. 8.2). Kir6.x is an inwardly rectifying K-channel [36–38] that forms the potassium-selective pore. Inward rectifiers conduct positive charge more easily in the inward direction across the membrane. This is due to the high-affinity block by endogenous polyamines and

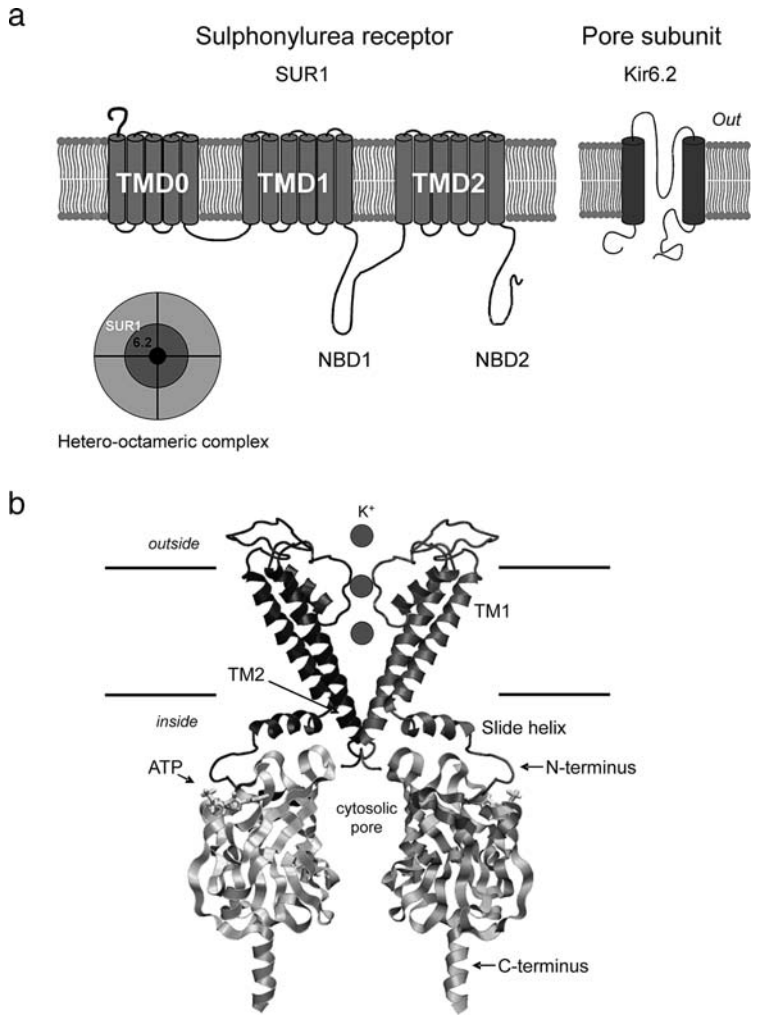

Fig. 8.2 The structure of the K_{ATP} channel. (**a**) Membrane topology of the sulphonylurea receptor (*left*) and Kir6.2 subunit (*right*). These subunits associate in a 4:4 octamer (*below left*). (**b**) Homology model of the Kir6.2 tetramer viewed from the side [60]. For clarity, the intracellular domains of 2 subunits and the transmembrane domains of 2 separate subunits are shown. ATP (denoted by *arrow* and shown in stick representation) is docked into its binding sites

magnesium ions at positive membrane potentials. There are two isoforms: Kir6.1, which is expressed in vascular smooth muscle [38] and Kir6.2 which is expressed more widely, including in the β-cell [37]. ATP-binding to the Kir6.2 subunit causes K_{ATP} channel closure [39].

The sulphonylurea receptor is a member of the ABC (ATP-Binding Cassette) superfamily [40]. This subunit plays a regulatory role. It confers sensitivity to:

(i) stimulation by Mg-nucleotides via two nucleotide-binding domains [41, 42]; (ii) activation by K channel openers such as diazoxide; and (iii) inhibition by sulphonylureas such as tolbutamide and glibenclamide [39, 40]. There are three isoforms of the sulphonylurea receptor. SUR1 is expressed in β-cells and neurons [40], SUR2A in skeletal and cardiac muscle [43, 44] and SUR2B in smooth muscle and brain [45–47]. The K_{ATP} channel found in β-cells is made up of four Kir6.2 subunits and four SUR1 subunits. Current evidence indicates that pancreatic α-cells and δ-cells also possess the β-cell type of K_{ATP} channel [15, 16].

Kir6.2 is unable to reach the membrane surface in the absence of SUR1 and vice versa. Both Kir6.2 and SUR1 contain an endoplasmic reticulum retention motif (RKR). This ensures that only fully functional K_{ATP} channels are trafficked to the plasma membrane, as these motifs are only masked when the two subunits associate together [48]. However, truncation at the C-terminus of Kir6.2 at residue 355 (Kir6.2ΔC) deletes the ER retention signal and allows independent surface expression of Kir6.2 [39, 48]. This allows the intrinsic properties of Kir6.2 to be assessed in the absence of SUR1.

Studies of Kir6.2ΔC have allowed specific functions to be assigned to Kir6.2 and SUR1. It is now clear that metabolic regulation of K_{ATP} channel activity is mediated by both Kir6.2 and SUR1, and that the two subunits are able to influence the function of each other. The ATP-binding site responsible for channel closure lies on Kir6.2 [39] whereas MgADP binding to SUR1 opens the channel [39, 41, 42]. MgATP can also stimulate K_{ATP} channel activity via SUR1, but it must first be hydrolysed to MgADP [49]. SUR1 therefore functions as a second metabolic sensor and, when combined with Kir6.2, creates a channel with exquisite sensitivity to changes in adenine nucleotide concentrations [12].

SUR1 has several other effects on Kir6.2 [39, 50, 51]. It increases the channel ATP sensitivity approximately 10-fold; the ATP concentration required to half-maximally close the channel (IC_{50}) decreases from ~100 μM to ~10 μM in the presence of SUR1 [39]. It enhances the open probability of the channel in the absence of nucleotide in excised membrane patches (Po[0]) from 0.1 to around 0.4. Additionally, it confers sensitivity to drugs such as sulphonylureas, which interact directly with SUR1. It appears that Kir6.2 also alters the function of SUR1. In the presence of Kir6.2 the K_m for ATP hydrolysis is greater, suggesting a lower affinity for the K_{ATP} channel complex compared to SUR1 alone [52, 53]. The K_{ATP} channel complex also has a higher turnover rate compared to SUR1 alone, which suggests that Kir6.2 may have an effect similar to substrate activation seen in other ABC transporters such as MRP1 [54, 55].

The IC_{50} for ATP inhibition of K_{ATP} channels in excised patches is ~10 μM, yet cytoplasmic ATP concentrations are millimolar, thus predicting that K_{ATP} channels are ~99% inhibited at physiological nucleotide concentrations. In contrast, estimates of the percentage of open channels at substimulatory glucose concentrations from whole-cell experiments appear to be much greater, ~5–25% [56]. Recently, the open-cell configuration was used to estimate the ATP sensitivity of K_{ATP} channels in intact cells [57]. It was found that channel sensitivity is substantially shifted to higher ATP concentrations, indicating that the excised patch data are not a reliable indicator of the ATP sensitivity of K_{ATP} channels in intact β-cells.

8.3.1 Recent Structural Advances

In order to understand where exactly the nucleotide and drug-binding sites are located on the channel, and how ligand binding leads to changes in channel gating, an atomic resolution structure of the K_{ATP} channel is required. Unfortunately at present the only published structure of the K_{ATP} channel is an electron microscopy map of the purified complex at 18Å resolution [52]. The channel is viewed as a tightly packed complex 13 nm in height and 18 nm in diameter. As expected, the K_{ATP} channel assembles as a central tetrameric Kir6.2 pore surrounded by four SUR1 subunits. However, at this resolution, little, if any, information can be gleaned about ligand-binding sites. A high-resolution structure of either the individual K_{ATP} channel subunits or the entire K_{ATP} channel complex is now essential to bridge the gap between structure and function.

Figure 8.2b shows a Kir6.2 homology model based on the crystal structures of the transmembrane domain of the bacterial KirBac1.1 channel [58] and the cytosolic domain of the eukaryotic GIRK1 channel [59]. The model lends some insight into the location of nucleotide and drug-binding sites on the K_{ATP} channel [60]. When combined with mutagenesis studies, this constitutes a powerful tool in the study of interaction sites on the K_{ATP} channel. The ATP-binding site was elucidated via automated docking. In agreement with a large body of mutagenesis data [5, 6, 61–63], the ATP-binding pocket was predicted to lie at the interface between the cytosolic domains of adjacent Kir6.2 subunits. The residues in the C-terminus of one subunit form the main binding pocket, and residues from the N-terminus of the adjacent subunit also contribute.

Information on the nucleotide-binding sites of SUR1 is also available. Similar to other ABC proteins, SUR1 has two cytosolic domains that contain consensus sequences for ATP binding and hydrolysis. Mutation of residues in the nucleotide-binding domains (NBDs) impair radiolabelled ATP binding and channel activation by Mg nucleotides [41]. Homology modelling of the complete SUR1 protein is not yet possible, due to a lack of high-resolution structures from the ABCC subfamily of ABC proteins, which could be used as a template. However, several models of the NBDs have been generated using other ABC protein structures as a template [64–66]. The high sequence conservation and overall folds of NBDs between ABC proteins suggests that homology models of the NBDs of SUR1 may be a good approximation to reality. Despite this, the transmembrane domains of SUR1 are too divergent from other ABC proteins to model accurately at present.

8.4 Congenital Hyperinsulinism of Infancy

Following cloning of the Kir6.2 and SUR1 genes in 1995, it was discovered that mutations in the two K_{ATP} channel subunits could cause congenital hyperinsulinism of infancy (CHI). This disorder is a clinically heterogeneous disease characterized by continuous, unregulated insulin secretion despite severe hypoglycaemia [67, 68]. Patients usually present with this disorder at birth or shortly afterwards. In the

absence of treatment, blood glucose levels can fall so low that irreversible brain damage results. Most cases of CHI are sporadic, but well-documented familial forms also exist. Sporadic forms have an incidence of around one in 50,000 live births [69] but in some isolated communities the incidence is higher [40, 69].

CHI is a heterogeneous disorder with mutations recorded in the K_{ATP} channel genes (*ABCC8* and *KCNJ11*); the glycolytic enzyme glucokinase (GCK); mitochondrial glutamate dehydrogenase (GLUD1); and short-chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD) [7, 8, 70–72]. CHI is also histologically heterogeneous, both diffuse and focal forms of CHI have been reported. The diffuse form affects all of the β-cells within the islets of Langerhans, whereas in the focal form only an isolated region of β-cells is affected and the surrounding tissue appears normal [68].

8.4.1 ABCC8 *and CHI*

All CHI mutations are loss of function mutations that lead to permanent depolarization of the β-cell membrane. This results in continuous Ca^{2+} influx and insulin secretion, irrespective of the blood glucose level. The most common cause of CHI is mutation of the gene encoding SUR1 (*ABCC8*). SUR1 is located within a region of chromosome 11p15.1 to which a severe form of persistent hyperinsulinemic hypoglycaemia of infancy was initially mapped [8]. Over 20 years after the first mutation was discovered, more than 100 CHI-causing mutations in SUR1, distributed throughout the gene, have now been described. These mutations can be divided into two categories: those that lead to a loss of protein at the membrane surface, and those that result in a permanently closed channel due to an impaired response to MgADP [5].

Many *ABCC8* mutations lead to reduced surface expression of K_{ATP} channels due to abnormal gene expression, protein synthesis, maturation, assembly or membrane trafficking [67, 73–75]. Such mutations are distributed throughout the protein and in general produce a severe phenotype. Other mutations act by reducing the ability of MgADP to activate the channel, so the channels remain closed in response to metabolic inhibition [42, 67, 76]. These mutations cluster within the NBDs of SUR1 where they impair nucleotide binding/hydrolysis. They have also been reported in other regions of SUR1 [77], where they could interfere with Kir6.2-SUR1 coupling or affect MgATP binding/hydrolysis allosterically. In general, mutations of this type result in a less severe phenotype, due to a residual response to MgADP, and some patients can be treated by the K-channel opener diazoxide [67, 76, 78]. However, there is no definite genotype–phenotype correlation and the same mutation can result in CHI of differing severity in different patients.

8.4.2 KCNJ11 *and CHI*

In contrast to SUR1, relatively few CHI mutations have been reported in *KCNJ11* [7, 70, 79]. The mutations that have been reported act by reducing or abolishing

K_{ATP} channel activity in the surface membrane [7, 70, 79]. Interestingly, an H259R mutation has been described that affects both the trafficking and function of the K_{ATP} channel [80].

8.4.3 Therapeutic Implications

In general, mutations in Kir6.2 and SUR1 cause a severe form of CHI that does not respond to diazoxide [67, 79] and requires subtotal pancreatectomy. This occurs due to the absence of K_{ATP} channels. CHI caused by mutations in GCK, GLUD1 or SCHAD respond well to diazoxide [67], as K_{ATP} channel properties are normal. In these patients diazoxide is able to open K_{ATP} channels, which hyperpolarizes the β-cell membrane, and reduces electrical activity and insulin secretion. Genotyping of CHI patients is therefore important in determining the correct therapy.

Interestingly, sulphonylureas and K-channel openers can act as chaperones and rectify trafficking defects associated with some SUR1 mutations [74, 75]. Sulphonylureas restored surface expression of SUR1-A116P and SUR1-V187D [75], and diazoxide corrected trafficking of SUR-R1349H [74]. The resulting K_{ATP} channels have normal nucleotide sensitivity, so drugs with similar chaperone properties, but without channel blocking activity, could be useful in treating some cases of CHI.

8.5 Neonatal Diabetes Mellitus

Neonatal diabetes mellitus (NDM) is defined as hyperglycaemia that presents within the first 3 months of life. It is a rare disorder that affects approximately one in 200,000 live births [81]. Around 50% of cases resolve within 18 months and are named transient neonatal diabetes mellitus (TNDM). The remaining cases require insulin treatment for life and are termed permanent neonatal diabetes mellitus (PNDM) [82]. The majority (~80%) of cases of TNDM are caused by abnormalities of an imprinted locus on chromosome 6q24 that results in the over-expression of a paternally expressed gene [83]. However, heterozygous mutations in Kir6.2 can produce a form of neonatal diabetes that resembles TNDM, that remits, but may subsequently relapse [84–86].

Until recently little was known about the genetic causes of PNDM, and indeed some clinicians denied that it existed at all [12]. It is now known that PNDM does exist and is caused by mutations in a number of genes. Homozygous and compound heterozygous mutations in glucokinase (GCK) have been reported to cause PNDM [87–89]. These are thought to act indirectly by a reduced metabolic generation of ATP, which therefore impairs K_{ATP} channel closure. Several rare syndromes that feature PNDM also exist, including X-linked diabetes mellitus, Wolcott-Rallison syndrome due to mutations in the EIF2AK3 gene, pancreatic agenesis due to mutations in IPF-1 (insulin promoter factor-1) and neonatal diabetes with cerebellar agenesis due to mutations in the PTF-1A gene [90–93].

8.5.1 KCNJ11 *and NDM*

It is now well established that the most common cause of PNDM is heterozygous activating mutations in the *KCNJ11* gene encoding Kir6.2 [5]. The majority of these mutations arise spontaneously. One class of mutations, such as R50P and R201H [6, 62] cause PNDM alone. Other mutations, such as Q52R and I296L, cause a severe phenotype in which PNDM is accompanied by neurological features such as developmental delay, muscle weakness and epilepsy; a condition known as DEND syndrome [6, 81, 94–98]. Intermediate DEND (i-DEND) is a less severe clinical syndrome in which patients show neonatal diabetes, developmental delay and/or muscle weakness, but not epilepsy [6, 81, 94–96, 98].

Early evidence for the role of Kir6.2 in PNDM came from the generation of a mouse model that over-expressed a mutant K_{ATP} channel in the pancreatic β-cells [99]. When the N-terminal deletion mutant Kir6.2[ΔN2-30] is expressed in COSm6 cells it results in a channel with 10-fold lower ATP sensitivity than wild-type K_{ATP} channels. Transgenic mice expressing this mutation in β-cells showed severe hyperglycaemia, hypoinsulinemia and ketoacidosis within 2 days of birth and died within 5 days.

To date, over thirty gain-of-function mutations in Kir6.2 associated with PNDM have been identified, the most common being at residues R201 and V59 [84, 86]. Strikingly, these mutations cluster around the predicted ATP-binding site, or are located in regions of the protein thought to be involved in channel gating such as the slide helix, the cytosolic mouth of the channel, or gating loops linking the ATP-binding site to the slide helix. They may also affect residues involved in interaction with SUR1.

A strong, but not absolute, correlation between genotype and phenotype appears to exist for Kir6.2 mutations. For example, of 24 patients with mutations at R201, all but three have non-remitting neonatal diabetes without neurological features. Of 13 patients with the V59M mutation, 10 have developmental delay and symptoms consistent with i-DEND syndrome [86]. Mutations that are associated with full DEND syndrome are not found in less severely affected patients. Conversely, two of four patients with the C42R mutation did not develop diabetes until early adulthood, one patient developed transient neonatal diabetes and one exhibited diabetes at 3 years of age [100]. Therefore, as observed for other types of monogenic diabetes, genetic background and environmental factors may influence the clinical phenotype [101, 102].

8.5.2 Location of NDM Mutations in the Kir6.2 Subunit

Residues in Kir6.2 that, when mutated, cause neonatal diabetes cluster in several distinct locations: (i) the putative ATP-binding site of Kir6.2 (R50, I192, R201 and F333); (ii) the interfaces between Kir6.2 subunits (F35, C42 and E332); (iii) the interface between Kir6.2 and SUR1 subunits (Q52, G53); and (iv) parts of the

channel implicated in channel gating (V59, C166, I197, I296). Most (but not all) mutations that cause additional neurological complications are located further away from the ATP-binding site. For example, Q52 lies within the cytosolic part of the N-terminal domain, which is thought to be involved in the coupling of SUR1 to Kir6.2 [98, 103, 104]. Residue G53 has been proposed to form a gating hinge, which permits flexibility of the N-terminus of the protein, allowing the induced fit of ATP at the ATP-binding site [105]. Residue V59 lies within the slide helix, a region of the protein implicated in the gating of the pore [58, 60, 98, 106]. C166 lies close to the helix bundle crossing, which is suggested to form an inner gate to the channel [107] and I197 is located within the permeation pathway, in an area thought to be involved in channel gating [60, 108]. Recently, a gating mutation at residue I296, which causes DEND syndrome, suggested the existence of a novel gate within the cytosolic pore of Kir6.2 [97]. This was further supported by recent structural data [109]. Mutations of the same residue may result in different phenotypes; for example, the R50Q mutation causes neonatal diabetes alone, while R50P causes DEND syndrome [62].

8.5.3 Functional Effects of Kir6.2 Mutations Causing NDM

The effects of more than 20 Kir6.2 NDM mutations on the properties of the K_{ATP} channel have been investigated by heterologous expression of recombinant channels, in systems such as *Xenopus* oocytes [5, 57, 61, 62, 85, 86, 97, 98, 105, 106, 110–113]. All NDM mutations are gain-of-function mutations that decrease the ability of MgATP to block the K_{ATP} channel. This reduction in ATP sensitivity means there is an increased K_{ATP} current at physiological concentrations of ATP (~1–5 mM). In β-cells, such an increase in K_{ATP} current is predicted to produce hyperpolarization, which suppresses electrical activity, calcium influx and insulin secretion. The greater the increase in K_{ATP} current, the more severely insulin secretion will be impaired.

Functional analysis reveals that all Kir6.2 mutations studied to date act by reducing the ATP sensitivity of Kir6.2 via two major mechanisms. These are schematically depicted using a simple allosteric channel-gating scheme in Fig. 8.3b. Mutations at residues within the Kir6.2 ATP-binding site are expected to reduce the inhibitory effect of nucleotides by impairing binding directly. Pure binding defects will reduce the binding constants of both open (K_O) and closed states (K_C) of the channel by equal factors (Fig. 8.3b, left) and produce a parallel shift of the ATP dose–response curve to the right of wild-type. Such mutations will have no effect on channel gating in the absence of the nucleotide (Fig. 8.3a, compare top and middle traces). Conversely, mutations in gating regions of the channel reduce the inhibitory effect of ATP indirectly, by biasing the channel towards the open state, and impairing its ability to close both in the absence (E_O) and presence (E_A) of bound ATP (Fig. 8.3b right, [97, 98]). A decrease in E_O enhances the open probability of the channel, $P_O(0)$ (Fig. 8.3a, bottom trace). ATP inhibition is diminished by both a

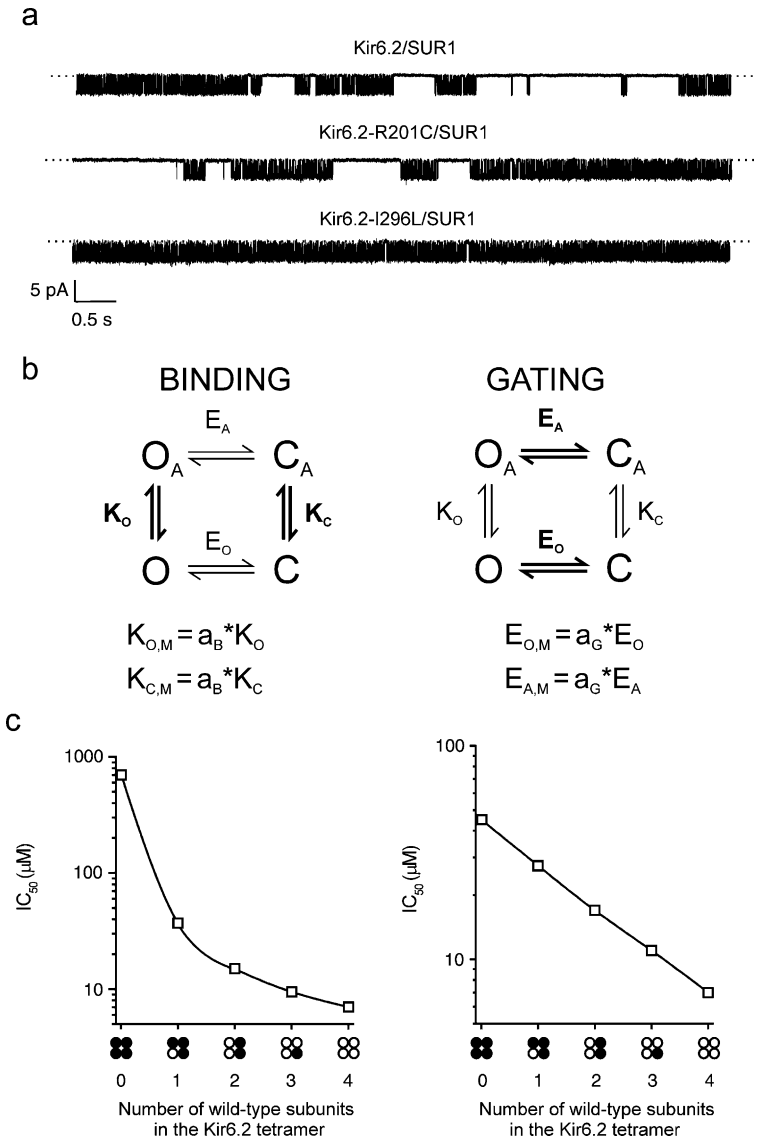

Fig. 8.3 Molecular mechanisms of NDM mutations in the Kir6.2 subunit. (**a**) Single K_{ATP} channel currents recorded from an inside-out patch at –60 mV in nucleotide-free solution (*top trace*) of wild-type (*top*), a mutant channel with a point mutation R201C that is predicted to lie within the ATP-binding site (*middle*) and a mutant channel with a gating mutation I296L that dramatically increases channel open probability $P_O(0)$ (*bottom*). Channel openings are facing downwards; the dotted line represents closed channel level. The open states are clustered into bursts of openings, separated by long closed interburst intervals. Transitions between states within bursts are thought to be governed by a "fast gate" of the channel and are little affected by nucleotides [147]. Transitions between burst and interburst states are thought to be governed by a separate "slow gate" (or gates) and are strongly modulated by nucleotides [148]. (**b**) Allosteric scheme for "slow" K_{ATP} channel

decrease in E_A, which reduces the destabilizing effect of ATP on the open state, and a decrease in E_O, which reduces the availability of closed states to which ATP binds with higher affinity ($K_C > K_O$).

Kir6.2 mutations can also reduce ATP inhibition via a third mechanism. They could alter the transduction of conformational changes in the ATP-binding pocket to the channel gate (in Fig. 8.3b, these mutations will alter the E_A/E_O and K_C/K_O ratios). For gain-of-function mutations, this would mean a relative increase in ATP binding to the open state of the channel, resulting in a detectable fraction of ATP-resistant current at very high ATP concentrations in Mg-free solutions. This effect has indeed been observed; for example, with mutations at K185, which is predicted to lie within the putative ATP-binding site (e.g. K185E, 111). Recently, a PNDM-causing mutation with similar properties has been found at this residue (K185Q, K. Shimomura, unpublished observations).

Most mutations that impair channel inhibition by ATP without altering channel open probability in nucleotide-free solutions ($P_O[0]$) are associated with neonatal diabetes alone (Fig. 8.4). These mutations lie within the predicted ATP-binding site of Kir6.2 [6, 61, 62, 98, 114, 115]. The electrophysiological data are consistent with this view, but biochemical studies are required for confirmation. Most mutations associated with neurological features affect ATP inhibition indirectly by altering channel gating [85, 97, 98, 110]. It is worth noting that some of these mutations may have additional effects to those on gating (i.e. on ATP-binding or on transduction); however, since the mechanism of channel gating is quite complex, it has not yet been determined whether this is the case.

Fig. 8.3 (continued) gating. For simplicity, all interburst closed states are lumped in a single closed state C and all burst states into a single open state, O. In the absence of the nucleotide, the channel alternates between open and closed states with a gating constant E_O. Both O and C states can bind ATP with corresponding binding constants K_O and K_C. In the ATP-bound form, the channel alternates between open and closed states with an altered gating constant E_A ($E_A = K_C \times E_O/K_O$). A binding mutation (left) affects binding constants for ATP to open (K_O) and closed states (K_C) by the same factor, a_B (for a decrease in ATP binding, $a_B < 1$). A gating mutation affects gating constants in the absence (E_O) and presence (E_A) of ATP by the same factor, a_G (for increase in $P_O(0)$, $a_G < 1$). Index M in all equations refers to mutant channels. (**c**), *Left:* Relationship between the IC_{50} for ATP inhibition and the number of wild-type subunits for heteromeric K_{ATP} channels composed of wild-type subunits or mutant subunits with impaired ATP binding ($a_B = 0.01$ in b) in the absence of Mg^{2+} using a simple concerted gating model (Monod-Wyman-Changeux, [56]). $P_O(0)$ of all channels is 0.4. The corresponding tetrameric channel species are shown schematically below (*open circles*, wild-type Kir6.2 subunits, *filled circles*, mutant Kir6.2 subunits). *Right:* Relationship between the IC_{50} for ATP inhibition and the number of wild-type subunits for heteromeric K_{ATP} channels composed of wild-type subunits ($P_O(0)$ of the wild-type was set to 0.4) or subunits of a gating mutant ($P_O(0)$ of the homomeric mutant was set to 0.82) in the absence of Mg^{2+} using a simple concerted gating model (Monod-Wyman-Changeux, [56]). The corresponding tetrameric channel species are shown schematically below (*open circles*, wild-type Kir6.2 subunits, *filled circles*, mutant Kir6.2 subunits). In all simulations, the K_O for ATP binding to the open state was assumed to be $0.003\,\mu M^{-1}$ [117]; for the closed states, K_C was determined from the IC_{50} of wild-type channels ($7\,\mu M$) with $P_O(0) = 0.4$ ($K_C = 0.05\,\mu M^{-1}$)

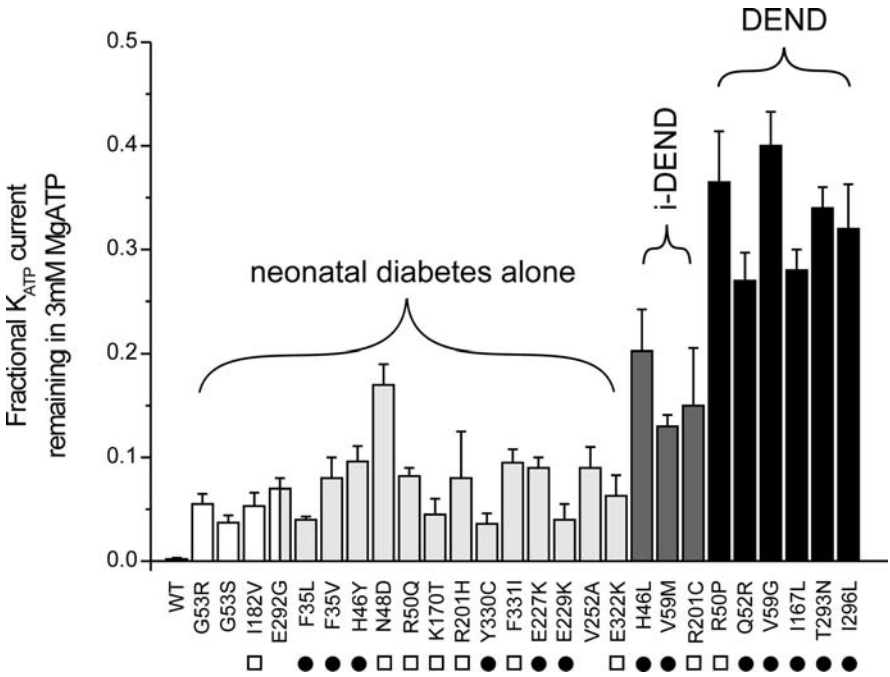

Fig. 8.4 ATP sensitivity correlates with disease severity but not molecular mechanism. Macroscopic current in 3 mM MgATP in excised patches expressed as a fraction of that in nucleotide-free solution of wild-type (WT) K_{ATP} channels and heterozygous K_{ATP} channels containing the indicated Kir6.2 mutations. For neonatal diabetes caused by Kir6.2 mutations, disease severity correlates with the extent of unblocked K_{ATP} current. Different phenotypes can be produced by the same molecular mechanism: i.e. impaired ATP binding (*open squares*) or changes in gating (*filled circles*). For those mutations without symbols, no single-channel kinetics have been measured and the molecular mechanism is unclear. *White bars* indicate mutations associated with TNDM; *pale grey bars* mutations causing PNDM; *dark grey bars* mutations causing i-DEND and *black bars* mutations producing DEND syndrome. Data are taken from [62, 63, 85, 97, 98, 110, 111, 149, 150]

So far we have only considered the effects of NDM mutations on ATP inhibition in Mg^{2+}-free solutions. Functional studies in the presence of Mg^{2+} demonstrated that a reduction of ATP inhibition due to Mg-nucleotide activation is much more pronounced in NDM mutant channels than in wild-type channels [106]. It is not clear whether this enhancement of the Mg-nucleotide activatory effect results from impaired ATP inhibition caused by NDM mutations, or whether these mutations also have a direct effect on channel activation by Mg-ATP/ADP. For mutations that are predicted to cause defects in ATP binding, the addition of Mg^{2+} predominantly produced a parallel shift of the ATP dose–response curve to the right ([99]; unless the channel was completely ATP insensitive in Mg-free solutions as seen for G334D and R50P [61, 62]). In contrast, for gating mutations the addition of Mg^{2+} could also dramatically increase the fraction of channel current insensitive to ATP [106].

8.5.4 Heterozygosity of Kir6.2 Mutations

All NDM patients with mutations in Kir6.2 are heterozygous. In functional studies, the heterozygous state is simulated by coexpression of wild-type and mutant Kir6.2 subunits with SUR1. Since Kir6.2 is a tetramer [34] there will exist a mixed population of channels, containing between zero and four mutant subunits. Assuming equal expression levels of wild-type and mutant Kir6.2 subunits and random mixing between them, the various channel species in the heterozygous mixture will follow a binomial distribution.

Functional studies have shown that, for binding mutations, the ATP sensitivity of the heterozygous population is close to that of the wild-type channel. In contrast, for gating mutations, the ATP sensitivity tends to be more intermediate between that of wild-type and homomeric mutant channels. This is consistent with our current understanding of the gating mechanism of the channel, that assumes one ATP molecule is able to close the channel [116] and that during gating the four Kir6.2 subunits move simultaneously in a concerted manner [117, 118]. Figure 8.3c shows predicted ATP inhibition IC_{50} values for heteromeric channel species composed of wild-type and mutant subunits with impaired binding (left) and gating (right) using a simple concerted model. It is clear that if a mutation affects ATP binding alone, only channels with four mutant subunits will have a markedly reduced ATP sensitivity (Fig. 8.3c, left). Homomeric mutant channels will account for only one-sixteenth of the channel population, thus the shift in ATP sensitivity compared to wild-type will be small. Heteromeric channels containing subunits with impaired gating have more evenly distributed IC_{50} values between that of the wild-type and homomeric mutant channel (Fig. 8.3c, right). Accordingly, the corresponding heterozygous mixture would have larger shift in ATP sensitivity with regard to that of the wild-type.

In the presence of Mg^{2+}, IC_{50} values for ATP inhibition of heterozygous channels with binding mutations are more dramatically increased (~10-fold) than those of the wild-type channel (~2-fold; [106]). Since the mechanism of channel activation by Mg-nucleotides and its interaction with the inhibitory action of ATP is poorly understood, this effect has not been addressed with modelling. In addition to an increase in the IC_{50}, heterozygous channels containing Kir6.2 subunits with impaired gating also show a substantial fraction of ATP-insensitive current [106, 111]. A similar effect is observed for heterozygous channels with defects in ATP binding that render homomeric mutant channels completely ATP insensitive [61, 62].

As illustrated in Fig. 8.4, all NDM-Kir6.2 mutations increase the current of heterozygous channels at 3 mM MgATP at least 20-fold, with DEND mutations having the greatest effect. There is no obvious correlation between the magnitude of the K_{ATP} current and whether the mutation causes permanent or relapsing–remitting neonatal diabetes. There is also no correlation between the phenotype and the molecular mechanism, as mutations causing defects in gating and binding can result in both NDM alone or more severe forms of NDM with neurological complications.

The importance of heterozygosity in determining the severity of a mutation appears to be a novel feature of K_{ATP} channelopathies. It is also worth noting that if mutant and wild-type Kir6.2 subunits were to express at different levels, or if they did not mix in a random fashion to form heteromers, the composition of the heterozygous population would deviate from a binomial distribution and thus influence the channel ATP sensitivity in a less quantitatively predictable fashion.

8.5.5 ABCC8 *and PNDM*

Activating mutations in SUR1 have also been shown to cause neonatal diabetes. Unlike Kir6.2 mutations, not all NDM patients with SUR1 mutations are heterozygous and some patients have compound, mosaic or homomeric mutations. These mutations are scattered throughout the protein sequence, but are particularly concentrated in the first five transmembrane helices (TMD0) and their connecting loops, in the CL3 linker, which is a long cytosolic loop connecting TMD0 to TMD1, and NBD2 [55]. SUR1 mutations can act in two main ways: (i) reducing the inhibition produced by ATP binding at Kir6.2 [119, 120], and (ii) enhancing channel activation by Mg-nucleotides [65, 121]. Both lead to a greater K_{ATP} current at a particular MgATP concentration [53, 61, 65, 122].

Mutations in SUR1 that decrease the amount of inhibition at Kir6.2 may do so in one of two ways. Firstly, they could reduce ATP binding directly. It is well established that the presence of SUR1 enhances ATP inhibition at Kir6.2, which suggests that SUR1 either contributes to the ATP-binding site itself, or influences it allosterically [39]. Disruption of this could reduce ATP binding directly, although it should be noted that no mutation has yet been shown to act in this way. Alternatively, SUR1 mutations could disrupt ATP inhibition indirectly by increasing the channel open probability. The F132L mutation has been shown to act in this way [119, 120]. F132 lies within the TMD0 region of SUR1, a region which is known to be involved in modulating channel gating [123]. Heterologous expression of F132L demonstrated that it increases the duration of K_{ATP} channel openings and decreases the duration and frequency of the inter-burst closed states. This shift in gating equilibrium towards the open state of the channel indirectly reduces ATP inhibition.

K_{ATP} channel activity is determined by both the extent of ATP block at Kir6.2 and Mg-nucleotide activation at the NBDs of SUR1. Hence gain-of-function SUR1 mutations may act to reduce the overall ATP inhibition by enhancing Mg-nucleotide activation. Many SUR1 mutations that lead to PNDM are found in NBD2 [121, 122, 124]. Only one SUR1-PNDM mutation is found in NBD1, and interestingly this mutation lies in the linker that is predicted to form part of NBD2 [65]. As predicted from their locations in NBD1 and NBD2 respectively, R826W and R1380L alter ATPase activity [65, 121]. The former reduces ATPase activity, whereas the latter increases it, yet they both increase MgATP activation of the K_{ATP} channel**.** How can this be resolved? It appears that both mutations increase the probability of SUR1 being in an MgADP-bound state, which enhances channel activity. R1380L

appears to accelerate the catalytic cycle, so that the protein spends less time in the pre-hydrolytic ATP-bound state [121]. R826W acts differently by slowing the rate at which P_i dissociates following ATP hydrolysis and thus halting the cycle in the MgADP-bound post-hydrolytic state [65]. Although it hasn't yet been demonstrated, SUR1 mutations may also enhance the way nucleotide binding to the NBDs of SUR1 is transduced to channel gate.

8.5.6 Mouse Models of PNDM

Mouse models often yield important insights into the molecular mechanisms of human disorders. Neonatal diabetes is no exception, and both gain- and loss-of-function K_{ATP} channel mouse models have been generated. These have allowed PNDM and CHI to be understood in far greater detail than is possible through expression of mutant K_{ATP} channels in heterologous systems.

As mentioned earlier, the first evidence that gain-of-function K_{ATP} channel mutations cause severe neonatal diabetes came from the generation of a mouse model that over-expresses the N-terminal deletion mutant Kir6.2 [ΔN2–30] in β-cells [99]. In these mice, no change in islet architecture, β-cell number or insulin content was observed. Nevertheless serum insulin levels were extremely low, as expected from the decreased ATP sensitivity of this mutant K_{ATP} channel [99]. As a result these mice show severe hypoglycaemia, and typically die within 2 days. Intriguingly mice in which the mutant gene was expressed in the heart had no obvious cardiac symptoms [125], as was subsequently found for human patients with gain-of-function K_{ATP} channel mutations [5, 6]. Additionally mice in which the mutant gene was expressed at a lower level did not develop PNDM, but instead had impaired glucose tolerance [126]. This provides evidence that mice, as well as humans, develop a spectrum of diabetes phenotypes that correlate with the extent of K_{ATP} channel activity.

In another mouse model a dominant-negative Kir6.2 mutation (Kir6.2-G132S) was introduced into pancreatic β-cells under the control of the human insulin promoter [127]. Animals in which K_{ATP} channel was functionally inactivated by this mutation initially exhibited hyperinsulinaemia, despite severe hypoglycaemia, indicating unregulated insulin secretion, which produces a phenotype resembling CHI. Subsequently, adult mice developed hyperglycaemia and glucose-induced insulin secretion was reduced due to substantial β-cell loss [127]. In the β-cells of transgenic mice the resting membrane potential and basal intracellular calcium concentration were significantly higher than in wild-type mice. Transgenic mice also appeared to have abnormal pancreatic islet architecture. In complete contrast, mice expressing a different dominant-negative Kir6.2 mutation ($Kir6.2^{132}A^{133}A^{134}A$) showed no β-cell loss and developed hyperinsulinism as adults [128]. It was suggested that the opposite phenotype of these mice might arise because K_{ATP} channel activity was only partially suppressed (30% of β-cells were unaffected). Patients with CHI usually undergo sub-total pancreatectomy as infants to control hyperinsulinism,

however, non-surgically treated patients often progress to glucose intolerance or diabetes [129]. The mouse models suggest that this may reflect a gradual β-cell loss.

Both Kir6.2 and SUR1 knockout mice have been generated [130, 131]. In the case of Kir6.2$^{-/-}$ mice, electrophysiological recordings showed that K_{ATP} channel activity was completely absent in pancreatic β-cells [130]. These mice showed transient hypoglycaemia as neonates, but adult mice had reduced insulin secretion in response to glucose and were normoglycaemic. It was suggested that the normoglycaemia could be due to an increased glucose lowering effect of insulin in these animals, but the precise mechanism remains unclear. SUR1$^{-/-}$ mice also had markedly reduced glucose-induced insulin secretion but normoglycaemia [131]. Both Kir6.2$^{-/-}$ and SUR1$^{-/-}$ mice showed a graded glucose-induced rise in intracellular Ca^{2+} and insulin exocytosis, indicating the presence of a K_{ATP}-independent amplifying pathway in glucose-induced insulin secretion [132–135].

Recently, a novel mouse model (β-V59M), which expresses one of the most common Kir6.2 mutations found in PNDM patients, was created [136]. In human patients, the V59M mutation is the most common cause of i-DEND syndrome [86]. Importantly, these mice express the V59M Kir6.2 subunit specifically in their pancreatic β-cells. They appear to express comparable levels of WT and V59M Kir6.2 mRNA in pancreatic islets, which is key, when considering the heterozygosity of human patients. The β-V59M mice develop severe diabetes soon after birth and by 5 weeks of age blood glucose levels are increased and insulin levels are undetectable. Islets isolated from these mice secreted less insulin and showed smaller increases in intracellular calcium concentrations in response to glucose, compared to wild-type mice. The data also showed that the pancreatic islets had a reduced percentage of β-cell mass, an abnormal morphology and lower insulin content.

A set of similar mouse models were generated by Remedi et al. in which an ATP-insensitive Kir6.2 mutant, K185Q-ΔN30, was expressed specifically in pancreatic β-cells either from birth or following induction by tamoxifen [137]. These mice develop severe glucose intolerance around 3 weeks of age, or within 2 weeks of tamoxifen injection and progress to severe diabetes. The disease state can be avoided by islet transplantation or early-onset sulphonylurea therapy.

Whilst the generation of mouse models of PNDM has provided insights into the patho-physiology of the pancreas in this disease, it has yielded little information about the extra-pancreatic symptoms associated with i-DEND and DEND syndrome. It is clear that the neurological features associated with K_{ATP} channel mutations constitute a distinct syndrome rather than a secondary consequence of diabetes. Evidence for this includes the fact that developmental delay is not a feature of neonatal diabetes from other causes [88, 92, 138]; that there is a strong genotype–phenotype relationship between the functional severity of mutations and the clinical phenotype observed; and that the neurological features are consistent with the tissue distribution of the K_{ATP} channel in muscle, neurons and the brain [9, 36]. However, it remains to be understood precisely how mutations in the K_{ATP} channel lead to muscle weakness, epilepsy and developmental delay. Investigation

into this remains a challenge for researchers in this field, and solving the mystery is likely to require further animal models.

8.5.7 Implications for Therapy

Prior to the discovery that PNDM can be caused by mutations in Kir6.2 and SUR1, many patients were assumed to be suffering from early-onset type 1 diabetes. Accordingly they were treated with insulin injections. Recognition that PNDM patients actually possess gain of function mutations in K_{ATP} channel genes rapidly led to a switch to sulphonylurea treatment. Sulphonylureas are drugs such as tolbutamide or glibenclamide that specifically block the K_{ATP} channel and thus stimulate insulin secretion. Fortunately, since sulphonylureas had been used to safely treat patients with type 2 diabetes for many years, no clinical trials were required.

To date, more than 100 patients with *KCNJ11* mutations have successfully transferred from insulin injections to sulphonylurea therapy [139]. More than 90% of all "insulin-dependent" patients with Kir6.2 mutations can be managed by sulphonylureas alone [139]. Not only does this improve their quality of life, it also appears to enhance their blood glucose control. Fluctuations in blood glucose are reported to be reduced [140] and there is a decrease in the HbA1C levels, which provide a measure of the average blood glucose level during the preceding weeks [139]. This improvement in glycaemic control is predicted to reduce the risk of diabetic complications [141, 142].

Interestingly, oral glucose is more effective than intravenous glucose at eliciting insulin secretion in nondiabetics and patients treated with sulphonylureas alike [139]. Oral glucose triggers the release of incretins such as gastrointestinal peptide (GIP) and glucagon-like-peptide (GLP-1) from the gut. These hormones do not activate insulin secretion alone, as they are unable to close K_{ATP} channels. If, however, intracellular calcium levels are elevated by prior closure of K_{ATP} channels, they are able to amplify insulin secretion [1]. Prior to sulphonylurea therapy, incretins have no effects in PNDM patients with K_{ATP} channel mutations, as their mutant K_{ATP} channels remain open at very high blood glucose levels [139]. Following treatment with sulphonylureas the mutant K_{ATP} channels close and incretins are able to amplify insulin secretion.

Sulphonylureas are very successful at treating patients with K_{ATP} channel mutations that cause PNDM without neurological complications. These mutations have little or no effect on sulphonylurea block of the K_{ATP} channel [139, 140]. As summarized in Fig. 8.5, in functional studies, these heterozygous channels remain almost as sensitive to tolbutamide inhibition as wild-type channels, being inhibited between 72 and 96% by 0.5 mM of the drug [139]. In contrast, patients with mutations that were blocked by <65% by tolbutamide (Fig. 8.5, open bars) did not respond to drug therapy. In most cases, sulphonylureas are not effective in DEND patients with Kir6.2 mutations that greatly enhance $P_O(0)$, because of the inability of sulphonylureas to sufficiently block the K_{ATP} channel. Similar to the effect on ATP

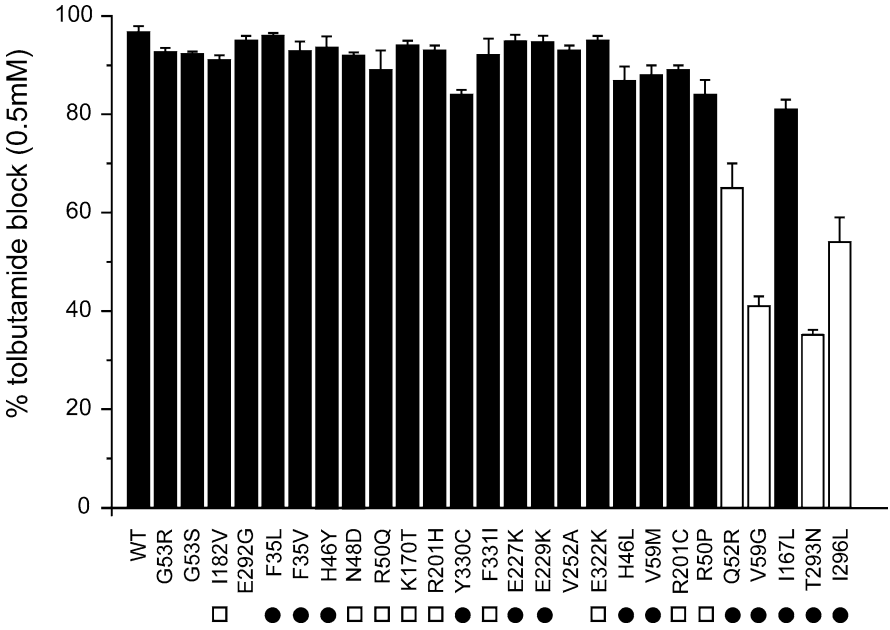

Fig. 8.5 The efficiency of sulphonylurea block of K_{ATP} channels with NDM mutations in the Kir6.2 subunit. An estimate of the percentage of the tolbutamide block (0.5 mM) of the whole-cell current of wild-type (WT) K_{ATP} channels and heterozygous K_{ATP} channels containing the indicated Kir6.2 mutations. Data were estimated by expressing the block in the presence of tolbutamide and 3 mM azide as a percentage of the current in azide alone. *Black bars* indicate mutant channels without significantly reduced sensitivity to tolbutamide. *White bars* indicate mutant channels with significantly impaired sulphonylurea sensitivity; patients carrying these mutations were unable to switch to treatment with sulphonylureas. Data are taken from [62, 63, 85, 97, 98, 110, 111, 149, 150]

block, K_{ATP} channel mutations that enhance the channel $P_O(0)$ also impair block by sulphonylureas, and patients with mutations that greatly enhance $P_O(0)$ are thus less likely to be able to transfer to sulphonylurea treatment [56, 139].

There is increasing evidence that sulphonylureas may be able to improve the muscle weakness found in patients with i-DEND syndrome [111, 143]. They may also be able to improve their motor and mental developmental delay. This is significant, since insulin cannot ameliorate the extra-pancreatic symptoms of i-DEND and DEND patients. Sulphonylureas may be able to do so by closing over-active K_{ATP} channels in the brain and muscle, as well as the pancreas.

8.5.8 Kir6.2 and Type 2 Diabetes

Given that mutations in Kir6.2 cause neonatal diabetes by decreasing insulin release, it follows that common genetic variations in the same gene, which produce smaller

functional effects, may lead to type 2 diabetes later in life. In fact the common Kir6.2 variant, E23K, is strongly linked to an increased risk of type 2 diabetes [144–146]. The increase in risk is modest, the odds ratio is 1.2, but the high prevalence of the K allele (34%) makes this a significant population risk. However, it remains unclear precisely how the E23K polymorphism enhances type 2 diabetes susceptibility.

8.6 Conclusions and Future Directions

Despite over 25 years of intense research into the K_{ATP} channel, many mysteries remain. Where exactly nucleotides and therapeutic drugs bind, and how this binding modulates K_{ATP} channel gating is still unclear. Insight into this requires high-resolution structural information on Kir6.2 and SUR1 and more detailed functional analyses. Further studies of naturally occurring mutations will be valuable in highlighting key K_{ATP} channel residues. Electrophysiological studies, in combination with biochemical experiments, on the intact K_{ATP} complex are required to understand how exactly these mutations function. The structure of the entire K_{ATP} channel complex would be even more valuable in elucidating how the interaction of nucleotides and drugs with SUR1 is communicated to Kir6.2.

At the clinical level, the discovery that Kir6.2 mutations cause neonatal diabetes has resulted in a major change in treatment for PNDM patients. Most patients are able to successfully transfer from insulin injections to sulphonylurea tablets, with the additional bonus of improving their glycaemic control upon doing so. It remains to be determined the extent to which sulphonylureas can improve the extra-pancreatic symptoms of patients with i-DEND and DEND syndrome and it is unclear why older patients respond less well to sulphonylurea treatment. Tying in with this, the mechanism by which severe Kir6.2 mutations cause muscle weakness, developmental delay and epilepsy remains to be elucidated. These questions are likely to require intense investigation and will not be easy to achieve. Nonetheless the quest for answers about the K_{ATP} channel promises to be an exciting and fruitful adventure.

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Chapter 9
Role of Mitochondria in β-cell Function and Dysfunction

Pierre Maechler, Ning Li, Marina Casimir, Laurène Vetterli, Francesca Frigerio, and Thierry Brun

Abstract Pancreatic β-cells are poised to sense glucose and other nutrient secretagogues to regulate insulin exocytosis, thereby maintaining glucose homeostasis. This process requires translation of metabolic substrates into intracellular messengers recognized by the exocytotic machinery. Central to this metabolism-secretion coupling, mitochondria integrate and generate metabolic signals, thereby connecting glucose recognition to insulin exocytosis. In response to a glucose rise, nucleotides and metabolites are generated by mitochondria and participate, together with cytosolic calcium, to the stimulation of insulin release. This review describes the mitochondrion-dependent pathways of regulated insulin secretion. Mitochondrial defects, such as mutations and reactive oxygen species production, are discussed in the context of β-cell failure that may participate to the etiology of diabetes.

Keywords Pancreatic β-cell · Insulin secretion · Diabetes · Mitochondria · Amplifying pathway · Glutamate · Reactive oxygen species

9.1 Introduction

The primary stimulus for pancreatic β-cells is in fact the most common nutrient for all cell types, i.e., glucose. Tight coupling between glucose metabolism and insulin exocytosis is required to physiologically modulate the secretory response. Accordingly, pancreatic β-cells function as glucose sensors with the crucial task of perfectly adjusting insulin release to blood glucose levels. Homeostasis depends on the normal regulation of insulin secretion from the β-cells and the action of insulin on its target tissues. The initial stages of type 1 diabetes, before β-cell destruction, are characterized by impaired glucose-stimulated insulin secretion. The

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large majority of diabetic patients are classified as type 2 diabetes, or noninsulin-dependent diabetes mellitus. The patients display dysregulation of insulin secretion that may be associated with insulin resistance of liver, muscle, and fat.

The exocytotic process is tightly controlled by signals generated by nutrient metabolism, as well as by neurotransmitters and circulating hormones. Through its particular gene expression profile, the β-cell is poised to rapidly adapt the rate of insulin secretion to fluctuation in the blood glucose concentration. This chapter describes the molecular basis of metabolism–secretion coupling in general and in particular how mitochondria function both as sensors and generators of metabolic signals. Finally, we will describe mitochondrial damages associated with β-cell dysfunction.

9.2 Overview of Metabolism–Secretion Coupling

Glucose entry within the β-cell initiates the cascade of metabolism–secretion coupling (Fig. 9.1). Glucose follows its concentration gradient by facilitative diffusion through specific transporters. Then, glucose is phosphorylated by glucokinase, thereby initiating glycolysis [1]. Subsequently, mitochondrial metabolism generates ATP, which promotes the closure of ATP-sensitive K+ channels (K_{ATP}-channel) and, as a consequence, depolarization of the plasma membrane [2]. This leads to Ca^{2+} influx through voltage-gated Ca^{2+} channels and a rise in cytosolic Ca^{2+} concentrations triggering insulin exocytosis [3].

Additional signals are necessary to reproduce the sustained secretion elicited by glucose. They participate in the amplifying pathway [4] formerly referred to as the K_{ATP}-channel-independent stimulation of insulin secretion. Efficient coupling of glucose recognition to insulin secretion is ensured by the mitochondrion, an organelle that integrates and generates metabolic signals. This crucial role goes far beyond the sole generation of ATP necessary for the elevation of cytosolic Ca^{2+} [5]. The additional coupling factors amplifying the action of Ca^{2+} (Fig. 9.1) will be discussed in this chapter.

9.3 Mitochondrial NADH Shuttles

In the course of glycolysis, i.e., upstream of pyruvate production, mitochondria are already implicated in the necessary reoxidation of NADH to NAD^+, thereby enabling maintenance of glycolytic flux. In most tissues, lactate dehydrogenase ensures NADH oxidation to avoid inhibition of glycolysis secondary to the lack of NAD^+ (Fig. 9.2). In β-cells, according to low lactate dehydrogenase activity [6], high rates of glycolysis are maintained through the activity of mitochondrial NADH shuttles, thereby transferring glycolysis-derived electrons to mitochondria [7]. Early evidence for tight coupling between glycolysis and mitochondrial activation came from studies showing that anoxia inhibits glycolytic flux in pancreatic islets [8].

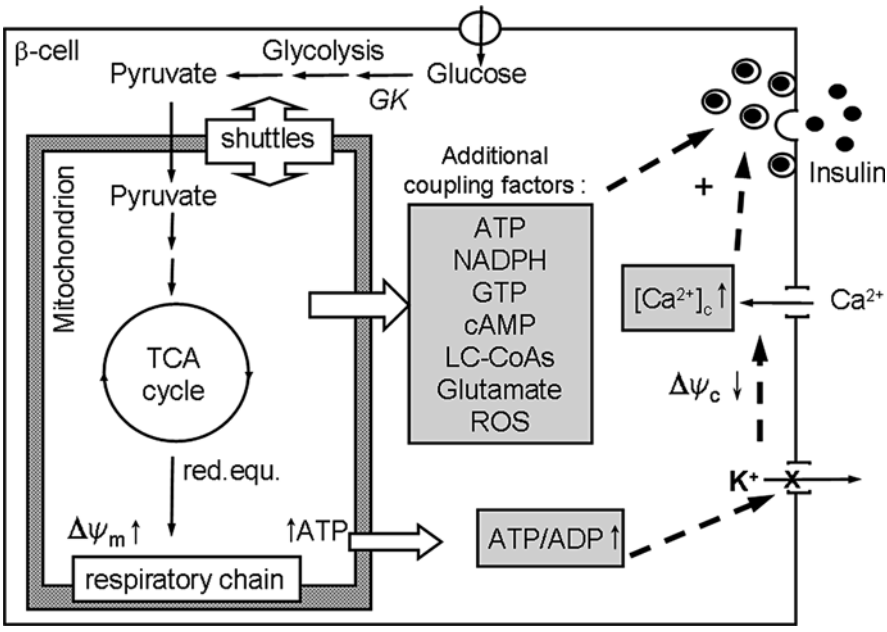

Fig. 9.1 Model for coupling of glucose metabolism to insulin secretion in the β-cell. Glucose equilibrates across the plasma membrane and is phosphorylated by glucokinase (GK). Further, glycolysis produces pyruvate, which preferentially enters the mitochondria and is metabolized by the TCA cycle. The TCA cycle generates reducing equivalents (red. equ.), which are transferred to the electron transport chain, leading to hyperpolarization of the mitochondrial membrane ($\Delta\Psi_m$) and generation of ATP. ATP is then transferred to the cytosol, raising the ATP/ADP ratio. Subsequently, closure of K_{ATP}-channels depolarizes the cell membrane ($\Delta\Psi_c$). This opens voltage-dependent Ca^{2+} channels, increasing cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), which triggers insulin exocytosis. Additive signals participate to the amplifying pathway of metabolism–secretion coupling

Therefore, NADH shuttle systems are necessary to couple glycolysis to activation of mitochondrial energy metabolism, leading to insulin secretion.

The NADH shuttle system is composed essentially of the glycerophosphate and the malate/aspartate shuttles [9], with its respective key members mitochondrial glycerol phosphate dehydrogenase and aspartate–glutamate carrier (AGC). Mice lacking mitochondrial glycerol phosphate dehydrogenase exhibit a normal phenotype [10], whereas general abrogation of AGC results in severe growth retardation, attributed to the observed impaired central nervous system function [11]. Islets isolated from mitochondrial glycerol phosphate dehydrogenase knockout mice respond normally to glucose regarding metabolic parameters and insulin secretion [10]. Additional inhibition of transaminases with aminooxyacetate, to nonspecifically inhibit the malate/aspartate shuttle in these islets, strongly impairs the secretory response to glucose [10]. The respective importance of these shuttles is indicated in islets of mice with abrogation of NADH shuttle activities, pointing

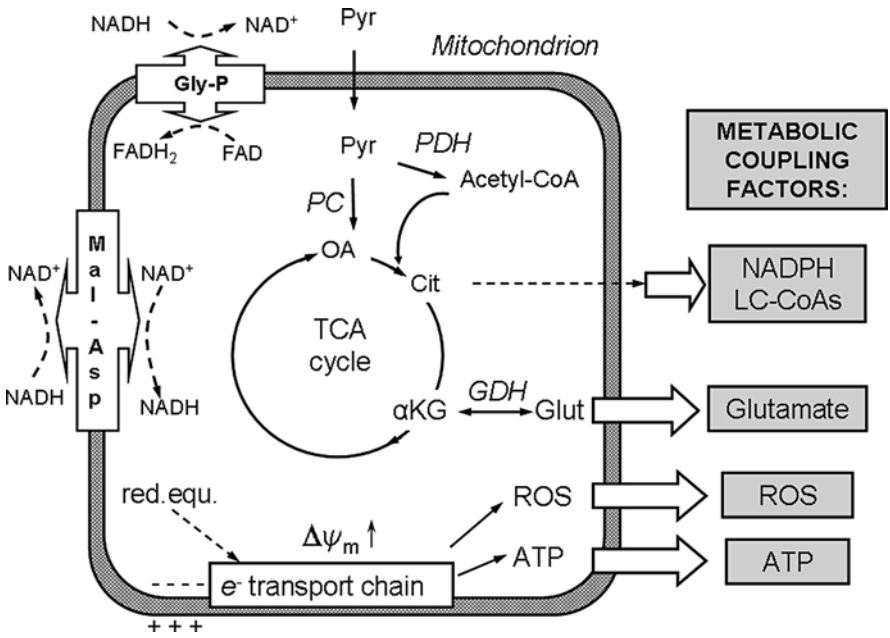

Fig. 9.2 In the mitochondria, pyruvate (Pyr) is a substrate both for pyruvate dehydrogenase (*PDH*) and pyruvate carboxylase (*PC*), forming, respectively, acetyl-CoA and oxaloacetate (OA). Condensation of acetyl-CoA with OA generates citrate (Cit) that is either processed by the TCA cycle or exported out of the mitochondrion as a precursor for long-chain acyl-CoA (LC-CoA) synthesis. Glycerophosphate (Gly-P) and malate/aspartate (Mal-Asp) shuttles as well as the TCA cycle generate reducing equivalents (red. equ.) in the form of NADH and $FADH_2$, which are transferred to the electron transport chain resulting in hyperpolarization of the mitochondrial membrane ($\Delta\Psi_m$) and ATP synthesis. As a by-product of electron transport chain activity, reactive oxygen species (ROS) are generated. Upon glucose stimulation, glutamate (Glu) can be produced from α-ketoglutarate (αKG) by glutamate dehydrogenase (GDH)

to the malate/aspartate shuttle as essential for both mitochondrial metabolism and cytosolic redox state.

Aralar1 (or aspartate–glutamate carrier 1, AGC1) is a Ca^{2+}-sensitive member of the malate/aspartate shuttle [12]. Aralar1/AGC1 and citrin/AGC2 are members of the subfamily of Ca^{2+}-binding mitochondrial carriers and correspond to two isoforms of the mitochondrial aspartate–glutamate carrier. These proteins are activated by Ca^{2+} acting on the external side of the inner mitochondrial membrane [12, 13]. We showed that adenoviral-mediated overexpression of Aralar1/AGC1 in insulin-secreting cells increases glucose-induced mitochondrial activation and secretory response [14]. This is accompanied by enhanced glucose oxidation and reduced lactate production. Therefore, aspartate–glutamate carrier capacity appears to set a limit for NADH shuttle function and mitochondrial metabolism. The importance of the NADH shuttle system also illustrates the tight coupling between glucose metabolism and the control of insulin secretion.

9.4 Mitochondria as Metabolic Sensors

Downstream of the NADH shuttles, pyruvate produced by glycolysis is preferentially transferred to mitochondria. The pyruvate imported into mitochondrial matrix is associated with a futile cycle that transiently depolarizes the mitochondrial membrane [15]. After its entry into the mitochondria, the pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase or to oxaloacetate by pyruvate carboxylase (Fig. 9.2). The pyruvate carboxylase pathway ensures the provision of carbon skeleton (i.e., anaplerosis) to the tricarboxylic acid (TCA) cycle, a key pathway in β-cells [16-19]. Importance of this pathway is highlighted in a study showing that inhibition of the pyruvate carboxylase reduces glucose-stimulated insulin secretion in rat islets [20]. The high anaplerotic activity suggests the loss of TCA cycle intermediates (i.e., cataplerosis), compensated for by oxaloacetate. In the control of glucose-stimulated insulin secretion, such TCA cycle derivates might potentially operate as mitochondrion-derived coupling factors [5].

Importance of mitochondrial metabolism for β-cell function is illustrated by stimulation with substrates bypassing glycolysis. This is the case for the TCA cycle intermediates succinate, or cell permeant methyl derivatives, that has been shown to efficiently promote insulin secretion in pancreatic islets [21-23]. Succinate induces hyperpolarization of the mitochondrial membrane, resulting in elevation of mitochondrial Ca^{2+} and ATP generation, while its catabolism is Ca^{2+} dependent [21].

Beside of its importance for ATP generation, the mitochondrion in general, and the TCA cycle in particular, is the key metabolic crossroad enabling fuel oxidation as well as provision of building blocks, or cataplerosis, for lipids and proteins [24]. In β-cells, approximately 50% of pyruvate is oxidized to acetyl-CoA by pyruvate dehydrogenase [17]. Pyruvate dehydrogenase is an important site of regulation as, among other effectors, the enzyme is activated by elevation of mitochondrial Ca^{2+} [25, 26] and, conversely, its activity is reduced upon exposures to either excess fatty acids [27] or chronic high glucose [28]. Oxaloacetate, produced by the anaplerotic enzyme pyruvate carboxylase, condenses with acetyl-CoA forming citrate, which undergoes stepwise oxidation and decarboxylation yielding α-ketoglutarate. The TCA cycle is completed via succinate, fumarate, and malate, in turn producing oxaloacetate (Fig. 9.2). The fate of α-ketoglutarate is influenced by the redox state of mitochondria. Low NADH to NAD^+ ratio would favor further oxidative decarboxylation to succinyl-CoA as NAD^+ is required as co-factor for this pathway. Conversely, high NADH to NAD^+ ratio would promote NADH-dependent reductive transamination forming glutamate, a spin-off product of the TCA cycle [24]. The latter situation, i.e., high NADH to NAD^+ ratio, is observed following glucose stimulation.

Although the TCA cycle oxidizes also fatty acids and amino acids, carbohydrates are the most important fuel under physiological conditions for the β-cell. Upon glucose exposure, mitochondrial NADH elevations reach a plateau after approximately 2 min [29]. In order to maintain pyruvate input into the TCA cycle, this new redox

steady state requires continuous reoxidation of mitochondrial NADH to NAD^+ primarily by complex I on the electron transport chain. However, as complex I activity is limited by the inherent thermodynamic constraints of proton gradient formation [30], additional NADH contributed by this high TCA cycle activity must be reoxidized by other dehydrogenases, i.e., through cataplerotic functions. Significant cataplerotic function in β-cells was suggested by the quantitative importance of anaplerotic pathway through pyruvate carboxylase [16, 17], as confirmed by use of NMR spectroscopy [18, 19, 31].

9.5 A Focus on Glutamate Dehydrogenase

The enzyme glutamate dehydrogenase (GDH) has been proposed to participate in the development of the secretory response (Fig. 9.2). GDH is a homohexamer located in the mitochondrial matrix and catalyses the reversible reaction, α-ketoglutarate + NH_3 + NADH ↔ glutamate + NAD^+; inhibited by GTP and activated by ADP [32, 33]. Regarding β-cell, allosteric activation of GDH has triggered most of the attention over the last three decades [34]. Numerous studies have used the GDH allosteric activator L-leucine or its nonmetabolized analog beta-2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) to question the role of GDH in the control of insulin secretion [34-37]. Alternatively, one can increase GDH activity by means of overexpression, an approach that we combined with allosteric activation of the enzyme [38]. To date, the role of GDH in β-cell function remains unclear and debated. Specifically, GDH might play a role in glucose-induced amplifying pathway through generation of glutamate [39-41]. GDH is also an amino acid sensor triggering insulin release upon glutamine stimulation in conditions of GDH allosteric activation [35, 37, 42].

Recently, the importance of GDH has been further highlighted by studies showing that SIRT4, a mitochondrial ADP-ribosyltransferase, downregulates GDH activity and thereby modulates insulin secretion [43, 44]. Clinical data and associated genetic studies also revealed GDH as a key enzyme for the control of insulin secretion. Indeed, mutations rendering GDH more active are responsible for a hyperinsulinism syndrome [45]. Mutations producing a less-active, or even nonactive, GDH enzyme have not been reported, leaving open the question if such mutations would be either lethal or asymptomatic. We recently generated and characterized transgenic mice (named ß*Glud1*$^{-/-}$) with conditional β-cell-specific deletion of GDH [46]. Data show that GDH accounts for about 40% of glucose-stimulated insulin secretion and that GDH pathway lacks redundant mechanisms. In ß*Glud1*$^{-/-}$ mice, the reduced secretory capacity resulted in lower plasma insulin levels in response to both feeding and glucose load while body weight gain and glucose homeostasis were preserved [46]. This demonstrates that GDH is essential for the full development of the secretory response in β-cells, being sensitive in the upper range of physiological glucose concentrations.

9.6 Mitochondrial Activation Results in ATP Generation

TCA cycle activation induces transfer of electrons to the respiratory chain resulting in hyperpolarization of the mitochondrial membrane and generation of ATP (Fig. 9.2). The electrons are transferred by the pyridine nucleotide NADH and the flavin adenine nucleotide FADH2. In the mitochondrial matrix, NADH is formed by several dehydrogenases, some of which being activated by Ca^{2+} [25], and FADH2 is generated in the succinate dehydrogenase reaction.

Electron transport chain activity promotes proton export from the mitochondrial matrix across the inner membrane, establishing a strong mitochondrial membrane potential, negative inside. The respiratory chain comprises five complexes, the subunits of which are encoded by both the nuclear and the mitochondrial genomes [47]. Complex I is the only acceptor of electrons from NADH in the inner mitochondrial membrane and its blockade abolishes glucose-induced insulin secretion [30]. Complex II (succinate dehydrogenase) transfers electrons to coenzyme-Q from $FADH_2$, the latter being generated both by the oxidative activity of the TCA cycle and the glycerophosphate shuttle. Complex V (ATP synthase) promotes ATP formation from ADP and inorganic phosphate. The synthesized ATP is translocated to the cytosol in exchange for ADP by the adenine nucleotide translocator (ANT). Thus, the work of the separate complexes of the electron transport chain and the adenine nucleotide translocator couples respiration to ATP supply.

NADH electrons are transferred to the electron transport chain, which in turn supplies the energy necessary to create a proton electrochemical gradient that drives ATP synthesis. In addition to ATP generation, mitochondrial membrane potential drives the transport of metabolites between mitochondrial and cytosolic compartments, including the transfer of mitochondrial factors participating in insulin secretion. Hyperpolarization of the mitochondrial membrane relates to the proton export from the mitochondrial matrix and directly correlates with insulin secretion stimulated by different secretagogues [30].

Accordingly, potentiation of glucose-stimulated insulin secretion by enhanced mitochondrial NADH generation is accompanied by increased glucose metabolism and mitochondrial hyperpolarization [14].

Mitochondrial activity can be modulated according to nutrient nature, although glucose is the chief secretagogue as compared to amino acid catabolism [48] and fatty acid beta-oxidation [49]. Additional factors regulating ATP generation include mitochondrial Ca^{2+} levels [25, 50], mitochondrial protein tyrosine phosphatase [51], mitochondrial GTP [52], and matrix alkalinization [53].

Mitochondrial function is also modulated by their morphology and contacts. Mitochondria form dynamic networks, continuously modified by fission and fusion events under the control of specific mitochondrial membrane anchor proteins [54]. Mitochondrial fission/fusion state was recently investigated in insulin-secreting cells. Altering fission by down regulation of fission-promoting Fis1 protein impairs respiratory function and glucose-stimulated insulin secretion [55]. The reverse experiment, consisting in overexpression of Fis1 causing mitochondrial fragmentation, results in a similar phenotype, i.e., reduced energy metabolism and secretory

defects [56]. Fragmented pattern obtained by dominant-negative expression of fusion-promoting Mfn1 protein does not affect metabolism–secretion coupling [56]. Therefore, mitochondrial fragmentation per se seems not to alter insulin-secreting cells at least in vitro.

9.7 The Amplifying Pathway of Insulin Secretion

The Ca^{2+} signal in the cytosol is necessary but not sufficient for the full development of sustained insulin secretion. Nutrient secretagogues, in particular glucose, evoke a long-lasting second phase of insulin secretion. In contrast to the transient secretion induced by Ca^{2+}-raising agents, the sustained insulin release depends on the generation of metabolic factors (Fig. 9.1). The elevation of cytosolic Ca^{2+} is a prerequisite also for this phase of secretion, as evidenced among others by the inhibitory action of voltage-sensitive Ca^{2+} channel blockers. Glucose evokes K_{ATP}-channel-independent stimulation of insulin secretion, or amplifying pathway [4], which is unmasked by glucose stimulation when cytosolic Ca^{2+} is clamped at permissive levels [57-59]. This suggests the existence of metabolic coupling factors generated by glucose.

9.8 Mitochondria Promote the Generation of Nucleotides Acting as Metabolic Coupling Factors

ATP is the primary metabolic factor implicated in K_{ATP}-channel regulation [60], secretory granule movement [61, 62], and the process of insulin exocytosis [63, 64].

Among other putative nucleotide messengers, NADH and NADPH are generated by glucose metabolism [65]. Single β-cell measurements of NAD(P)H fluorescence have demonstrated that the rise in pyridine nucleotides precedes the rise in cytosolic Ca^{2+} concentrations [66, 67] and that the elevation in the cytosol is reached more rapidly than in the mitochondria [68]. Cytosolic NADPH is generated by glucose metabolism via the pentose phosphate shunt [69], although mitochondrial shuttles being the main contributors in β-cells [70]. The pyruvate/citrate shuttle has triggered attention over the last years and has been postulated as the key cycle responsible for the elevation of cytosolic NADPH [70]. As a consequence of mitochondrial activation, cytosolic NADPH is generated by NADP-dependent malic enzyme and suppression of its activity was shown to inhibit glucose-stimulated insulin secretion in insulinoma cells [71, 72]. However, such effects have not been reproduced in primary cells in the form of rodent islets [73], leaving the question open.

Regarding the action of NADPH, it was proposed as a coupling factor in glucose-stimulated insulin secretion based on experiments using toadfish islets [74]. A direct effect of NADPH was reported on the release of insulin from isolated secretory granules [75], NADPH being possibly bound or taken up by granules [76]. More recently, the putative role of NADPH, as a signaling molecule in β-cells, has been

substantiated by experiments showing direct stimulation of insulin exocytosis upon intracellular addition of NADPH [77].

Glucose also promotes the elevation of GTP [78], which could trigger insulin exocytosis via GTPases [63, 79]. In the cytosol, GTP is mainly formed through the action of nucleoside diphosphate kinase from GDP and ATP. In contrast to ATP, GTP is capable of inducing insulin exocytosis in a Ca^{2+}-independent manner [63]. An action of mitochondrial GTP as positive regulator of the TCA cycle has been mentioned above [52].

The universal second messenger cAMP, generated at the plasma membrane from ATP, potentiates glucose-stimulated insulin secretion [80]. Many neurotransmitters and hormones, including glucagon as well as the intestinal hormones glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide, increase cAMP levels in the β-cell by activating adenyl cyclase [81]. In human β-cells, activation of glucagon receptors synergistically amplifies the secretory response to glucose [82]. Glucose itself promotes cAMP elevation [83] and oscillations in cellular cAMP concentrations are related to the magnitude of pulsatile insulin secretion [84]. Moreover, GLP-1 might preserve β-cell mass, both by induction of cell proliferation and inhibition of apoptosis [85]. According to all these actions, GLP-1 and biologically active-related molecules are of interest for the treatment of diabetes [86].

9.9 Fatty Acid Pathways and the Metabolic Coupling Factors

Metabolic profiling of mitochondria is modulated by the relative contribution of glucose and lipid products for oxidative catabolism. Carnitine palmitoyltransferase I, which is expressed in the pancreas as the liver isoform (LCPTI), catalyzes the rate-limiting step in the transport of fatty acids into the mitochondria for their oxidation. In glucose-stimulated β-cells, citrate exported from the mitochondria (Fig. 9.2) to the cytosol reacts with coenzyme-A (CoA) to form cytosolic acetyl-CoA that is necessary for malonyl-CoA synthesis. Then, malonyl-CoA derived from glucose metabolism regulates fatty acid oxidation by inhibiting LCPTI. The malonyl-CoA/long-chain acyl-CoA hypothesis of glucose-stimulated insulin release postulates that malonyl-CoA derived from glucose metabolism inhibits fatty acid oxidation, thereby increasing the availability of long-chain acyl-CoA for lipid signals implicated in exocytosis [16]. In the cytosol, this process promotes the accumulation of long-chain acyl-CoAs such as palmitoyl-CoA [87, 88], which enhances Ca^{2+}-evoked insulin exocytosis [89].

In agreement with the malonyl-CoA/long-chain acyl-CoA model, overexpression of native LCPTI in clonal INS-1E β-cells was shown to increase beta-oxidation of fatty acids and to decrease insulin secretion at high glucose [49], although glucose-derived malonyl-CoA was still able to inhibit LCPTI in these conditions. When the malonyl-CoA/CPTI interaction is altered in cells expressing a malonyl-CoA–insensitive CPTI, glucose-induced insulin release is impaired [90].

Over the last years, the malonyl-CoA/long-chain acyl-CoA model has been challenged, essentially by modulating cellular levels of malonyl-CoA, either up or down. Each ways resulted in contradictory conclusions, according to the respective laboratories performing such experiments. First, malonyl-CoA decarboxylase was overexpressed to reduce malonyl-CoA levels in the cytosol. In disagreement with the malonyl-CoA/long-chain acyl-CoA model, abrogation of malonyl-CoA accumulation during glucose stimulation does not attenuate the secretory response [91]. However, overexpression of malonyl-CoA decarboxylase in the cytosol in the presence of exogenous free fatty acids, but not in their absence, reduces glucose-stimulated insulin release [92]. The second approach was to silence ATP-citrate lyase, the enzyme that forms cytosolic acetyl-CoA leading to malonyl-CoA synthesis. Again, one study observed that such maneuver reduces glucose-stimulated insulin secretion [71], whereas another group concluded that metabolic flux through malonyl-CoA is not required for the secretory response to glucose [72].

The role of long-chain acyl-CoA derivatives remains a matter of debate, although several studies indicate that malonyl-CoA could act as a coupling factor regulating the partitioning of fatty acids into effector molecules in the insulin secretory pathway [93]. Moreover, fatty acids stimulate the G-protein-coupled receptor GPR40/FFAR1 that is highly expressed in β-cells [94]. Activation of GPR40 receptor results in enhancement of glucose-induced elevation of cytosolic Ca^{2+} and consequently insulin secretion [95].

9.10 Mitochondrial Metabolites as Coupling Factors

Acetyl-CoA carboxylase catalyzes the formation of malonyl-CoA, a precursor in the biosynthesis of long-chain fatty acids. Interestingly, glutamate-sensitive protein phosphatase 2A-like protein activates acetyl-CoA carboxylase in β-cells [96]. This observation might link two metabolites proposed to participate in the control of insulin secretion. Indeed, the amino acid glutamate is another discussed metabolic factor proposed to participate in the amplifying pathway [39, 40, 97]. Glutamate can be produced from the TCA cycle intermediate α-ketoglutarate or by transamination reactions [33, 48, 98]. During glucose stimulation total cellular glutamate levels have been shown to increase in human, mouse, and rat islets as well as in clonal β-cells [18, 38, 39, 41, 99-101], whereas one study reported no change [102].

The finding that mitochondrial activation in permeabilized β-cells directly stimulates insulin exocytosis [5] initiated investigations that identified glutamate as a putative intracellular messenger [39, 40]. In the in situ pancreatic perfusion, increased provision of glutamate using a cell permeant precursor results in augmentation of the sustained phase of insulin release [103]. The glutamate hypothesis was challenged by the overexpression of glutamate decarboxylase (GAD) in β-cells to reduce cytosolic glutamate levels [99]. In control cells, stimulatory glucose concentrations increased glutamate concentrations, whereas the glutamate response was significantly reduced in GAD overexpressing cells. GAD overexpression also

blunted insulin secretion induced by high glucose, showing direct correlation between the glutamate changes and the secretory response [99]. In contrast, it was reported by others that the glutamate changes may be dissociated from the amplification of insulin secretion elicited by glucose [100]. Recently, we abrogated GDH, the enzyme responsible for glutamate formation, specifically in the β-cells of transgenic mice. This resulted in a 40% reduction of glucose-stimulated insulin secretion [46]. Moreover, silencing of the mitochondrial glutamate carrier GC1 in β-cells inhibits insulin exocytosis evoked by glucose stimulation, an effect rescued by the provision of exogenous glutamate to the cell [104].

The use of selective inhibitors led to a model where glutamate, downstream of mitochondria, would be taken up by secretory granules, thereby promoting Ca^{2+}-dependent exocytosis [39, 40]. Such a model was strengthened by the demonstration that clonal β-cells express two vesicular glutamate transporters (VGLUT1 and VGLUT2) and that glutamate transport characteristics are similar to neuronal transporters [105]. The mechanism of action inside the granule could possibly be explained by glutamate-induced pH changes, as observed in secretory vesicles from pancreatic β-cells [106]. An alternative mechanism of action at the secretory vesicle level implicates glutamate receptors. Indeed, clonal β-cells have been shown to express the metabotropic glutamate receptor mGlu5 in insulin-containing granules, thereby mediating insulin secretion [107].

Another action of glutamate has been proposed. In insulin-secreting cells, rapidly reversible protein phosphorylation/dephosphorylation cycles have been shown to play a role in the rate of insulin exocytosis [108]. It has also been reported that glutamate, generated upon glucose stimulation, might sustain glucose-induced insulin secretion through inhibition of protein phosphatase enzymatic activities [101]. An alternative or additive mechanism of action would be the activation of acetyl-CoA carboxylase [96] as mentioned above. Finally, glutamate might serve as a precursor for related pathways, such as GABA (gamma-aminobutyric acid) metabolism that could then contribute to the stimulation of insulin secretion through the so-called GABA shunt [109].

Several mechanisms of action have been proposed for glutamate as a metabolic factor playing a role in the control of insulin secretion. However, we lack a consensus model and further studies should dissect these complex pathways that might be either additive or cooperative.

Among mitochondrial metabolites, succinate has been proposed to control insulin production. Indeed, it was reported that succinate and/or succinyl-CoA are metabolic stimulus-coupling factors for glucose-induced proinsulin biosynthesis [110]. Later, an alternative mechanism has been postulated regarding succinate stimulation of insulin production. Authors showed that such stimulation was dependent on succinate metabolism via succinate dehydrogenase, rather than being the consequence of a direct effect of succinate itself [111].

Citrate export out of the mitochondria has been described as a signal of fuel abundance that contributes to β-cell stimulation in both the mitochondrial and

the cytosolic compartments [70]. In the cytosol, citrate contributes to the formation of NADPH and malonyl-CoA, both proposed as metabolic coupling factors as discussed in this review.

9.11 Reactive Oxygen Species Participate to β-Cell Function

Reactive oxygen species (ROS) include superoxide ($O_2^-\bullet$), hydroxyl radical (OH•), and hydrogen peroxide (H_2O_2). Superoxide can be converted to less-reactive H_2O_2 by superoxide dismutase (SOD) and then to oxygen and water by catalase (CAT), glutathione peroxidase (GPx), and peroxiredoxin, which constitute antioxidant defenses. Increased oxidative stress and free radical-induced damages have been proposed to be implicated in diabetic state [112]. However, metabolism of physiological nutrient increases ROS without causing deleterious effects on cell function. Recently, the concept emerged that ROS might participate to cell signaling [113]. In insulin-secreting cells, it has been reported that ROS, and probably H_2O_2 in particular, is one of the metabolic coupling factor in glucose-induced insulin secretion [114]. Therefore, ROS fluctuations may also contribute to physiological control of β-cell functions. However, uncontrolled increase of oxidants, or reduction of their detoxification, may lead to free radical-mediated chain reactions ultimately triggering pathogenic events [115].

9.12 Mitochondria Can Generate ROS

Mitochondrial electron transport chain is the major site of ROS production within the cell. Electrons from sugar, fatty acid, and amino acid catabolism accumulate on the electron carriers NADH and $FADH_2$ and are subsequently transferred through the electron transport chain to oxygen, promoting ATP synthesis. ROS formation is coupled to this electron transportation as a by-product of normal mitochondrial respiration through the one-electron reduction of molecular oxygen [116, 117]. The main sub-mitochondrial localization of ROS formation is the inner mitochondrial membrane, i.e., NADH dehydrogenase at complex I and the interface between ubiquinone and complex III [118]. Increased mitochondrial free radical production has been regarded as a result of diminished electron transport occurring when ATP demand declines or under certain stress conditions impairing specific respiratory chain complexes [119, 120]. This is consistent with the observation that inhibition of mitochondrial electron transport chain by mitochondrial complex blockers, antimycin A and rotenone, lead to increased ROS production in INS-1 β-cells [114].

9.13 Mitochondria are Sensitive to ROS

Mitochondria not only produce ROS but are also the primary target of ROS attacks. The mitochondrial genome is more vulnerable to oxidative stress and consecutive damages are more extensive than those in nuclear DNA due to the lack of protective histones and low repair mechanisms [121, 122]. Being in close proximity to the site of free radical generation, mitochondrial inner membrane components are at a high risk for oxidative injuries, eventually resulting in depolarized mitochondrial membrane and impaired ATP production. Such sensitivity has been shown for mitochondrial membrane proteins such as the adenine nucleotide transporter and ATP synthase [123, 124]. In the mitochondrial matrix, aconitase was also reported to be modified in an oxidative environment [125].

Furthermore, mitochondrial membrane lipids are highly susceptible to oxidants, in particular the long-chain poly-unsaturated fatty acids. ROS may directly lead to lipid peroxidation and the production of highly reactive aldehyde species exerts further detrimental effects [126]. The mitochondrion membrane-specific phospholipid cardiolipin is particularly vulnerable to oxidative damages, altering the activities of adenine nucleotide transporter and cytochrome c oxidase [127].

9.14 ROS May Trigger β-Cell Dysfunction

ROS may have different actions according to cellular concentrations being either below or above a specific threshold, i.e., signaling or toxic effects, respectively. Robust oxidative stress caused either by direct exposure to oxidants or secondary to gluco-lipotoxicity has been shown to impair β-cell functions [128–130]. In type 1 diabetes, ROS participate in β-cell dysfunction initiated by autoimmune reactions and inflammatory cytokines [131]. In type 2 diabetes, excessive ROS impair insulin synthesis [132–134] and activate β-cell apoptotic pathways [132, 135].

Hyperglycemia induces generation of superoxide at the mitochondrial level in endothelial cells and triggers of a vicious cycle of oxidative reactions implicated in the development of diabetic complications [118]. In the rat Zucker diabetic fatty model of type 2 diabetes, direct measurements of superoxide in isolated pancreatic islets revealed ROS generation coupled to mitochondrial metabolism and perturbed mitochondrial function [136].

Short transient exposure to oxidative stress is sufficient to impair glucose-stimulated insulin secretion in pancreatic islets [128]. Specifically, ROS attacks in insulin-secreting cells result in mitochondrial inactivation, thereby interrupting transduction of signals normally coupling glucose metabolism to insulin secretion [128]. Recently, we observed that one single acute oxidative stress induces β-cell dysfunction lasting over days, explained by persistent damages in mitochondrial components accompanied by subsequent generation of endogenous ROS of mitochondrial origin [137].

The degree of oxidative damages also depends on protective capability of ROS scavengers. Mitochondria have a large set of defense strategies against oxidative injuries. Superoxide is enzymatically converted to H_2O_2 by the mitochondrion-specific manganese SOD [138]. Other antioxidants like mitochondrial GPx, peroxiredoxin, vitamin E and Coenzymes Q, and various repair mechanisms contribute to maintain redox homeostasis in mitochondria [139, 140]. However, β-cells are characterized by relatively weak expression of free radical-quenching enzymes SOD, CAT, and GPx [141]. Overexpression of such enzymes in insulin-secreting cells inactivates ROS attacks [142]. Beside ROS inactivation, the uncoupling protein (UCP) 2 was shown to reduce cytokine-induced ROS production, an effect independent of mitochondrial uncoupling [143].

9.15 Mitochondrial DNA Mutations and β-Cell Dysfunction

Mitochondrial DNA (mtDNA) carries only 37 genes (16,569 bp) encoding 13 polypeptides, 22 tRNAs and 2 ribosomal RNAs [47]. Mitochondrial protein biogenesis is determined by both nuclear and mitochondrial genomes, and the few polypeptides encoded by the mtDNA are all subunits of the electron transport chain [144]. Transgenic mice lacking expression of the mitochondrial genome specifically in the β-cells are diabetic and their islets exhibit impaired glucose-stimulated insulin secretion [145]. Moreover, mtDNA-deficient β-cell lines are glucose unresponsive and carry defective mitochondria, although they still exhibit secretory responses to Ca^{2+}-raising agents [146–148].

Mitochondrial inherited diabetes and deafness (MIDD) is often associated with mtDNA A3243G point mutation on the tRNA (Leu) gene [149, 150], usually in the heteroplasmic form, i.e., a mixture of wild-type and mutant mtDNA in patient cells. Mitochondrial diabetes usually appears during adulthood with maternal transmission and often in combination with bilateral hearing impairment [151]. The aetiology of diabetes may not be primarily associated with β-cells, rendering the putative link between mtDNA mutations and β-cell dysfunction still hypothetical [152]. Moreover, pancreatic islets of such patients may carry low heteroplasmy percentage of the mutation [153] and, accordingly, the pathogenicity of this mutation is hardly detectable in the endocrine pancreas [153, 154].

Some clinical studies strongly suggest a direct link between mtDNA mutations and β-cell dysfunction. Diabetic patients carrying mtDNA mutations exhibit marked reduction in insulin release upon intravenous glucose tolerance tests and hyperglycemic clamps compared to noncarriers [155–157]. It is hypothesized that mtDNA mutations could result in mitochondrial impairment associated with β-cell dysfunction as a primary abnormality in carriers of the mutation [155]. Alternatively, impaired mitochondrial metabolism in cells of individuals carrying mtDNA mutations might rather predispose for β-cell dysfunction, explaining late onset of the disease. Due to technical limitation of β-cell accessibility in individuals, the putative impact of mtDNA mutations on insulin secretion still lacks direct demonstration.

In cellular models, direct investigation of β-cell functions carrying specific mtDNA mutations also faces technical obstacles. Indeed, as opposed to genomic DNA, specific mtDNA manipulations are not feasible. The alternative commonly used is to introduce patient-derived mitochondria into cell lines by fusing enucleated cells carrying mitochondria of interest with cells depleted of mtDNA (ρ^o cells), resulting in cytosolic hybrids, namely cybrids.

Mitochondria derived from patients with mtDNA A3243G mutation were introduced into a human ρ^o osteosarcoma cell line. The resulting clonal cell lines contained either exclusively mutated mtDNA or wild-type mtDNA from the same patient [158]. The study shows that mitochondrial A3243G mutation is responsible for defective mitochondrial metabolism associated with impaired Ca^{2+} homeostasis [159]. The A3243G mutation induces a shift to dominantly glycolytic metabolism while glucose oxidation is reduced [159]. The levels of reducing equivalents in the form of NAD(P)H are not efficiently elevated upon glucose stimulation in mtDNA-mutant cells, reflecting the impact of this mutation on the electron transport chain activity [158]. As a metabolic consequence we observed a switch to anaerobic glucose utilization accompanied by increased lactate generation [159]. Accordingly, ATP supply is totally dependent on high glycolytic rates, enabling the mtDNA-mutant cells to only reach basal normal ATP levels at the expense of stimulatory glucose concentrations. Such a phenotype is well known to dramatically impair glucose-stimulated insulin secretion in β-cells.

9.16 Conclusion

Mitochondria are key organelles that generate the largest part of cellular ATP and represent the central crossroad of metabolic pathways. Metabolic profiling of β-cell function identified mitochondria as sensors and generators of metabolic signals controlling insulin secretion. Recent molecular tools available for cell biology studies shed light on new mechanisms regarding the coupling of glucose recognition to insulin exocytosis. Delineation of metabolic signals required for β-cell function will be instrumental in therapeutic approaches for the management of diabetes.

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Chapter 10
Basement Membrane in Pancreatic Islet Function

Martin Kragl and Eckhard Lammert

Abstract Clinical treatment of diabetic patients by islet transplantation faces various complications. At present, in vitro expansion of islets occurs at the cost of their essential features, which are insulin production and release. However, the recent discovery of blood vessel/β-cell interactions as an important aspect of insulin transcription, secretion, and proliferation might point us to ways of how this problem could be overcome.

The correct function of β-cells depends on the presence of a basement membrane, a specialized extracellular matrix located around the blood vessel wall in mouse and human pancreatic islets. In this chapter, we summarize how the vascular basement membrane influences insulin transcription, insulin secretion, and β-cell proliferation. In addition, a brief overview about basement membrane components and their interactions with cell surface receptors is given.

Keywords Basement membrane · β1-integrin · Laminin · Collagen · Blood vessels

10.1 Introduction

Basement membranes are imaged by transmission electron microscopy as sheet-like structures with an average thickness of 50–100 nm [1–4]. They are found in every tissue adjacent to epithelia, endothelia, peripheral nerve axons, fat and muscle cells and are linked to the cytoskeleton via cell surface receptors [5, 6]. They serve important functions in conferring mechanical stability and compartmentalization in tissues as well as in regulating cell behavior [5, 7]. In every organ, basement membranes exhibit different characteristics, which are vital for correct function. For example, the basement membrane encasing muscle fibers is specialized to support the fibers in response to the extreme mechanical forces, a feature that distinguishes it from

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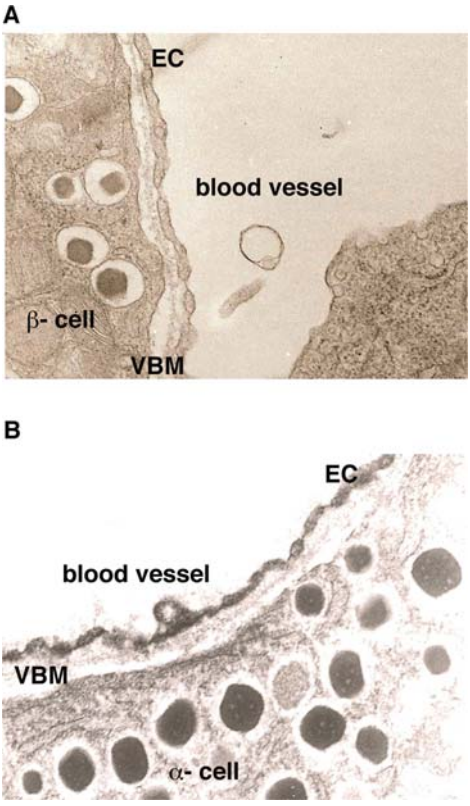

Fig. 10.1 Endocrine cells adjacent to a vascular basement membrane. (**A**) Electron micrograph of a β-cell next to a blood vessel. Endothelial cell (EC), β-cell, and vascular basement membrane (VBM) are indicated. (**B**) Electron micrograph of an α-cell next to a blood vessel. Endothelial cell (EC), α-cell, and vascular basement membrane (VBM) are indicated

basement membranes found in other organs. In contrast, in mouse pancreatic islets, a specialized basement membrane is largely formed by endothelial cells and is implicated in insulin production and release as well as β-cell proliferation (Fig. 10.1).

The most prominent components of basement membranes are collagen IV, laminins, heparan sulfate proteoglycans (HSPGs) such as perlecan and agrin, and nidogen/entactin [5, 6, 8] (Fig. 10.2). These molecules can exist as different isoforms, which can be glycosylated in different manners. In addition, their tissue-specific combination in basement membranes is important for any given tissue [9–12].

Before addressing the role of the vascular basement membrane in β-cell function, we briefly introduce the molecules of the basement membrane and their cell surface receptors.

10.2 Basement Membrane Components

10.2.1 Collagen IV

Collagen IV comprises a major part of all basement membranes and is also abundant in the vascular basement membrane of pancreatic islets [13, 14].

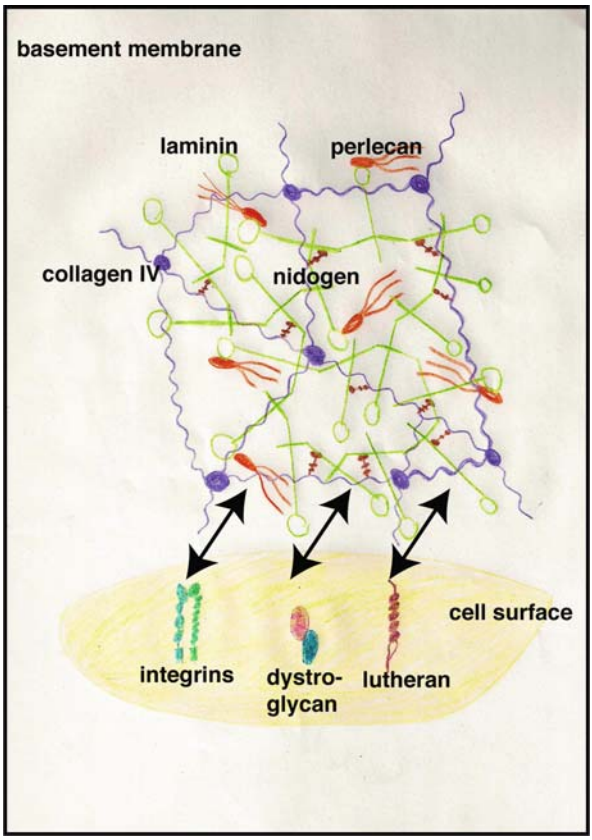

Fig. 10.2 Basement membrane components and their receptors. Basement membranes are sheet-like structures adjacent to epithelia, endothelia, nerves, muscle, or fat cells. They influence tissue stability and cell behavior via cell surface receptors. The scheme shows the major components of BMs: collagen IV, laminins, perlecan (a heparan sulfate proteoglycan), and nidogen, which exist as various glycosylated isotypes and isoforms that can potentially form networks and interact with cell surface receptors, such as integrins, dystroglycan, or lutheran

Collagen IV has been proposed to exist as a network of protomers in basement membranes [15, 16]. Protomers of collagen IV form from combinations of three α-chains. There are six genes coding for different α-chains, α1(IV)–α6(IV), and three different combinations of protomers have been identified in vivo so far: α1.α1.α2(IV), α3α4α5(IV), and α5α5α6(IV) [11, 12, 17, 18].

The major collagen IV isoform is α1.α1.α2(IV), and deletion of both α-chains causes early embryonic lethality due to defects in basement membrane stability [19]. In invertebrates, mutations or reduced expression of collagen IV-related genes is embryonic lethal due to the failure of muscle attachment to the basement membrane [20, 21]. In islets, collagen IV has been suggested to regulate insulin secretion [13, 22].

10.2.2 Laminin

Laminins are heterotrimeric glycoproteins that, according to the current model, assemble from an α-, β-, and γ-chain to form a trimer [9, 23–25]. In mammals, there are 5 genetically different α, 4 β, and 3 γ-chains, and 15 different laminin trimers have been found so far [26].

The different chain compositions define the nomenclature of laminin isoforms: for example, laminin-411 is composed of the α4, β1, γ1 chains, whereas laminin-511 is a trimer of the α5, β1, γ1 chains [26]. Laminins have a cross- or T-like shape and bind other matrix components including collagen IV, nidogen-1, perlecan, and cell surface receptors [27–33]. Some laminin trimers such as laminin-111 and laminin-511 can undergo polymerization [9, 27, 30–34].

Laminins are essential for vitality of an organism. For example, laminin α5-chain knockout mice are not viable; they die during embryogenesis at E16.5 with exencephaly, syndactyly, small or absent kidneys and eyes, defects in lung and tooth morphogenesis, and hair growth that come along with abnormalities in basement membrane assembly, structure, and integrity in these tissues [35–37]. In contrast, laminin α4-chain knockout mice are viable. However, they display defects in vessels, neuromuscular junctions, and the peripheral nerve system [38–40].

In islets, laminin-411 and laminin-511 are expressed and have been suggested to play an important role in β-cell proliferation and insulin transcription [14].

10.2.3 Nidogen/Entactin

Nidogen is a component of basement membranes [41] and exists as two isoforms: nidogen-1 and nidogen-2. Both are elongated molecules composed of three globular domains (G1, G2, and G3) connected by a flexible, protease-sensitive link, and a rigid rod-like domain [42–44]. Both nidogens are present in the vascular basement membrane of the islets [45]. Several in vitro studies suggest that nidogen facilitates the interaction between collagen IV and laminin [42, 44, 46, 47]. Its in vivo role has been controversial, since nidogen-1 and nidogen-2 knockout mice did not display severe defects [48, 49]. Interestingly, mice in which both isoforms were deleted developed until birth but died soon after birth with heart defects and impaired lung development, and deposition of basement membrane compounds in these organs appeared to be reduced. Surprisingly, defects in kidney development and glomerular basement membrane were less severe in these mutants [50].

At present, it is unknown whether nidogens are involved in β-cell function or not.

10.2.4 Heparan Sulfate Proteoglycans (HSPGs)

Most HSPGs are giant proteins with branched glycosyl residues and multiple binding sites for other matrix components and cell surface receptors. Due to their

branched structure and charged sugar residues, they affect the distribution of FGFs (fibroblast growth factors), VEGFs (vascular endothelial growth factors), HGF (hepatocyte growth factor), and other molecules and their diffusion within the extracellular space [51–53].

One of the most abundant HSPG is perlecan [54], which contains domains homologous to growth factors and cell adhesion molecules and interacts with laminins and collagen IV [31, 55]. Homozygous knockout mice die during embryogenesis due to BM defects [56, 57].

Although HSPGs have been poorly studied in the context of β-cell function, it is possible that they affect insulin transcription, secretion, or cell proliferation in islets. Perlecan is expressed in the intra-islet vascular basement membranes [45], and several growth factors whose diffusion and distribution is mediated by HSPGs have been reported to affect β-cell function, including VEGFs [14, 58, 59], FGFs [60–62], and HGF [63, 64].

10.3 Cell Surface Receptors

10.3.1 Integrins

Integrins were the first receptors identified to mediate BM/cell contacts in epithelium [65–68] and are also expressed on β-cells [13, 14].

Integrins are transmembrane proteins with large globular extracellular and smaller cytosolic domains. They undergo interactions with the BM as heterodimers of an α- and a β-integrin chain. The composition of the heterodimer defines specificity of the integrin for components of the basement membrane [69]. To interact with extracellular factors, integrins need to become activated, either by intracellular or extracellular factors. Upon activation and ligand binding, integrins influence various cellular processes, such as cytoskeletal rearrangements, cell proliferation, and cell survival [69–71] (Fig. 10.3A).

One of the most abundant integrin classes are those containing the β1-chain. Knockout of β1-integrin and members of some of its cytosolic partners resulted in embryonic lethality [72–76].

In islets, heterodimers containing β1-integrin have been suggested to affect insulin transcription and secretion as well as β-cell proliferation [13, 14].

10.3.2 Dystroglycan

Another cell surface receptor for basement membranes is dystroglycan. It is part of the dystrophin–glycoprotein complex in muscle fibers. However, it is also expressed in many other tissues [77].

Dystroglycan is a heterodimer composed of an extracellular α-subunit and a transmembrane β-subunit containing an intracellular signaling domain. Both

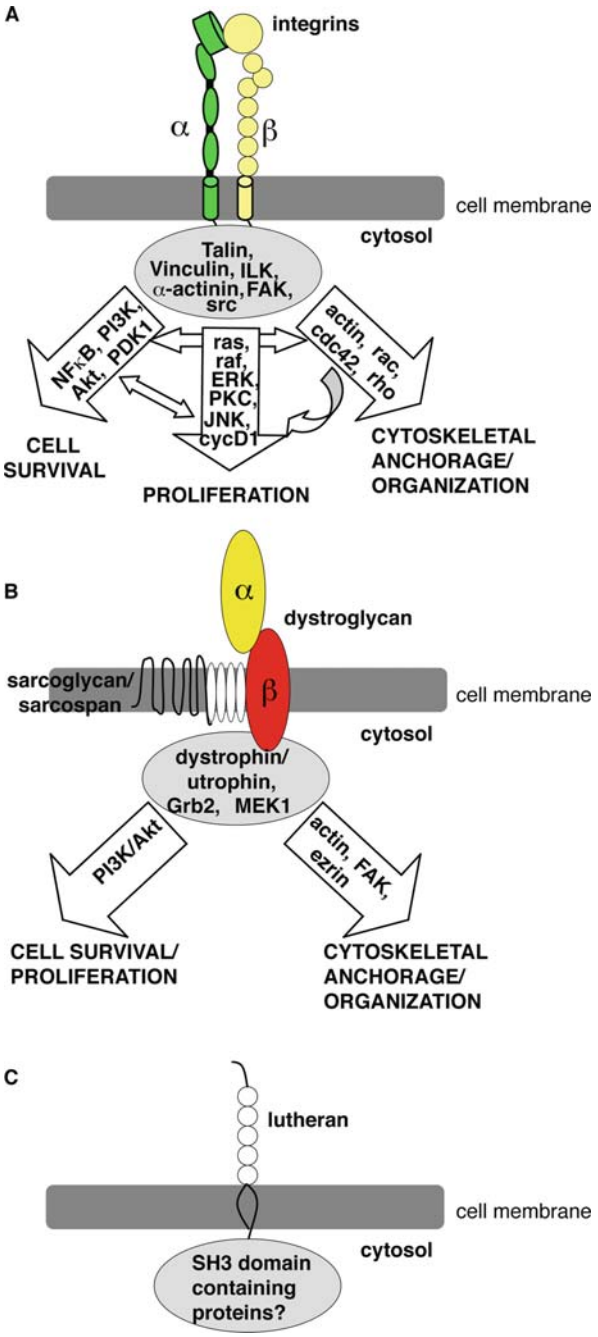

Fig. 10.3 (continued)

subunits are encoded by one gene and are a product of posttranslational cleavage [78, 79].

The extracellular α-subunit is highly glycosylated. The glycosylation patterns differ among cell types, suggesting that glycosylation of the protein confers tissue-specific interactions between the basement membrane and the cell surface [80].

The extracellular α-subunit has been shown to interact with laminin-111 and laminin-211 as well as perlecan [80–84], whereas the β-subunit binds to dystrophin in muscle fibers [85, 86] or utrophin in other tissues [87], thus linking matrix components to the actin cytoskeleton. Dystroglycan has also been co-purified with Grb2 and FAK [88] and might be an adaptor for several other intracellular signaling molecules, including c-Src, Fyn, caveolin-1, MEK1, ERK, and ezrin [89–92], suggesting that it might be involved in cell proliferation and cell motility (Fig. 10.3B).

Although dystroglycan has been suggested to play a role in laminin-induced β-cell differentiation [93], this molecule has been poorly studied in the context of diabetes and β-cell function. However, it has been shown to be an important regulator of interactions between basement membranes and cells. For example, abnormal glycosylation is associated with several congenital muscular dystrophies and impaired neural development [94–96]. Moreover, its targeted deletion in the brain resulted in a less organized extracellular matrix and a reduced laminin-binding activity [97]. Thus, it might modulate the communication between β-cells and the vascular basement membrane.

10.3.3 Lutheran Glycoprotein

The lutheran glycoprotein, a member of the Ig superfamily, has been long known for being one of the blood group antigens in red blood cells. Recently, it has been shown to be a laminin receptor with a specific affinity for the laminin α5 chain [98–100]. Interestingly, this molecule is expressed in human pancreatic β-cells, while it is absent in the β-cells of rodents [101, 102].

Fig. 10.3 Major cell surface receptors binding to BM components. (**A**) Integrins form heterodimers of an α- and β-chain and bind different forms of laminin, collagen IV, and perlecan. Integrins are upstream of various intracellular signaling pathways. Binding partners of the cytoplasmic tails (within the *gray oval circle*) link the integrins to these signaling pathways. For further details, readers are referred to several excellent reviews [69, 71, 115–118]. (**B**) Dystroglycan consists of an extracellular α- and a transmembrane β-subunit that undergo a non-covalent interaction. The α-subunit interacts with various BM components. Dystroglycan is upstream of various intracellular signaling pathways. Binding partners of the cytoplasmic tail of the β-subunit (within the *gray oval circle*) link dystroglycan to these pathways. For further details, readers are referred to several excellent reviews [119–121]. (**C**) Lutheran is a cell surface receptor that belongs to the Ig superfamily. It has been mainly known as a blood group antigen. Apart from being expressed in red blood cells, it is also present in various tissues and is shown to be a laminin receptor. Its cytoplasmic tail contains an SH3 (Src homology 3)-binding domain, but its role in bridging the BM to the cytosol is currently unknown

There are two splice forms of the lutheran glycoprotein: one version has a short cytoplasmic domain containing an SH3-binding motif [103, 104] (Fig. 10.3C). Thus, proteins containing an SH3 domain, e.g., the tyrosine kinases c-Src and Fyn might interact and provide a link between lutheran and important intracellular signaling processes. The shorter version of the protein (called B-CAM for basal cell adhesion molecule) lacks this domain. However, this short form is not expressed in human islets [102].

Although the role of lutheran in β-cell function has not been investigated, in vitro adhesion experiments with dispersed human β-cells revealed lutheran as one of the molecules that bind effectively to laminin-511 [102]. It will be interesting to study the role of lutheran in β-cell function.

10.4 The Vascular Basement Membrane and its Role in Pancreatic Islets

In islets, blood vessels play an important role, since they are required for forming the vascular basement membrane. Although most epithelial cells can form basement membrane, mouse pancreatic β-cells require blood vessels for basement membrane formation. The importance of blood vessels for β-cells is reflected by islet physiology: Islets are highly vascularized [105] (Fig. 10.4), and each β-cell is in contact with an endothelial cell-derived basement membrane. In contrast to rodents, blood vessels in human islets are surrounded by two layers of basement membrane – one probably derived from endothelial cells, the other one coming from another islet cell type, for example, pericytes or the β-cells themselves [101, 102].

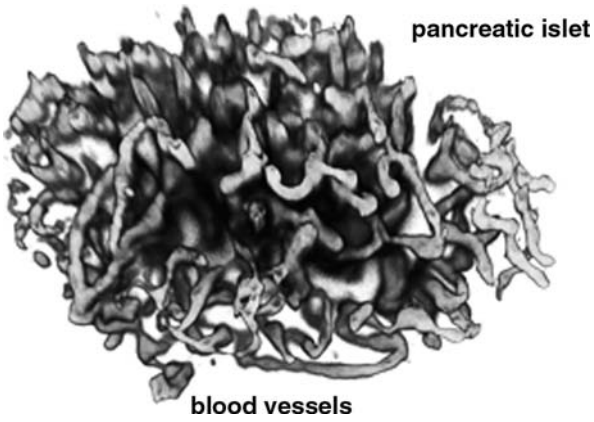

Fig. 10.4 Islets are highly vascularized. The 3D reconstruction of the vasculature in a mouse pancreatic islet reconstructed from images taken with a single plane illumination microscope (SPIM). The image shows the high density of blood vessels found within pancreatic islets
From Lammert, E.: The Vascular Trigger of Type II Diabetes Mellitus. Exp Clin Endocrinol Diabetes 2008; 116: S21–S25. Printed with permission of the Georg Thieme Verlag, Stuttgart

A crucial factor for the communication between β-cells and endothelial cells is VEGF-A, which is secreted by β-cells. VEGF-A-depleted islets display a reduced degree of islet vasculature in a dose-dependent manner, and when transplanted into normal hosts, such islets were only inefficiently revascularized when compared to control islets [58].

In addition, islets depleted of VEGF-A have a reduced number of capillaries and exhibit several defects in β-cell function, including insulin transcription [14], insulin content, and first-phase insulin secretion [58, 106], glucose tolerance [58, 59], and β-cell proliferation [14].

These defects clearly show that the presence of endothelial cells is required for β-cell function, but do not explain on its own why blood vessels are so important. As mentioned above, endothelial cells are required for forming a basement membrane within the islets [14], and the lack of vascular basement membrane significantly contributes to impaired β-cell behavior in VEGF-A-deficient islets.

10.5 Control of β-Cell Function by Vascular Basement Membrane

Evidence for the vascular basement membrane being implicated in islet function came from in vitro experiments using purified rat β-cells plated on the so-called 804G-extracellular matrix [107–111]. This matrix is formed by a rat bladder carcinoma cell line and contains essential basement membrane components such as collagens and laminins. Under these conditions, β-cells secreted more insulin in response to glucose and exhibited a better survival rate when compared to appropriate controls [107].

Interestingly, it could also be shown that the 804G-matrix enhanced insulin secretion via NFκB as well as the Rho/ROCK pathway [109, 110]. Furthermore, this matrix stimulated the activation of the ERK and Akt/PKB pathways, further suggesting that basement membrane components can influence cell survival and proliferation [107, 108].

What are the specific basement membrane components and cell surface receptors that influence insulin production and secretion as well as β-cell proliferation? Parnaud et al. [111] showed that interaction of laminin-332 and $\beta 1$-integrin affects insulin secretion in this experimental system. In the following, we discuss more examples of basement membrane/integrin interactions implicated in β-cell function.

10.6 Specific Basement Membrane/Cell Surface Interactions That Control β-Cell Function

10.6.1 Laminin/α6β1-Integrin Interaction and Insulin Transcription

Nikolova et al. [14] showed that laminins positively influence insulin transcription by performing rescue experiments on cultured VEGF-$A^{-/-}$ islets and in vitro studies

using MIN6 cells, a mouse tumor cell line derived from pancreatic β-cells, plated on laminins [112].

When MIN6 cells were plated on different basement membrane components, including laminins, collagen IV, or fibronectin, the transcriptional levels of both insulin genes were upregulated compared to controls. The strongest effect was observed, when cells were plated on laminin-111, laminin-411, and laminin-511. Specific knockdown of α6- or β1-integrin by siRNA and the use of a blocking antibody against β1-integrin showed that the α6/β1 integrin heterodimer is one laminin receptor that promotes insulin gene transcription.

Experiments on VEGF-A$^{-/-}$ islets, which do not harbor an intra-islet vascular basement membrane and exhibit reduced levels of insulin transcription, lead to a similar conclusion: soluble laminin-111 partially restored the transcriptional activity, and this rescue effect could be blocked by an antibody directed against β1-integrin [14].

Surprisingly, another study showed that the culture of primary human β-cells on collagen IV or vitronectin negatively affects insulin transcription, whereas laminin had neither a positive nor a negative effect [22]. This difference to the above experiments might be due to the differences between human and mouse cells or due to the fact that the mouse studies used intact islets, whereas the human β-cells were dissociated prior to culture.

10.6.2 Collagen IV/α1β1-Integrin Interaction and Insulin Secretion

Experiments on cultured primary human β-cells plated on various matrices showed that collagen IV could enhance insulin secretion [13, 22]. Furthermore, the use of a blocking antibody directed against the α1β1-integrin heterodimer abolished this effect, suggesting that the specific interaction between α1β1-integrin and collagen IV improves insulin secretion [13].

10.6.3 Laminin and β-Cell Proliferation

Studies on MIN6 cells and VEGF-A$^{-/-}$ islets suggested that laminin positively influences β-cell proliferation. When plated on a laminin matrix, BrdU assays showed that the percentage of MIN6 cells undergoing S-phase was higher, when compared to cells plated on other matrices. This laminin effect could be blocked by the application of an antibody directed against β1-integrin or, alternatively, knockdown of β1-integrin.

Most importantly, soluble laminin-111 partially rescued the frequency of mitotic cells in VEGF-A$^{-/-}$ islets, further suggesting that laminin specifically supports β-cell proliferation [14].

10.7 Conclusion

Blood vessels are attracted to invade pancreatic islets via VEGF-A secreted by β-cells. The blood vessels, in turn, initiate the formation of the vascular basement membrane, a specialized extracellular matrix that controls β-cell function. Although we are far away from understanding the complex network of communication between the vascular basement membrane and the β-cells, a few specific basement membrane/cell surface receptor interactions could already be identified, which are implicated in insulin production and secretion as well as β-cell proliferation (Fig. 10.5).

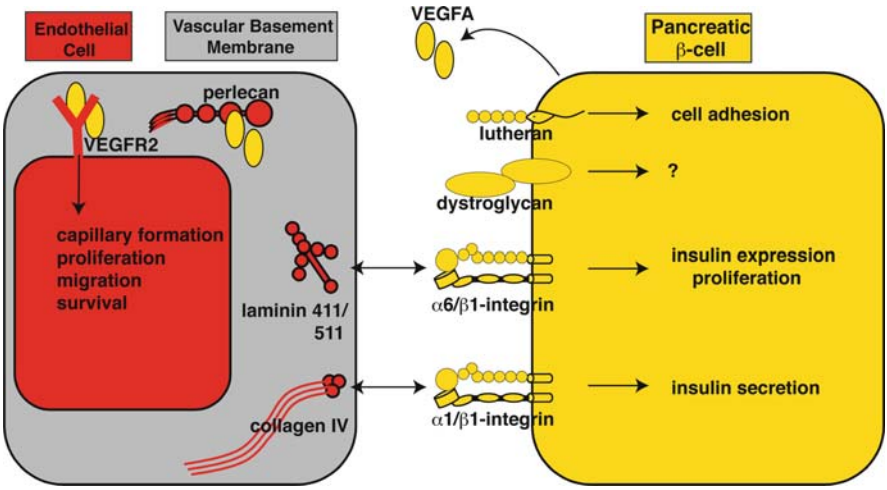

Fig. 10.5 Schematic view on the communication between blood vessels and β-cells and the role of the vascular basement membrane. VEGF-A, secreted by β-cells, attracts blood vessels to invade the islet. The presence of blood vessels in the islet is crucial for β-cell function as it depends on components of the specialized vascular basement membrane, which communicate with cell surface receptors on β-cells. The interaction between laminin and α6β1-integrin affects insulin gene transcription, whereas binding of collagen IV to α1β1-integrin influences insulin secretion. Laminin binding to β1-integrin receptors accounts for stimulation of β-cell proliferation

10.8 Outlook

It is important to understand the interactions between vascular basement membrane and β-cells in order to design more efficient diabetes therapies. In particular, inducing proliferation without affecting β-cell quality could improve the success of islet transplantation.

Various problems have been reported related to the transplantation of islets. One complication is that transplanted islets are opposed by the host immune system, leaving an insufficient number of islets to deal with glucohomeostasis. Therefore,

restoring a proper basement membrane in islets may increase the viability of islets and at the same time lower their antigenicity. In addition, a few insulin-producing β-cells were observed in patients with diabetes [113]. However, it is unknown how to expand these β-cells in these patients. Therefore, studies aiming to elucidate how the vascular basement membrane affects β-cell proliferation as well as autoimmune destruction of islets may help to improve the regenerative potential of islets.

Another problem of islet transplantation is to obtain sufficient numbers of healthy islets from donors, and a major goal is to culture and expand islets in vitro. However, it is difficult to stimulate β-cell proliferation without losing the β-cell's ability to secrete sufficient levels of insulin in response to glucose. Therefore, understanding the molecular networks underlying the communication between the vascular basement membrane and β-cell surface receptors may help to reveal how the mass of functional insulin-producing and insulin-secreting β-cells can be increased.

Furthermore, it would be interesting to make artificial islets by generating a scaffold of basement membrane and populate this scaffold by β-cells or their progenitors. In this regard it is noteworthy that a recent study showed that decellularized heart matrices could be repopulated by cardiocytes and endothelial cells [114]. Thus, studies on the vascular basement membrane may open new avenues for generating artificial and functional islets.

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Chapter 11
Calcium Signaling in the Islets

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Abstract Easy access to rodent islets and insulinoma cells and the ease of measuring Ca^{2+} by fluorescent indicators have resulted in an overflow of data that have clarified minute details of Ca^{2+} signaling in the rodent islets. Our understanding of the mechanisms and the roles of Ca^{2+} signaling in the human islets, under physiological conditions, has been hugely influenced by uncritical extrapolation of the rodent data obtained under suboptimal experimental conditions. More recently, electrophysiological and Ca^{2+} studies have elucidated the ion channel repertoire relevant for Ca^{2+} signaling in the human islets and have examined their relative importance. Many new channels belonging to the transient receptor potential (TRP) family are present in the β-cells. Ryanodine receptors, nicotinic acid adenine dinucleotide phosphate channel, and Ca^{2+}-induced Ca^{2+} release add new dimension to the complexity of Ca^{2+} signaling in the human β-cells. A lot more needs to be learnt about the roles of these new channels and CICR, not because that will be easy but because that will be difficult. Much de-learning will also be needed. Human β-cells do not have a resting state in the normal human body even under physiological fasting conditions. Their membrane potential under physiologically relevant resting conditions is ~−50 mV. Biphasic insulin secretion is an experimental epiphenomenon unrelated to the physiological pulsatile insulin secretion into the portal vein in the human body. Human islets show a wide variety of electrical activities and patterns of $[Ca^{2+}]_i$ changes, whose roles in mediating pulsatile secretion of insulin into the portal vein remain questionable. Future studies will hopefully be directed toward a better understanding of Ca^{2+} signaling in the human islets in the context of the pathogenesis and treatment of human diabetes.

Keywords CICR · Transient receptor potential channels · Calcium oscillation · Depolarization · TRP channels · TRPV1 · Ryanodine receptor · TRPV4 · Basal

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calcium · RyR1 · TRPM2 · TRPV2 · TRPM3 · RyR2 · KATP channel · RyR3 · Membrane potential · Calcium-induced calcium release

11.1 Introduction

Changes in the concentration of the free Ca^{2+} in the cytoplasm ($[Ca^{2+}]_i$) or in subcellular compartments can act as signals for many cellular processes. Increase in $[Ca^{2+}]_i$ may be local (e.g., Ca^{2+} "sparks"), which may give rise to global $[Ca^{2+}]_i$ changes [1]. $[Ca^{2+}]_i$ changes take the forms of oscillations and propagating waves. Generation and shaping of the Ca^{2+} signals require participation of different membranes, channels, pumps, stores, other organelle, as well as many Ca^{2+}-binding-proteins. $[Ca^{2+}]_i$ changes are often loosely termed "Ca^{2+} signals", although it is likely that all $[Ca^{2+}]_i$ changes do not have a signaling role. Ca^{2+} signals control events such as exocytosis that take place in seconds and events like gene transcription that take place over minutes to hours. In this review, I shall not attempt to compile a catalog of all of the molecules and phenomena that are known in connection with Ca^{2+} signaling in the islets; instead, I shall depict some emerging and intriguing areas and give my views. The review is structured and dimensioned to deliver selected messages rather than to dilute them by writing a complete treatise on Ca^{2+} signaling.

When it comes to Ca^{2+} signaling in the islets, the literature is dominated by data obtained from in vitro experiments that have used islets or insulinoma cells from rodents. We, therefore, understand how rodent islets behave in Petri dishes or in in vitro perfusion systems, better than we understand how human islets behave in their native environment in the pancreas in the normal human body. If we want to learn how mountain gorillas behave, we can do that by poking a monkey in a cage or by watching real mountain gorillas in their social and natural environments in Rwanda. In practice, however, we tend to draw far-reaching conclusions not only about the function of normal human islets but also about the dysfunctions of human islets in diabetes, from in vitro studies done on rodent islets. It is therefore important that we carefully examine what key experiments were done, what conditions were used in those experiments, and what results were obtained. This may enable us to reinterpret the existing data and draw our own conclusions about some fundamental issues some of which are illustrated in the following paragraphs.

11.2 Human β-Cells as a Group Are Never Resting

The notion that β-cells have a "resting" state is a myth arising from in vitro experimental protocols. In vitro experimentalists find it convenient to work with a stable low rate of insulin secretion and a stable low basal $[Ca^{2+}]_i$ at the beginning of an experiment. They want to ensure that the $[Ca^{2+}]_i$ or insulin curves show a stable baseline, which reviewers like to see. To achieve this, investigators expose islets to

low concentrations of glucose (often 2–3 mM, sometimes 0–1 mM), and no other nutrients are included in the solution. Human islets are incubated in zero glucose for as long as an hour to force them to rest [2]. Under such conditions, β-cells are largely depleted of energy, and consequently, a high proportion of K_{ATP} channels are open. β-Cells in the human body, however, even after overnight fast, are bathed in ~4–6 mM glucose, other nutrients like the amino acids, and the hormone glucagon that is present in high concentration in the fasting plasma. The availability of these nutrients ensures that human β-cells, even under fasting conditions, are not energy depleted. The K_{ATP} channels of many β-cells in the human islets are thus mostly in a closed state even under fasting conditions. In in vitro experiments, human β-cells secrete insulin even when they are exposed to only 3–5 mM glucose as the only nutrient [2]. When human β-cells are exposed to only 5–6 mM glucose, as the sole nutrient, they keep firing action potentials from a baseline membrane potential of ~−50 to ~−45 mV, at rates ranging from one every 4 s to one every 2 s [3]. Complex patterns of membrane potential oscillations are seen in human islets even when they are exposed to only 2.8 mM glucose (and no other nutrients) and even when the experiments are performed at 34°C [4]. (To the cell biologists 34°C or even 21°C is O.K.; to the clinicians, a patient with 34°C body temperature poses a real emergency). Thus, in the normal human body, β-cells are not resting even after overnight fast. Under fasting conditions, the concentration of insulin in the portal vein of human is 440 ± 25 pmol/L [5]. Under such conditions, islets secrete not only insulin but also glucagon, which protects against hypoglycemia. This is evident from the observations that total pancreatectomy in human leads not only to diabetes but also to a rather more difficult complication, namely hypoglycemia due to the lack of glucagon [6].

It is accepted that the $[Ca^{2+}]_i$ of "resting" β-cells is ~25–100 nM and that the membrane potential of "resting" β-cells is ~−70 mV. These values are obtained from experiments where β-cells are forced to artificial "resting conditions" that are different from the physiological resting conditions. If β-cells are, instead, kept in a solution that mimics the human plasma after an overnight fast (i.e., physiologically relevant resting condition), then their resting membrane potentials will be different (perhaps ~−50 to ~−45 mV). Consequently, their resting $[Ca^{2+}]_i$ will also be different (perhaps ~300 nM and perhaps in the form of oscillations). In other words, β-cells in the normal human body spend most of their life-time with a much higher $[Ca^{2+}]_i$ and secretory activity than can be guessed from conventional experiments.

11.3 Biphasic Insulin Secretion Is an Experimental Epiphenomenon

In experiments where β-cells are first forced to rest (often by incubating in ~2–3 mM glucose, as the only nutrient), and then *suddenly* exposed to a high concentration of glucose (often >10 mM, sometimes 30 mM!), continuously for a prolonged period, then one sees what has been called "biphasic insulin secretion" over the past

decades. Biphasic refers to two phases of insulin secretion: the first phase consists of the initial large insulin secretion that peaks at 5–6 min after increasing the concentration of glucose and the second phase consists of the subsequent lower rate of insulin secretion that remains stable or slowly rises as long as the glucose concentration remains high (over a period of 1–2 h or more) (see Fig. 6.3). (Electrophysiologists have a different definition of "biphasic," their first phase peaking in <500 ms! [7].) Human β-cells in normal human body encounter conditions of stimulations that are substantially different from the experimental conditions that are used to elicit the biphasic nature of insulin secretion. As mentioned before, normal human β-cells are not in a resting state even under fasting conditions. They are seldom subjected to a sudden increase of glucose to a very high concentration (or sudden increase of $[Ca^{2+}]_i$ to 30 μM by UV flash [7]). They are usually triggered by lower concentrations of glucose (usually by ~7–<10 mM glucose after a mixed meal), and normally glucose concentrations in the plasma do oscillate. The result is that normal insulin secretion in the human portal vein is oscillatory and not biphasic as elicited by artificial experimental conditions. Experiments that are designed to demonstrate biphasic nature of insulin secretion are not usually designed to detect oscillations of insulin secretion (e.g., samples for insulin assay are not collected at 1 min or more frequent intervals). Thus normal secretion in normal human being during fasting states and after mixed meals may employ a set of molecular mechanisms that may be substantially different from those involved in mediating biphasic insulin secretion elicited by experimental protocols described above.

11.4 Glucose Increases Insulin Secretion by Increasing $[Ca^{2+}]_i$ and by Providing ATP in the Face of Energy-Consuming Processes Triggered by Ca^{2+} Influx Through the Voltage-Gated Ca^{2+} Channels (VGCC)

When 30 mM KCl is applied to islets in the presence of low concentration of glucose (or zero glucose [8], and no other nutrients are included in the solution), there is an increase of both $[Ca^{2+}]_i$ and insulin secretion with a biphasic time course [2, 8]. A large and persistent increase of $[Ca^{2+}]_i$ in a cell that is kept at 1 mM glucose (and no other nutrients) reduces cytoplasmic [ATP] [9]. This is due to the fact that plasma membrane Ca^{2+}-ATPase and other Ca^{2+}-sensitive biochemical cascades that link Ca^{2+} influx to insulin secretion consume ATP of the cell which is kept in only 1 mM glucose (and which has a high-K_m glucokinase to phosphorylate the sugar) [10]. In fact, in the later part of the second phase, $[Ca^{2+}]_i$ increases slowly since the cell can no longer pump out Ca^{2+} adequately because of energy deficiency [8]. Consequently, Ca^{2+}-mediated insulin secretion (which is an energy-consuming process) is progressively reduced in the second phase of prolonged $[Ca^{2+}]_i$ increase by KCl [8]. If one now applies 15 mM glucose (and thereby improves energy status of the cells) to these "$[Ca^{2+}]_i$-clamped" islets, a larger amount of secretion is obtained [2, 8]. So, to recapitulate, in the first scenario, insulin secretion increases because

of an increase of $[Ca^{2+}]_i$, but the magnitude of the increase is low and it declines further over time because of inadequate energy availability to support secretion. In the second scenario, glucose does what it is supposed to do, i.e., it performs its universal fuel function by supplying energy to the cells and thereby it increases insulin secretion further. Of course, glucose metabolism produces many other molecules too, e.g., cAMP (via ATP), which can increase insulin secretion [11].

That glucose can stimulate insulin secretion from human β-cells in vivo, without inducing further closure of K_{ATP} channels, is evident from cases of severe poisoning with sulfonylureas. In these patients, the K_{ATP} channels are presumably completely closed and $[Ca^{2+}]_i$ of β-cells is certainly high. However, when glucose is infused into such patients (as an attempt to correct hypoglycemia), the β-cells secrete even more insulin, making the hypoglycemia recurrent and difficult to treat [12]. Similarly, people with $SUR1^{-/-}$, who do not respond to tolbutamide, do respond to glucose by insulin secretion [13].

Thus, while investigating signaling roles of glucose, the more universal role of glucose as a fuel needs to be considered explicitly. In experimental conditions where glucose is the only nutrient, its role as a fuel becomes even more critical. If concentration of glucose in the human plasma is reduced to less than 3 mM (and all other nutrients are kept normal), one will become unconscious within seconds, a vivid example of the role of glucose as a fuel in the central neurons. Similarly, if there is no glucose or only very low glucose in the perfusion medium (and no other nutrients are present), muscle cells will eventually fail to contract, heart will stop beating, and not surprisingly, islets will fail to secrete insulin properly.

11.5 Mechanism of Initial Depolarization of β-Cells by Glucose

Initial depolarization of plasma membrane to the thresholds for activation of voltage-gated Ca^{2+} channels is one of the most critical signaling events leading to Ca^{2+} signaling and insulin secretion. The most important function of β-cells is to prevent death due to hypoglycemia. If your fasting plasma glucose concentration is raised from 5 mM to 8 mM (i.e., you have diabetes), you will not die immediately. You may not even feel for years that your glucose is high. On the other hand if your fasting plasma glucose drops from 5 mM to 3 mM, you will have hypoglycemic symptoms and you may become unconscious and die. Other hormones in the body are not like insulin; if your pituitary or adrenal hormones are acutely low, it will not kill you immediately. β-Cells, thus, secrete a hormone that is potentially a killer. Nature has, therefore, equipped β-cells with powerful brakes to immediately stop insulin secretion, when glucose concentration is inappropriately low. Key elements of this brake system are the high-K_m glucokinase and the K_{ATP} channels. When plasma glucose concentration is reduced to near hypoglycemic levels, there is less glucose metabolism via glucokinase, leading to a reduced cytoplasmic MgATP/MgADP, opening of the K_{ATP} channels, and repolarization of plasma membrane potential (see chapter by Clark and Proks in this book). Thus, K_{ATP} channels

play a crucial role in stopping insulin secretion quickly, and its main function is to mediate quick repolarization of plasma membrane potential. Defects in these two brake systems, namely inactivating mutations of the K_{ATP} channels or activating mutations of glucokinase, lead to hypoglycemia [14].

At low glucose concentration (provided that no other nutrients are present), a high proportion of the K_{ATP} channels are in the open state. This situation occurs only in in vitro experiments that are often done at ~21°C [15] and by using cells or tissues that are to a varying degree "metabolically stunned." It has no resemblance to any in vivo situation in any living human being, where β-cells are at 37°C and are constantly bathed in a variety of nutrients including 20 different amino acids and fatty acids, even under normal fasting conditions when plasma glucose concentration is ~4–6 mM. In vivo, a healthy β-cell, thus, has enough ATP to keep almost 100% of the K_{ATP} channels closed. In the normal human body, where plasma glucose concentration changes only between ~4 mM in the fasting conditions and ~8 mM after meals, further closure of the K_{ATP} channels is thus not the likely mechanism for bringing about depolarization to the threshold for the activation of VGCCs. Glucose depolarizes β-cells in Sur1 or Kir6.2 knocked-out mice [16, 17]. Thus, under normal fasting conditions, the input resistance of β-cells is high and depolarization to the thresholds for the activation of VGCCs is brought about by various inward depolarizing currents mainly carried by Na^+. Here we are talking about tiny winy currents which are difficult to measure in native β-cells. Thus, mere anticipation, sight, or smell of food will depolarize β-cells and stimulate insulin secretion by vagus-mediated acetylcholine-induced depolarizing Na^+ current [18, 19]. Similarly, after a mixed meal, the incretin hormone GLP-1 depolarizes β-cells by triggering a cAMP-activated Na^+ current [20]. There is an urgent need to elucidate the molecular identity of the channels that mediate inward depolarizing currents in the β-cells. In this respect, there is currently, legitimate interest in the transient receptor potential (TRP) channels, which is the topic of the next paragraphs.

11.6 TRP Channels

More and more TRP channels are being identified in the β-cells. It is thought that these channels may account for the background depolarizing current (often called "leak" current) carried mostly by Na^+. Activation of some of these channels leads to an increase of $[Ca^{2+}]_i$ directly or by way of membrane depolarization. Examples of Ca^{2+}-permeable TRP channels in the islets include TRPC1, TRPC4, TRPV1, TRPV2, TRPV4, TRPV5, TRPM2, and TRPM3. TRPs are tetrameric ion channels and many form heterotetramers giving rise to a variety of ion channels with a variety of regulatory mechanisms. Expression of some TRP channels in the native cells is often low and their regulation is often studied in heterologous systems where the channels are overexpressed, an approach not entirely without problem. In the following paragraphs, I will write a few lines about each of the TRP channels that have been described in the β-cells.

Examination of formalin-fixed paraffin-embedded tissue shows strong TRPC1 immunoreactivity in the human islets (www.hpr.se). By RT-PCR, TRPC1 mRNA can be readily detected in mouse islets, MIN6 cells, INS-1 cells, and rat β-cells [21, 22]. TRPC1 is the only TRPC channel that is expressed at high level in MIN6 cells and mouse islets [22]. In contrast, another mouse insulinoma cell line βTC3 does not express TRPC1 mRNA. The only TRPC channel that can be detected by Northern blot in βTC3 cells is TRPC4 [23]. TRPC4 is also abundant in INS-1 cells and rat β-cells [21]. TRPC4 has two abundant splice variants: the full-length TRPC4α and a shorter TRPC4β that lacks 84 amino acids in the C-terminus. In INS-1 cells, TRPC4α is the dominant isoform, whereas in rat β-cells, TRPC4β dominates [21]. TRPC4α is inhibited by phosphatidylinositol 4,5-bisphosphate (PIP2) [24]. TRPC1 and TRPC4 are nonspecific cation channels with about equal permeability to Na^+ and Ca^{2+}. As alluded to earlier, it is possible that TRPC1 and other TRP channels mediate the inward depolarizing currents in β-cells. TRPC1 and TRPC4 are also molecular candidates for nonselective cation currents activated by Gq/PLC-coupled receptors or by store depletion [25]. From studies in other cells, it appears that TRPC1 together with STIM1 and Orai1 can mediate store-operated Ca^{2+} entry (SOCE), but the issue remains controversial [26]. TRPC5, which is closely related to TRPC4, is not expressed in mouse islets [23].

In the islets, TRPV1 is present mainly in a subset of sensory nerve fibers that are thought to be involved in mediating local islet inflammation in autoimmune diabetes [27, 28]. The TRPV1-expressing fibers secrete calcitonin gene-related peptide, which inhibits insulin secretion. In Zucker diabetic rats, it has been demonstrated that ablation of the TRPV1-expressing fibers by capsaicin treatment improves insulin secretion [27]. Insulinoma cell lines RIN and INS-1 express TRPV1. The picture is less clear for primary β-cells. TRPV1 has been demonstrated in primary β-cells of Sprague Dawley rats [29] but not in those of Zucker diabetic rats [27] or NOD mice [28]. It remains unknown whether the human β-cells express TRPV1.

Studies on TRPV2 channel in different cells show that it is activated by physical stimuli like noxious heat (>52°C), membrane stretch, and osmotic swelling. The roles of such stimuli in the regulation of TRPV2 channel of β-cells are unclear. TRPV2 channel of β-cells is in fact constitutively active [30]. It may thus be one of the channels responsible for the background depolarizing current in β-cells. Another remarkable feature of the TRPV2 channel of β-cells is its ability to translocate from cytoplasm to the plasma membrane, upon stimulation by insulin [30]. Such translocation of TRPV2 and the resulting increase in Ca^{2+} entry through the channel increases insulin secretion and β-cell growth. This observation implies that hyperinsulinemia, which is common in type 2 diabetes, may act as a positive feed-back to increase insulin secretion further. High concentration of glucose also induces translocation of TRPV2 to the plasma membrane. It appears that while glucose closes K_{ATP} channel by increasing cytoplasmic MgATP/MgADP ratio, it, at the same time, increases inward depolarizing current through TRPV2 channel by inducing translocation of the channel to the plasma membrane.

Immunohistochemistry of formalin-fixed paraffin-embedded tissues shows that the TRPV4 protein is highly expressed in the human islets, in contrast to the

pancreatic acinar cells, where it is almost absent (www.hpr.se). Even though TRPV4 is known to be a plasma membrane channel, the immunoreactivity is mostly in the cytoplasm, a situation apparently similar to that of TRPV2 in the β-cells. TRPV4 acts as a mechano-sensor and osmo-sensor, but it can be activated by various ligands including 4α-Phorbol 12,13-didecanoate, anandamide, arachidonic acid, and epoxyeicosatrienoic acids. Aggregated human islet amyloid polypeptide (hIAPP) induces changes in the plasma membrane leading to the activation of TRPV4, membrane depolarization, increase in $[Ca^{2+}]_i$, induction of ER stress, and apoptosis [31]. hIAPP-induced $[Ca^{2+}]_i$ changes and β-cell death are reduced by siRNA against TRPV4 [31].

By immunohistochemistry it has been shown that TRPV5 (formerly called ECaC1) protein is expressed in rat β-cells but not in the α-cells nor in the pancreatic acinar cells [32]. In the β-cells, the TRPV5 immunoreactivity is mostly in the secretory granules. TRPV5 is highly selective for Ca^{2+} and the tissues where it is typically expressed are the ones that are responsive to 1,25-dihydroxyvitamin D_3 (e.g., kidney and intestine), where it plays crucial roles in transepithelial (re)absorption of Ca^{2+} [33]. It is noteworthy that β-cells are also 1,25-dihydroxyvitamin D_3-responsive (see chapter by Leung and Cheng in this book). The antiaging protein klotho deglycosylates TRPV5 and thereby entraps the channel in the plasma membrane and ensures durable channel activity [34]. Islets of klotho mutant mice have decreased insulin content and secrete reduced amount of insulin [35]. However, there is so far only one study which has shown TRPV5 protein in the β-cells and the data, apparently, cannot be reproduced [32]. Native TRPV5 current has not been demonstrated in β-cells or indeed in any other cells because of low expression of the channel. TRPV5 protein has not been demonstrated in human β-cells.

The presence of functional TRPM2 channels in rodent insulinoma cells as well as in primary β-cells is well established [36]. In human islets, there are at least two main isoforms of the channel: the full-length form (TRPM2-L) and a short form (TRPM2-S), where the four C-terminal transmembrane domains, the putative pore region, and the entire C-terminus are deleted [37]. TRPM2-S does not form a functional channel. There are other splice variants of TRPM2 which form channels and are differentially regulated [38]. TRPM2 is activated by intracellular ADP ribose, β-NAD^+, nitric oxide, H_2O_2, free radicals, and Ca^{2+}. ADP ribose formed by the degradation of NAD^+ by poly(ADP ribose) polymerase is an important activator of the TRPM2 channel. The nonselective cation channel activated by the diabetogenic agent alloxan is probably TRPM2 [39]. The channel can be gated also by warm temperature (>35°C). Arachidonic acid, which is produced on stimulation of β-cells by glucose, is a positive modulator of TRPM2 channel [40–42]. Cyclic ADP ribose potentiates activation of the channel [43], but this is not a universal observation [44]. Perhaps the most important physiological regulator of TRPM2 is Ca^{2+}. All of the splice forms of TRPM2 that form a channel are activated by Ca^{2+}; Ca^{2+} released from the intracellular stores can activate the channel [45]. TRPM2 is located also on the lysosomal membranes and activation of intracellular TRPM2 releases Ca^{2+} from the lysosomes [36]. The role of TRPM2 channels in the regulation of insulin secretion and in mediating β-cells death in diabetes is an active area of research.

TRPM2 knock-out mice are apparently not diabetic [46]. This may mean that the channel is not important in mice β-cells or that other ionic mechanisms compensate for its absence in the knock-out mice. The channel may provide a mechanism for eliminating β-cells that have been severely damaged by oxidative stress [47].

The TRPM3 channel has many splice variants which differ in their functional properties including their permeabilities for divalent cations [48]. Micromolar concentrations of the steroid pregnenolone directly activate TRPM3 channel of β-cells leading to increase of $[Ca^{2+}]_i$ and augmentation of glucose-stimulated insulin secretion [49]. The channel is activated by nifedipine, commonly used as a blocker of L-type VGCCs.

TRPM4 is permeable to monovalent cations but not to Ca^{2+} [50]. It is activated by elevated $[Ca^{2+}]_i$ and its activity is regulated by voltage. Immunohistochemistry shows that TRPM4 protein is present in human β-cells [51]. In rodent insulinoma cells, increased $[Ca^{2+}]_i$ activates TRPM4 and generates a large depolarizing membrane current [52]. An increase in $[Ca^{2+}]_i$ in β-cells upon stimulation by glucose or activation of PLC-linked receptors activates TRPM4 channel [51]. An important regulator of TRPM4 is PIP2, which sensitizes the channel to the activation by $[Ca^{2+}]_i$, whereas depletion of PIP2 inhibits the channel [53]. Glucose, by increasing cytoplasmic MgATP/MgADP ratio, increases the concentration of PIP2 in the plasma membrane of β-cells [54]. This is a potential mechanism by which glucose may sensitize TRPM4 channel. On the other hand, glucose increases cytoplasmic [ATP], which has inhibitory effect on TRPM4 channel [55]. Amino acid sequence of TRPM4 shows two motifs that look like ABC transporter signature motif [56]. Consistent with this, TRPM4 is inhibited by glibenclamide [57]. Another voltage-modulated intracellular Ca^{2+}-activated monovalent-specific cation channel, which is closely related to the TRPM4 channel, is the TRPM5 channel [58]. Compared with TRPM4, TRPM5 is even more sensitive to activation by $[Ca^{2+}]_i$, but in contrast to TRPM4, it is not inhibited by ATP [55]. TRPM5 mRNA is present in MIN6 cells, INS-1 cells, and in whole human islets [58]. Reportedly, glucose-induced insulin secretion is reduced in TRPM5 knock-out mice. TRPM4 and TRPM5 may mediate Na^+ entry into the β-cells by sulfonamides, muscarinic agonists, and glucose and thereby depolarize membrane potential.

11.7 Store-Operated Ca^{2+} Entry (SOCE)

The filling state of the ER Ca^{2+} store may trigger Ca^{2+} entry across the plasma membrane in β-cells as in many other cells [59]. Thus, depletion of ER Ca^{2+} pools by SERCA inhibitors induces Ca^{2+} entry and depolarizes the plasma membrane potential of β-cells [60]. The ER Ca^{2+} store thus plays a role in the regulation of membrane potential [61, 62]. Two important molecular players involved in SOCE are stromal interaction molecule (STIM) and Orai1. STIM1 has an intraluminal EF-hand domain which enables it to act as a sensor of $[Ca^{2+}]$ in the ER lumen. STIM1, by its association with Orai1 or TRPC, regulates SOCE in some cells. Pancreatic

islets express STIM1. In MIN6 cells, it has been shown that EYFP-STIM1 is delivered to the peri-plasma membrane location when the ER Ca^{2+} pool is depleted [63]. 2-aminoethoxydiphenyl borate (2-APB) prevents SOCE and translocation of STIM1 to peri-plasma membrane locations. It is not known whether STIM1 interacts with Orai1 or TRPC channels in β-cells. The roles of TRPCs and the roles of STIM1 and Orai1 in mediating SOCE remain unsettled. Some results support the view that STIM1-Orai1-TRPC1 complex provides an important mechanism for SOCE [26]; others demonstrate that TRPC channels operate by mechanisms that do not involve STIM1 [64]. It should be noted that in β-cells, activation of muscirinic receptors leads to the activation of nonselective cation currents that have a store-operated and a store-independent component [19]. We demonstrated that activation of RyRs of β-cells leads to Ca^{2+} entry through TRP-like channels by mechanisms that apparently do not involve store depletion [65].

11.8 Voltage-Gated Ca^{2+} Channels of β-Cells

In β-cells, the most robust mechanism for the entry of extracellular Ca^{2+} across the plasma membrane is the Ca^{2+} entry through VGCCs. Opening of VGCCs leads to a large increase of $[Ca^{2+}]_i$ in microdomains near the plasma membrane and triggers exocytosis of insulin [66]. Both high-voltage-activated (HVA) and low-voltage-activated (LVA) Ca^{2+} currents are detected in human β-cells [67, 68]. The major component of the HVA current is L-type that is blocked by dihydropyridine antagonists and enhanced by BAYK8644. A second component of HVA current is resistant to inhibition by dihydropyridines and ω-conotoxin GVIA, an inhibitor of N-type Ca^{2+} channel but is blocked by P/Q channel blocker ω-agatoxin IVA. Consistent with this, 80–100% of glucose-induced insulin secretion from human islets is blocked by saturating concentration of dihydropyridine antagonists [68, 69]. Such dramatic inhibition is thought to be due to the fact that the L-type channels play essential role in the generation of electrical activity (however, these inhibitors also block NAADP receptor). In contrast, their roles in mediating exocytosis are less pronounced [69]. The L-type Ca^{2+} current in human β-cells is mediated mainly by $Ca_v1.3$ (α_{1D}) channel and to a lesser extent by $Ca_v1.2$ (α_{1C}). Compared to $Ca_v1.2$, the $Ca_v1.3$ channels activate at lower membrane potential (~−55 mV), which suggests that the latter may be the more important isoform in human β-cells. This is in contrast to mouse β-cells where $Ca_v1.2$ plays a central role in insulin secretion [70]. Compared to the $Ca_v1.2$ channels, the $Ca_v1.3$ channels are less sensitive to the dihydropyridine antagonists [71]. Identical de novo mutation (G406R) in this channel causes prolonged inward Ca^{2+} currents and causes episodic hypoglycemia [72].

The P/Q type Ca^{2+} channels ($Ca_v2.1$, α_{1A}) account for 45% of integrated whole-cell Ca^{2+} current in human β-cells. These channels are blocked by ω-agatoxin IVA. Compared to the L-type Ca^{2+} channels, the P/Q type Ca^{2+} channels are more tightly coupled to exocytosis.

The LVA current is of T-type which is activated at −50 mV and reaches a peak between −40 and −30. It inactivates within less than 1 s of sustained depolarization to −40 mV. The T-type current in human β-cells is mediated by $Ca_V3.2$ (α_{1G}). T-type channels are involved in insulin release induced by 6 mM but not by 20 mM glucose [69]. T-type current is blocked by NNC 55-0396.

If all of these ion channels are present in a given β-cell, one can envisage that closure of the K_{ATP} channels depolarizes membrane potential to above −55 mV, which then leads to the activation of T-type Ca^{2+} channels (which open at voltage above −60 mV) and then to the activation of the L-type Ca^{2+} channels (which open at voltage above −50 mV), which generates the action potential. Further depolarization occurs due to the activation of the voltage-gated Na^+ channels (which open at above −40 mV) leading finally to the activation of P/Q type Ca^{2+} channels (which opens at above −20 mV) [69].

R-type Ca^{2+} channels ($Ca_v2.3$, α_{1E}) are not present in human β-cells [69]. Mice lacking the R-type Ca^{2+} channels exhibit impaired insulin secretion. In this context, it is noteworthy that polymorphisms in the gene encoding the R-type Ca^{2+} channels $Ca_v2.3$ (CACNA1E) are associated with impaired insulin secretion and type-2 diabetes in human too [73, 74]. It is possible that, in human, R-type Ca^{2+} channels are involved in insulin secretion by operating other glucose-sensing cells like central neurons or GLP-1-producing L-cells in the gut [75].

11.9 Intracellular Ca^{2+} Channels of β-Cells

Among the channels that release Ca^{2+} from the ER or the secretory vesicles, the roles of the inositol 1,4,5-trisphosphate receptors (IP_3R) in the β-cells are well accepted. From immunohistochemistry pictures of paraffin-embedded formalin-fixed human tissues in the human protein atlas (www.hpr.se), it is evident that human islets express mainly the IP_3R2 and to a lesser extent the IP_3R3 but no IP_3R1. INS-1 and rat β-cells express predominantly IP_3R3 and IP_3R2 and to a lesser extent IP_3R1 [21]. It is evident from the same atlas that the tissue distribution of RyRs is wider than that of the IP_3Rs. In fact all of the three RyRs (i.e., RyR1, RyR2, and RyR3) are expressed to a variable degree, in almost all human tissues examined. All of the three RyRs are present also in the human islets. By RT-PCR, the mRNAs of the three types of RyRs can be detected in whole human islets [76]. β-cells certainly express the RyR2 and probably also the RyR1 isoform [76–78]. By RT-PCR, mRNA for RyR1 was not detectable in INS-1 cells and rat islets, whereas mRNA for RyR2 was readily detected [21]. By immunofluorescence using a monoclonal antibody that detects RyR1 and RyR2, Johnson et al. show that RyRs are present in ~80% of β-cells in dispersed human islets [79, 80]. Earlier studies on the RyRs in the β-cells and regulation of these channels have been reviewed [81].

In MIN6 cells, it has been shown that RyR1 is located mainly on the insulin-containing dense-core secretory vesicles, whereas RyR2 is located mainly on the ER [78]. Dantrolene, a blocker of RyR1, inhibits Ca^{2+} release from the vesicles and

inhibits insulin secretion [78]. By using a variety of approaches, including siRNA technology, Rosker et al. show that RINm5F cells express RyR2 also on the plasma membrane [82]. These putative plasma membrane RyR channels have conductance properties that are different from those reported for RyR2 in the literature, which makes one speculate that it could be a different nonspecific cation channel [83].

Low concentration of ryanodine (e.g., 1 nM) increases $[Ca^{2+}]_i$ and stimulates insulin secretion from human β-cells [80]. Another activator of RyR, 9-Methyl-7-bromoeudistomin D increases insulin secretion in a glucose-dependent manner [84]. Four molecules of FKBP12.6 are tightly associated with the four RyR2 protomers, whereby it stabilizes and modulates activity of the channel [85]. In FKBP12.6 knock-out mice, glucose-induced insulin secretion is impaired [86]. Among the glycolytic intermediates, fructose 1,6 diphosphate activates RyR2 [87]. Stimulation of β-cells by glucose increases the concentration of arachidonic acid which can activate RyRs [40]. Other molecules that can link glucose metabolism to the RyRs are cADPR, long chain Acyl CoA, and of course ATP [88].

A mathematical model to explain mechanism of glucose-induced changes in membrane potential of β-cells postulates that RyR stimulation changes the pattern from "bursting" to "complex bursting" [89]. The term "complex" or "compound" bursting refers to cyclic variations in the duration of the slow waves of depolarization and repolarization intervals observed in some islets, when they are stimulated by glucose [90, 91]. In mouse islets, compound bursting gives rise to mixed $[Ca^{2+}]_i$ oscillations (i.e., rapid $[Ca^{2+}]_i$ oscillations superimposed on slow ones) [91]. If Ca^{2+} release from the ER (through RyRs or IP_3Rs) is responsible for compound bursting and consequent mixed $[Ca^{2+}]_i$ oscillations, then both of them should be abolished if the ER Ca^{2+} pool is kept empty. In fact that is exactly what happens. Thus if the ER Ca^{2+} pool is emptied by thapsigargin in the normal mice, or by knocking out SERCA3, then there is no compound bursting and no mixed $[Ca^{2+}]_i$ oscillations [91]. Analysis of electrical activity shows a higher percentage of active phases in SERCA3$^{-/-}$ mice [91], which suggests that Ca^{2+} release (through RyRs or IP_3Rs) from SERCA3-equipped ER Ca^{2+} pool terminates the active phase (for instance, by activating Kca channels).

Glinides are a group of drugs used to stimulate insulin secretion in the treatment of type 2 diabetes. These drugs stimulate exocytosis even in SUR1 knock-out mice [92]. One of the mechanisms by which glinides induce insulin secretion is activation of the RyRs [93]. GLP-1 stimulates insulin secretion by cAMP-dependent mechanisms that include sensitization of RyR-mediated CICR [94].

11.10 Cyclic ADP Ribose (cADPR) and Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP)

These two intracellular messengers are formed from β-NAD^+ and $NADP^+$ by several ADP ribosyl cyclases including CD38 [95]. These messengers release Ca^{2+} from intracellular stores. While cADPR releases Ca^{2+} from the ER, NAADP

releases Ca^{2+} from acidic Ca^{2+} stores like lysosomes and even from insulin secretory vesicles [78]. Several groups have reported important roles for cADPR and NAADP in the regulation of Ca^{2+} signaling and insulin secretion. In β-cells, cADPR not only releases Ca^{2+} from the ER but also triggers Ca^{2+} entry across the plasma membrane by activating the TRPM2 channel [43]. High concentrations of glucose increase cADPR level in the β-cells. PKA phosphorylation activates CD38 and thereby increases formation of cADPR [96]. Thus, incretins like GLP-1 lead to an increased formation of cADPR [97]. Abscisic acid is a proinflammatory cytokine released by β-cells upon stimulation by glucose. It acts in an autocrine/paracrine fashion on a putative receptor that is coupled to a pertussis-toxin sensitive G protein and increases cAMP level which via PKA phosphorylation of CD38 increases formation of cADPR. Nanomolar concentration of abscisic acid increases glucose-stimulated insulin secretion from human islets [96].

Glucose increases NAADP level in MIN6 cells and uncaging of microinjected caged NAADP increases $[Ca^{2+}]_i$ in these cells by releasing Ca^{2+} from a thapsigargin-insensitive pool [98]. NAADP-induced Ca^{2+} release is blocked by nifedipine and some other blockers of L-type VGCCs. One of the organelle that constitutes the NAADP-sensitive Ca^{2+} stores in these cells is the dense-core insulin secretory vesicles [78]. Microinjection of NAADP into human β-cells induces Ca^{2+} release from intracellular stores in an oscillatory manner [99]. Insulin increases $[Ca^{2+}]_i$ in about 30% of human β-cells by a NAADP-dependent mechanism [99]. It is not known whether insulin increases NAADP level in human β-cells. It does not increase NAADP in mouse β-cells [100]. NAADP releases Ca^{2+} by activating a relatively new group of voltage-gated ion channels called "two-pore channels" (TPCs also termed TPCNs) [101]. TPC2 is located on the lysosomal membranes and releases Ca^{2+} when activated by low nanomolar concentration of NAADP. Micromolar concentration of NAADP inhibits the channel. As expected, in TPC2 knock-out mice, NAADP fails to release Ca^{2+} from the intracellular stores of β-cells [101].

The most well-known enzyme that synthesizes cADPR and NAADP is CD38. However, studies using CD38-knock-out mice suggest that CD38 does not play an essential role in glucose stimulation of Ca^{2+} signals or insulin secretion. In CD38-knock-out mice, the islets are more susceptible to apoptosis suggesting that CD38/cADPR/NAADP system may be important for β-cell survival [102].

11.11 Ca^{2+}-Induced Ca^{2+} Release (CICR)

Just as there are voltage-gated Ca^{2+} channels (VGCC) in the plasma membrane, there are Ca^{2+}-gated Ca^{2+} channels (CGCC) on the intracellular Ca^{2+} stores. Both IP_3Rs and RyRs are CGCCs [103, 104] and both can mediate CICR, making the process a universal one [105]. It is easy to study VGCCs on the plasma membrane by patch clamp. Nevertheless, to activate a given VGCC, one has to carefully choose the holding potential, the voltage jump, and its duration depending on which VGCC

one is looking for. Availability of potent and specific inhibitors of VGCCs has made it further easier to study these channels. This is why the literature on Ca^{2+} signaling in the islets is hugely dominated by VGCCs. The situation is far more difficult when it comes to the study of CGCCs. In analogy with VGCCs, for triggering CGCCs by Ca^{2+}, one has to carefully choose the magnitude and the duration of the Ca^{2+} trigger [106]. In practice, this is not easy. Activation of CGCCs is further dependent on the filling state of the Ca^{2+} store, phosphorylation status, and co-agonists, e.g., IP_3 and cADPR. The pharmacology of CGCCs is also more complex than that of VGCCs. Thus, low nanomolar concentration of ryanodine activates RyRs and high concentration of ryanodine irreversibly locks the RyRs in a subconductance state. Inhibition of Ca^{2+} release by ryanodine is a use-dependent process and needs attention to appropriate protocols [107].

Measurement of spatially averaged $[Ca^{2+}]_i$ by using nonlinear Ca^{2+} indicators like fura-2 and indo-1 is not particularly suitable for quantitative studies of CICR, which takes the form of transient rises of $[Ca^{2+}]_i$ in discrete locations in the cytoplasm [108]. Moreover, these indicators act as mobile buffers that bind the triggering Ca^{2+} with high affinity and snatch it away from the site of action [109]. In this respect, lower affinity brighter indicators like fluo-3 which can be used at lower concentrations are less of a problem. The global increase of $[Ca^{2+}]_i$ that one sees in a β-cell upon stimulation by glucose plus incretin hormones (e.g., GLP-1) is a net result of Ca^{2+} that enters through the plasma membrane and Ca^{2+} that is released from the stores by the process of CICR (provided the conditions for engaging CICR mechanism are in place). However, direct visualization of the CICR component may be difficult because of cell-wide increase of $[Ca^{2+}]_i$. One trick we employed was to use Sr^{2+} instead of Ca^{2+} as the trigger and exploited the differences in the fluorescence properties of Ca^{2+}- and Sr^{2+}-bound fluo-3. By this way one can show Sr^{2+}-induced Ca^{2+} release and assume that it is equivalent to CICR [110]. Another trick is to use verapamil which reduces the probability of opening of the L-type VGCCs and thereby reduces their contribution to the $[Ca^{2+}]_i$ increase. This enables better visualization of the $[Ca^{2+}]_i$ increase that is attributable to CICR. The rationale of such approach is based on the facts that verapamil does not reduce the amplitude of the single channel current; it reduces only the frequency of the triggering events but not their effectiveness in eliciting CICR [111]. In the experiment illustrated in Fig. 11.1, we stimulated a human β-cell first by 30 mM KCl which resulted in an increase of $[Ca^{2+}]_i$ to ~400 nM. We then applied verapamil which reduced the $[Ca^{2+}]_i$ to the baseline. We then washed away KCl and added instead glucose plus GLP-1. Glucose depolarized the β-cells but the expected sustained $[Ca^{2+}]_i$ increase was absent because of verapamil. Nevertheless, the L-type VGCC-mediated trigger events (which were now less frequent because of verapamil), did elicit large $[Ca^{2+}]_i$ transients by activating CICR. These $[Ca^{2+}]_i$ transients are too large to be explained by Ca^{2+} entry through the L-type VGCCs per se. These are due to synchronous activation of RyRs in clusters. In this protocol glucose facilitates CICR by increasing the ER Ca^{2+} content and by providing ATP and fructose 1,6 diphosphate, all of which sensitizes the RyRs. GLP-1 was included in this protocol since it facilitates CICR by PKA-dependent phosphorylation of the RyRs [77, 112]. In addition,

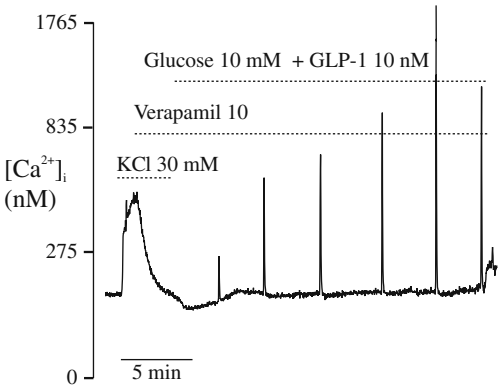

Fig. 11.1 CICR in human β-cells. $[Ca^{2+}]_i$ was measured by microfluorometry in fura-2-loaded single human β-cells. The cell was depolarized by KCl (25 mM) which increased $[Ca^{2+}]_i$. Verapamil (10 μM) was then added which lowered $[Ca^{2+}]_i$ to the baseline. (For rationale of using verapamil, please see the text and the references.) KCl was then removed and the cell was activated by glucose (10 mM) plus GLP-1 (10 nM). This protocol allowed visualization of CICR in the form of large Ca^{2+} transients

cAMP-regulated guanine nucleotide exchange factors (Epac) can also activate CICR via RyRs in human β-cells [113].

One important function of CICR in the β-cells is that it amplifies Ca^{2+}-dependent exocytosis [114, 115]. Secretory vesicle-associated RyRs are thought to play a role in exocytosis by increasing local Ca^{2+} concentration [78]. It may be noted that stimulation of β-cells by glucose alone (without cAMP-elevating agents) does not engage RyRs and thus glucose-induced insulin-secretion from human β-cells is not sensitive to inhibition or stimulation by ryanodine specially when protocols for use-dependent inhibition of RyRs by ryanodine are not employed [80]. CICR takes the form of large local Ca^{2+} transients and their function depends on the subcellular location of the transients. One possibility is that a large Ca^{2+} transient caused by CICR repolarizes plasma membrane potential by activating Kca channels. Thus a CICR event can end a burst of electrical activity and bring back the membrane potential from plateau depolarization to the baseline repolarized state and thereby increase the frequency of membrane potential oscillations. This view is supported by the observations that β-cells of $SERCA3^{-/-}$ mice as well as thapsigargin-treated β-cells (both of which would be unable to trigger CICR) spend a higher proportion of time in depolarized state and have lower frequency of membrane potential oscillation [91]. One may speculate that at early stages of development of type 2 diabetes, β-cell failure can be predominantly a depolarization failure or a repolarization failure. This view is akin to two forms of heart failure where one can have predominantly systolic failure or predominantly diastolic failure. Repolarization failure of β-cells (failure of β-cells to "relax") will lead to hyperinsulinemia and disturb the pulsatility of insulin secretion, all too well-known features of early stages of diabetes. In terms of Ca^{2+} signaling, such repolarization failure can be attributed to failure of CICR,

which can in principle be corrected by GLP-1 an established therapeutic agent for type 2 diabetes (see chapter by Leung and Cheng in this book).

11.12 $[Ca^{2+}]_i$ Oscillation in β-Cells

In the normal human body, β-cells are stimulated by glucose, the concentration of which oscillates at ~4 min interval. However, in most experiments, β-cells are stimulated by a constantly elevated concentration of glucose. In the normal human body β-cells are supplied with glucose (and other nutrients, hormones) through a rich network of capillaries; in most in vitro experiments, glucose is not delivered to the islet cells through capillaries. As mentioned earlier, human islets secrete insulin in the form of pulses at ~5 min intervals both in the fasting and in the fed states. One would expect that $[Ca^{2+}]_i$ in the human islets would change in the form of oscillations with one $[Ca^{2+}]_i$ peak every ~5 min; $[Ca^{2+}]_i$ would return to the baseline in between the peaks. This expectation is based on the observations made in mouse islets, where glucose induces baseline $[Ca^{2+}]_i$ oscillations and corresponding pulses of insulin secretion [116]. However, stimulation of human islets by glucose shows many types of $[Ca^{2+}]_i$ responses [117]. In many islets, $[Ca^{2+}]_i$ is increased and remains persistently elevated, and in others there are some high frequency sinusoidal oscillations of $[Ca^{2+}]_i$ on top of the $[Ca^{2+}]_i$ plateau [117–121]. Such sinusoidal oscillations of $[Ca^{2+}]_i$ on top of a $[Ca^{2+}]_i$ plateau have been described also in islets obtained from a subject with impaired glucose tolerance [119]. As early as in 1992, Misler et al. wrote: "four of 11 islets showed little or no response to 10 mM glucose while still responding to 20 μM tolbutamide. The pattern of glucose response of glucose-sensitive islets was also variable. Four islets displayed glucose-induced oscillations superimposed on a plateau. Two islets displayed a slow rise to a plateau without oscillations. The remaining islets showed an increasing frequency of short transients on an unchanging baseline; these transients ultimately coalesced into a prominent spike-like rise" [122]. Note that these are not bad islets; in fact these are islets of such good quality that they could be used for transplantation into human body for the cure of diabetes. Investigators know that stimulation of human islets by glucose often leads to persistent elevations of $[Ca^{2+}]_i$, rather than baseline oscillations of $[Ca^{2+}]_i$. To increase chances of obtaining oscillatory changes in $[Ca^{2+}]_i$, investigators sometimes replace extracellular Ca^{2+} by Sr^{2+} [123]. This maneuver yields nicer oscillatory changes in $[Sr^{2+}]_i$ and pulsatile insulin secretion from human islets [123]. But again, nature has chosen Ca^{2+} and not Sr^{2+} for signaling.

Some islet researchers assume that normal human islets should respond by $[Ca^{2+}]_i$ increase in the form of baseline $[Ca^{2+}]_i$ oscillations and that persistent $[Ca^{2+}]_i$ elevation is a sign of subtle damage to the islets or suboptimal experimental conditions [123]. At first sight, this seems to be a fair argument: for instance, some Ca^{2+} laboratories receive islets from a human islet isolation facility located next door; others receive islets via transatlantic flights. Ca^{2+} measurement techniques that use UV light and fura-2 acetoxymethyl esters (or similar probes) can damage

islets whose metabolism is often stunned and whose microcirculation and neural connections are lost. In fact, many individual islets obtained from normal subjects do not show any Ca^{2+} response at all to any stimulus [119]. Investigators select, consciously or subconsciously, the experiments that show nice $[Ca^{2+}]_i$ oscillations (because the islets that do not show oscillations are presumed to be the bad ones). In fact, they select the very islet that they choose to examine. There are up to several millions of islets in a human pancreas and they differ in their sizes, structures, and cellular make-up (see Chapter by In't Veld and Marichal in this book). They look different even to the naked eyes and under the microscope. Some look like "nice" encapsulated islets and others look like small aggregates of loosely associated cells, both types being normal. Investigators choose the "nice" ones for their experiments but still get different kinds of $[Ca^{2+}]_i$ responses. It is noteworthy that most such studies did not employ any cAMP-elevating agents, making CICR impossible.

$[Ca^{2+}]_i$ responses of single human β-cells to glucose are also extremely heterogeneous. Nevertheless, when single human β-cells are stimulated by glucose (in the absence of other nutrients, hormones, or neurotransmitters), many of them do respond by $[Ca^{2+}]_i$ changes in the form of slow oscillations, whereby $[Ca^{2+}]_i$ reaches to peaks every 2–5 min and then return to the baseline. Some investigators show that when $[Ca^{2+}]_i$ oscillations occur in one human β-cell, the neighboring β-cells in an aggregate or in an islet show $[Ca^{2+}]_i$ oscillation in a synchronized manner [117, 123]. This is due to coupling between β-cells via gap junctions made of connexin36 [116, 124]. Other investigators report that synchrony of $[Ca^{2+}]_i$ oscillation between groups of β-cells occur in mouse islets but not in human islets [4, 118]. Experiments using expressed fluorescent vesicle cargo proteins and total internal reflection fluorescence microscopy show that stimulation of single human β-cells by glucose gives rise to bursts of insulin vesicle secretion (at intervals of 15–45 s) that coincides with transient increase of $[Ca^{2+}]_i$ [125]. However, it needs to be pointed out that glucose-induced baseline $[Ca^{2+}]_i$ oscillations in single β-cells that we are talking about occur only in Petri dishes and are unlikely to occur in vivo. In vivo, hormones (e.g., glucagon and incretins) and amino acids (e.g., glycine and many others) are likely to transform the oscillatory $[Ca^{2+}]_i$ changes to a persistent elevation of $[Ca^{2+}]_i$ [121]. Thus, in the human β-cells and islets, persistent increase of $[Ca^{2+}]_i$ in response to glucose is a rule rather than exception. The underlying cause of glucose-induced baseline $[Ca^{2+}]_i$ oscillations in β-cells is thought to be the electrical bursts (clusters of large amplitude brief action potentials; one burst accounting for one episode of $[Ca^{2+}]_i$ increase). Study of β-cells from large mammals (e.g., dogs), however, shows that bursts occur only during the initial period of stimulation by glucose. In the later part of stimulation, bursts disappear; instead, there is sustained plateau depolarization to −35 to −20 mV and sustained increase of $[Ca^{2+}]_i$ to 500–1000 nM which causes tonic exocytosis [126]. Furthermore, at least some studies claim that insulin secretion is pulsatile even when $[Ca^{2+}]_i$ is stably elevated [127, 128]. It should be noted that stimulation of β-cells by glucose increases concentration of many molecules in the β-cells in an oscillatory manner (e.g., ATP [129] and cAMP [11]). Of these, oscillations of $[Ca^{2+}]_i$ are the easiest one to record and have, therefore, been adopted for modeling studies. It is thus not

surprising that pulsatility of insulin secretion from human islets in vivo has been modeled based on data obtained from in vitro experiments done on mice islets (see chapter by Bertram et al. in this book). This is in spite of the fact that the kind of electrical bursts and baseline $[Ca^{2+}]_i$ oscillations that occur in mouse islets have not been reproducibly demonstrated in human islets. This is not because of scarcity of human islets. In fact, during recent years it has become easier to obtain human islets for basic researches [130]. At present it appears that human islets show a wide variety of electrical activities and patterns of $[Ca^{2+}]_i$ changes which cannot explain the pulsatile insulin secretion into the human portal vein. Other less obvious factors that are unrelated to $[Ca^{2+}]_i$ oscillations, e.g., islet-liver interaction, may well constitute part of the mechanisms that determine pulsatile insulin secretion into the portal vein under normal conditions [131].

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Chapter 12
Electrical Bursting, Calcium Oscillations, and Synchronization of Pancreatic Islets

Richard Bertram, Arthur Sherman, and Leslie S. Satin

Abstract Oscillations are an integral part of insulin secretion and are ultimately due to oscillations in the electrical activity of pancreatic β-cells, called bursting. In this chapter we discuss islet bursting oscillations and a unified biophysical model for this multi-scale behavior. We describe how electrical bursting is related to oscillations in the intracellular Ca^{2+} concentration within β-cells and the role played by metabolic oscillations. Finally, we discuss two potential mechanisms for the synchronization of islets within the pancreas. Some degree of synchronization must occur, since distinct oscillations in insulin levels have been observed in hepatic portal blood and in peripheral blood sampling of rats, dogs, and humans. Our central hypothesis, supported by several lines of evidence, is that insulin oscillations are crucial to normal glucose homeostasis. Disturbance of oscillations, either at the level of the individual islet or at the level of islet synchronization, is detrimental and can play a major role in type 2 diabetes.

Keywords Bursting · Insulin secretion · Islet · Pulsatility · Oscillations

Like nerve and many endocrine cells, pancreatic β-cells are electrically excitable, producing electrical impulses in response to elevations in glucose. The electrical spiking pattern typically comes in the form of bursting, characterized by periodic clusters of impulses followed by silent phases with no activity (Fig. 12.1). In this chapter we discuss the different types of bursting observed in islets, some potential biophysical mechanisms for the bursting, and potential mechanisms for synchronizing activity among a population of uncoupled islets.

Bursting electrical activity is important since it leads to oscillations in the intracellular free Ca^{2+} concentration [1, 2], which in turn lead to oscillations in insulin secretion [3]. Oscillatory insulin levels have been measured in vivo [4–7], and sampling from the hepatic portal vein in rats, dogs, and humans shows large oscillations with period of 4–5 minutes [8, 9]. Deconvolution analysis demonstrates that the

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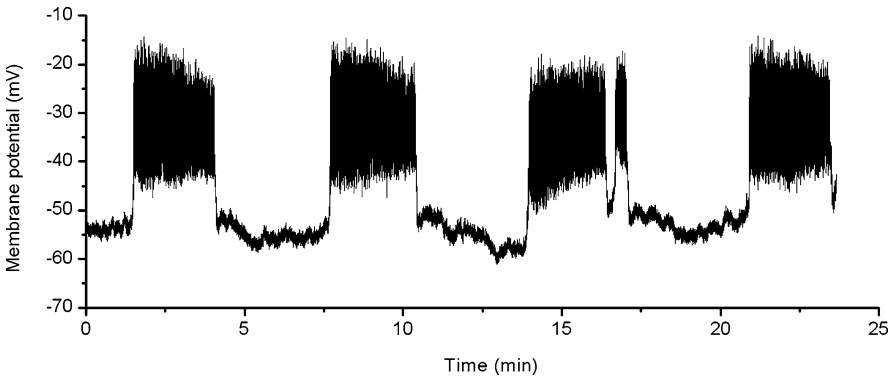

Fig. 12.1 Slow electrical bursting recorded from a mouse islet. Provided by J. Ren and L.S. Satin

oscillatory insulin level is due to oscillatory secretion of insulin from islets [8, 10], and in humans at least 75% of insulin secretion is from insulin pulses [10]. In humans, the amplitude of insulin oscillations in the peripheral blood is ~100 times smaller than that in the hepatic portal vein [9]. This attenuation is confirmed by findings of hepatic insulin clearance of ~50% in dogs [11] and ~40–80% in humans [12, 13]. It has also been demonstrated that the hepatic insulin clearance rate itself is oscillatory, corresponding to portal insulin oscillations. That is, the insulin clearance rate is greater during the peak of an insulin oscillation than during the trough [13]. This illustrates that insulin oscillations are treated differently by the liver than non-pulsatile insulin levels and thus suggests an important role for oscillations in the hepatic processing of insulin and, presumably, of glucose. In fact, coherent insulin oscillations are disturbed or lost in patients with type 2 diabetes and their near relatives [14–17], and this will most likely affect insulin clearance by the liver [13].

Oscillations in insulin have also been observed in the perifused pancreas [18] and in isolated islets [2, 3, 19–21]. The oscillations have two distinct periods; the faster oscillations have a period of 1–2 minutes [3, 5, 22, 23], while the slower oscillations have a period of 4–6 minutes [4, 5, 7]. In one recent study, insulin measurements were made in vivo in mice, and it was shown that some mice exhibit insulin oscillations with period of 3–5 minutes (the "slow mice"), while others exhibit much faster insulin oscillations with period of 1–2 minutes (the "fast mice"). Surprisingly, most of the islets examined in vitro from the "fast mice" exhibited fast Ca^{2+} oscillations with similar period, while most of those examined from the "slow mice" exhibited either slow or compound Ca^{2+} oscillations (fast oscillations clustered together into slow episodes) with similar period [24]. Thus, the islets within a single animal have a relatively uniform oscillation period which is imprinted on the insulin profile in vivo. As we describe later, the two components of oscillatory insulin secretion and their combinations can be explained by the two timescales of electrical bursting.

12.1 The Role of Calcium Feedback

Ca^{2+} enters β-cells through Ca^{2+} channels during the active phase of a burst during which it accumulates and activates Ca^{2+}-dependent K^+ channels [25, 26]. The resulting hyperpolarizing current can itself terminate the active phase of the burst, and the time required to deactivate the current can set the duration of the silent phase of the burst [27]. The endoplasmic reticulum (ER) plays a major role here, taking up Ca^{2+} during the active phase of a burst when Ca^{2+} influx into the cytosolic compartment is large and releasing Ca^{2+} during the silent phase of the burst. These filtering actions have a significant impact on the time dynamics of the cytosolic Ca^{2+} concentration, and thus on the period of bursting. The influence of the ER on cytosolic free Ca^{2+} dynamics was convincingly demonstrated using pulses of KCl to effectively voltage clamp the entire islet [28; 29]. Using 30-second pulses, similar to the duration of a medium burst, it was shown that the amplitude of the Ca^{2+} response to depolarization was greater when the ER was drained of Ca^{2+} by pharmacologically blocking ER Ca^{2+} pumps (SERCA). In addition, the slow decline of the cytosolic Ca^{2+} concentration, which followed the depolarization in control islets and which follows a burst in free-running islets, was absent when SERCA pumps were blocked. The mechanisms for these effects were investigated in a mathematical modeling study [30]. This study also showed that Ca^{2+}-induced Ca^{2+} release (CICR) is inconsistent with data from [28, 29]. CICR did occur in single β-cells in response to cyclic AMP, but in this case, electrical activity and Ca^{2+} oscillations are out of phase [32, 33], which is in contrast to the in-phase oscillations observed in glucose-stimulated islets [1, 2].

In addition to the direct effect on Ca^{2+}-activated K^+ channels, intracellular Ca^{2+} has two opposing effects on glucose metabolism in β-cells. Ca^{2+} enters mitochondria through Ca^{2+} uniporters, depolarizing the mitochondrial inner membrane potential and thus reducing the driving force for mitochondrial ATP production [34–37]. Once inside mitochondria, free Ca^{2+} stimulates pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase [38, 39], resulting in increased production of NADH, which can increase the mitochondrial ATP production. Thus, Ca^{2+} has two opposing effects on the ATP/ADP ratio: one may dominate under some conditions, while the other action dominates in different conditions.

The ATP/ADP ratio is relevant for islet electrical activity due to the presence of ATP-sensitive K^+ channels [40]. Variations in the nucleotide ratio result in variation in the fraction of open K(ATP) channels. Thus, oscillations in the intracellular Ca^{2+}concentration can lead to oscillations in the ATP/ADP ratio, which can contribute to bursting through the action of the hyperpolarizing K(ATP) current [41–44]. However, K(ATP) channels are not the whole story, since bursting and Ca^{2+} oscillations persist in islets from mice with the sulfonylurea receptor *Sur1* gene knocked out or the pore-forming *Kir6.2* gene knocked out [45–47]. Thus it is likely that another channel contributes to bursting, at least in the case of K(ATP)-knockout mutant islets.

Figure 12.2 uses a mathematical model [42] to demonstrate the dynamics of the variables described above. (Other models have recently been developed, postulating

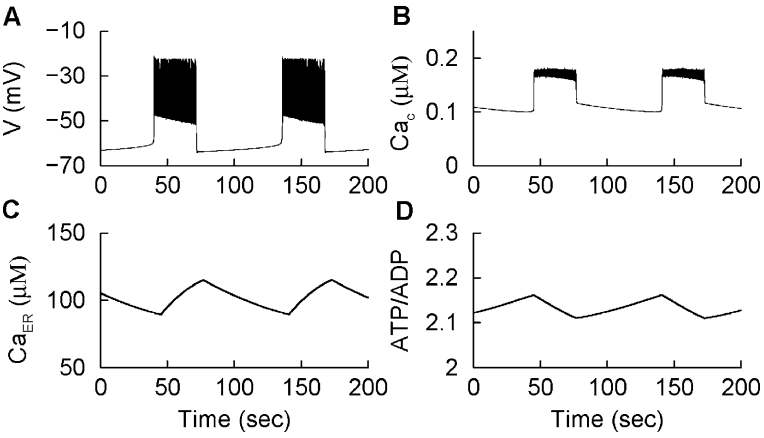

Fig. 12.2 Model simulation of bursting, illustrating the dynamics of membrane potential (*V*), free cytosolic Ca^{2+} concentration (Ca_c), free ER Ca^{2+} concentration (Ca_{ER}), and the ATP/ADP concentration ratio. The model is described in [42] and the computer code can be downloaded from www.math.fsu.edu/~bertram/software/islet

different burst mechanisms and highlighting other biochemical pathways [48, 49].) Two bursts are shown in Fig. 12.2A and the cytosolic free Ca^{2+} concentration (Ca_c) is shown in Fig. 12.2B. At the beginning of an active phase, Ca_c quickly rises to a plateau that persists throughout the burst. Simultaneously, the ER free Ca^{2+} concentration (Ca_{ER}) slowly increases as SERCA activity begins to fill the ER with Ca^{2+} (Fig. 12.2C). In contrast, the ATP/ADP ratio during a burst declines (Fig. 12.2D), since in this model the negative effect of Ca^{2+} on ATP production dominates the positive effect. Both K(Ca) and K(ATP) currents, concomitantly activated by the phase of increased Ca^{2+} and decreased ATP/ADP, respectively, combine to eventually terminate the burst, after which Ca_c slowly declines. This slow decline reflects the release of Ca^{2+} from the ER during the silent phase of the burst along with the removal of Ca^{2+} from the cell by Ca^{2+} pumps in the plasma membrane. Also, ATP/ADP increases during the silent phase, slowly turning off the K(ATP) current. The combined effect of reducing K(Ca) and K(ATP) currents eventually leads to the initiation of a new active phase and the cycle restarts.

12.2 Metabolic Oscillations

As described above and illustrated in Fig. 12.2, there will be metabolic oscillations due to the effects of Ca^{2+} on the mitochondria. In addition, there is considerable evidence for Ca^{2+}-independent metabolic oscillations, reviewed in [50, 51]. One hypothesis is that glycolysis is oscillatory and is the primary mechanism underlying pulsatile insulin secretion from β-cells [50]. The M-type isoform of the glycolytic enzyme phosphofructokinase 1 (PFK-1) is known to exhibit oscillatory activity

in muscle extracts, as measured by oscillations in the levels of the PFK-1 substrate fructose 6-phosphate (F6P) and product fructose 1,6-bisphosphate (FBP) [52, 53]. The period of these oscillations, 5–10 minutes, is similar to the period of slow insulin oscillations [50]. The mechanism for the oscillatory activity of this isoform, which is the dominant PFK-1 isoform in islets [54], is the positive feedback of its product FBP on phosphofructokinase activity and subsequent depletion of its substrate F6P [55–57]. While there is currently no direct evidence for glycolytic oscillations in β-cells, there is substantial indirect evidence for it. This comes mainly from measurements of oscillations in several key metabolic variables, such as oxygen consumption [19, 58–60], ATP or ATP/ADP ratio [61–63], mitochondrial inner membrane potential [34], lactate release [64], and NAD(P)H levels [65]. Additionally, it has been demonstrated that patients with homozygous PFK-1-M deficiency are predisposed to type 2 diabetes [66], and in a study on humans with an inherited deficiency of PFK-1-M it was shown that oscillations in insulin secretion were impaired [67]. An alternate hypothesis for Ca^{2+}-independent metabolic oscillations is that the oscillations are inherent in the citric acid cycle, based on data showing citrate oscillations in isolated mitochondria [38].

There is a long history of modeling of glycolytic oscillations, notably in yeast. Our model has a similar dynamical structure based on fast positive feedback and slow negative feedback to some of those models but differs in the identification of sources of feedback. In the models of Sel'kov [68] and Goldbeter and Lefever [69], ATP was considered the substrate, whose depletion provided the negative feedback as F6P does in our model, and ADP was considered the product, which provided the positive feedback as FBP does in our model.

Such models can also combine with electrical activity to produce many of the patterns described here [70], but the biochemical interpretation is different. In our view, ATP acts rather as a negative modulator, which tends to shut down glycolysis when energy stores are replete, and ADP acts as a positive modulator, which activates glycolysis when ATP production falls behind metabolic demand. More fundamentally, we argue that β-cells, as metabolic sensors, differ from primary energy-consuming tissues such as muscle in that they need to activate metabolism whenever glucose is present even if the cell has all the ATP it needs. In this view, ATP and ADP are not suitable to serve as essential dynamic variables but do play significant roles in regulating activity.

12.3 The Dual Oscillator Model for Islet Oscillations

Recent islet data provide the means to disentangle the influences of Ca^{2+} feedback and glycolysis on islet oscillations. Figure 12.3A shows "compound" Ca^{2+} oscillations, recorded from islets in 15 mM glucose. There is a slow component (period ~5 minutes) with much faster oscillations superimposed on the slower plateaus. These compound oscillations have been frequently observed by a number of research groups [2, 71–73] and reflect compound bursting oscillations, where fast bursts are

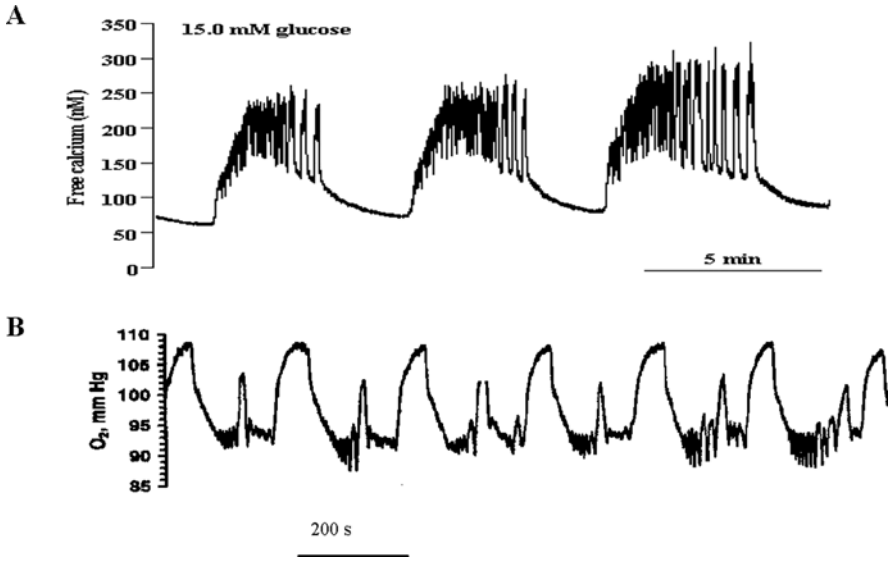

Fig. 12.3 **A** Compound islet Ca^{2+} oscillations measured using fura-2/AM. The oscillations consist of slow episodes of fast oscillations. Reprinted with permission from [79]. **B** Slow oxygen oscillations with superimposed fast "teeth." Reprinted with permission from [76]

clustered together into slower episodes [74, 75]. Figure 12.3B shows measurements of islet oxygen levels in 10 mM glucose [76]. Again there are large-amplitude slow oscillations (period of 3–4 minutes) with superimposed fast oscillations or "teeth." Similar compound oscillations have been observed in intra-islet glucose and in insulin secretion [77, 78], as assayed by Zn^{2+} efflux from β-cells. These data showing compound oscillations in a diversity of cellular variables suggest that compound oscillations are fundamental to islet function.

We have hypothesized that the slow component of the compound oscillations is due to oscillations in glycolysis, while the fast component is due to Ca^{2+} feedback onto ion channels and metabolism. This hypothesis has been implemented as a mathematical model, which we call the "dual oscillator model" [79, 80]. The strongest evidence for this model is its ability to account for the wide range of time courses of Ca^{2+} and metabolic variables observed in glucose-stimulated islets in vitro and in vivo. One behavior frequently observed in islets is fast oscillations, which do not have an underlying slow component. An example is shown in Fig. 12.4A. The dual oscillator model reproduces this type of pattern (Fig. 12.4B) when glycolysis is non-oscillatory (Fig. 12.4C). The fast oscillations are mainly due to the effects of Ca^{2+} feedback onto K^+ channels as discussed earlier. Compound oscillations (Fig. 12.4D) are also produced by the model (Fig. 12.4E) and occur when both glycolysis and electrical activity are oscillatory (Fig. 12.4F) and become phase locked. The glycolytic oscillations provide the slow envelope and electrically driven Ca^{2+} oscillations produce the fast pulses of Ca^{2+} that ride on the slow wave. Note

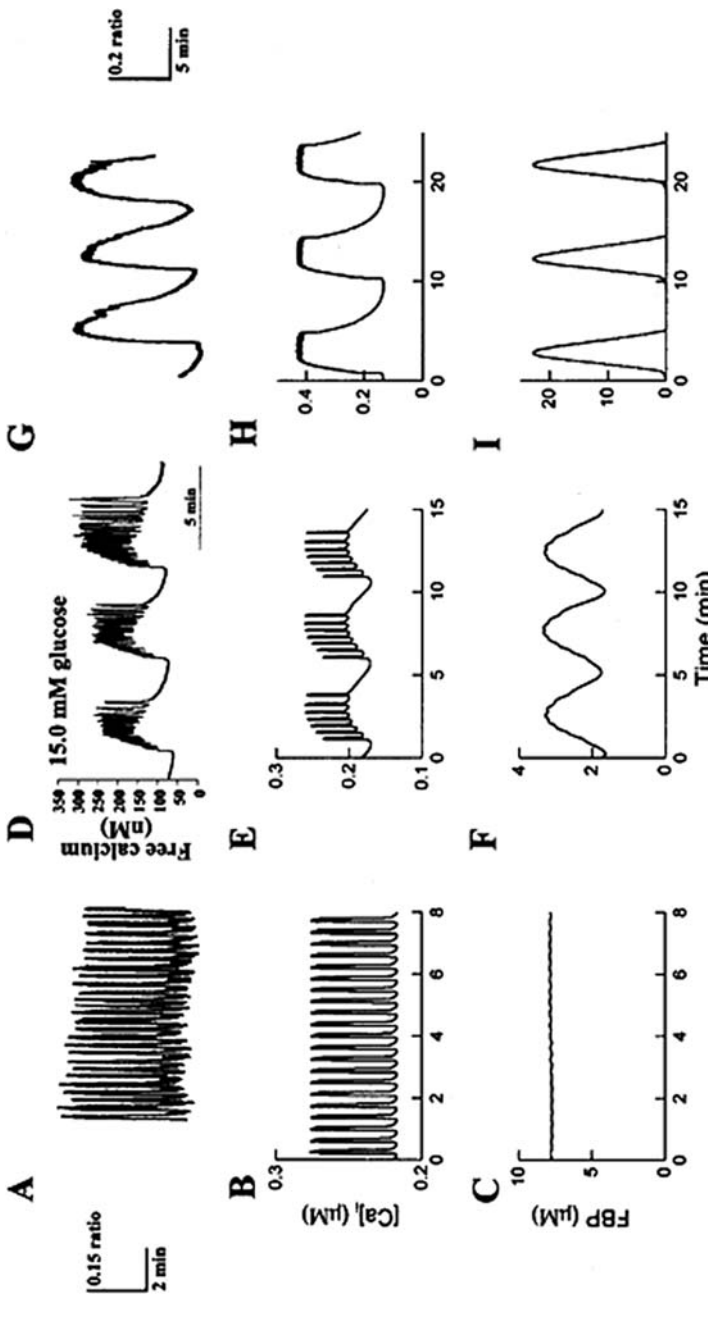

Fig. 12.4 Three types of oscillations typically observed in islets. *Top row* of panels is from islet measurements of Ca^{2+} using fura-2/AM. *Middle row* shows simulations of Ca^{2+} oscillations using the dual oscillator model. *Bottom row* shows simulations of the glycolytic intermediate fructose 1,6-bisphosphate (FBP), indicating that glycolysis is either stationary (**C**) or oscillatory (**F**, **I**). Reprinted with permission from [24, 51, 79]

that this pattern, while resembling the bursting of Fig. 12.2 on a slower timescale, is fundamentally different in that the fast bursts are sometimes observed to occur during the valleys of the glycolytic envelope, albeit with lower plateau fraction, and thus are modulated by rather than strictly dependent on the surge in FBP. This pattern ("accordion bursting") has been observed in membrane potential, Ca^{2+}, and oxygen [72, 74, 75, 81].

Compound oscillations also produce slow O_2 oscillations with "teeth," as in Fig. 12.3B. The slow oscillations in the flux of metabolites from glycolysis to the mitochondria result in oscillations in O_2 consumption by the mitochondrial electron transport chain. The Ca^{2+} feedback onto mitochondrial respiration also affects O_2 consumption, resulting in the faster and smaller O_2 teeth. A third pattern often observed in islets is a purely slow oscillation (Fig. 12.4G). The model reproduces this behavior (Fig. 12.4H) when glycolysis is oscillatory (Fig. 12.4I) and when the cell is tonically active during the peak of glycolytic activity. Thus, a model that combines glycolytic oscillations with Ca^{2+}-dependent oscillations can produce the three types of oscillatory patterns typically observed in islets, as well as faster oscillations in the O_2 time course when in compound mode.

Accordion bursting, like compound bursting, is accompanied by O_2 oscillations with fast teeth, but now is present at all phases of the oscillation in both the model [79] and in experiments [81]. The model thus suggests that the compound and accordion modes are just quantitative variants of the same underlying mechanisms. The former can be converted into the latter by reducing the conductance of the K(ATP) current, limiting its ability to repolarize the islets. It also supports the notion that β-cells have two oscillators that interact but can also occur independently of each other.

12.4 Glucose Sensing in the Dual Oscillator Framework

The concept of two semi-independent oscillators can be captured in a diagrammatic scheme (Fig. 12.5) representing how the two subsystems respond to changes in glucose. Depending on the glucose concentration, glycolysis can be low and steady, oscillatory, or high and steady. Similarly, the electrical activity can be off, oscillatory due to Ca^{2+} feedback, or in a continuous-spiking state. The two oscillators thus have glucose thresholds separating their different activity states. Increasing the glucose concentration can cause both the glycolytic and the electrical subsystems to cross their thresholds, but not necessarily at the same glucose concentrations.

The canonical case is for the two oscillators to become activated in parallel. For example, in Case 1 of Fig. 12.5, when the islet is in 6 mM glucose, both the glycolytic oscillator (GO) and the electrical oscillator (EO) are in their low activity states. When glucose is raised to 11 mM, both oscillators are activated, yielding slow Ca^{2+} oscillations. In this scenario, the electrical burst duty cycle or the plateau fraction of the slow oscillation, a good indicator of the relative rate of insulin secretion, increases with glucose concentration, as seen in classical studies of fast bursting

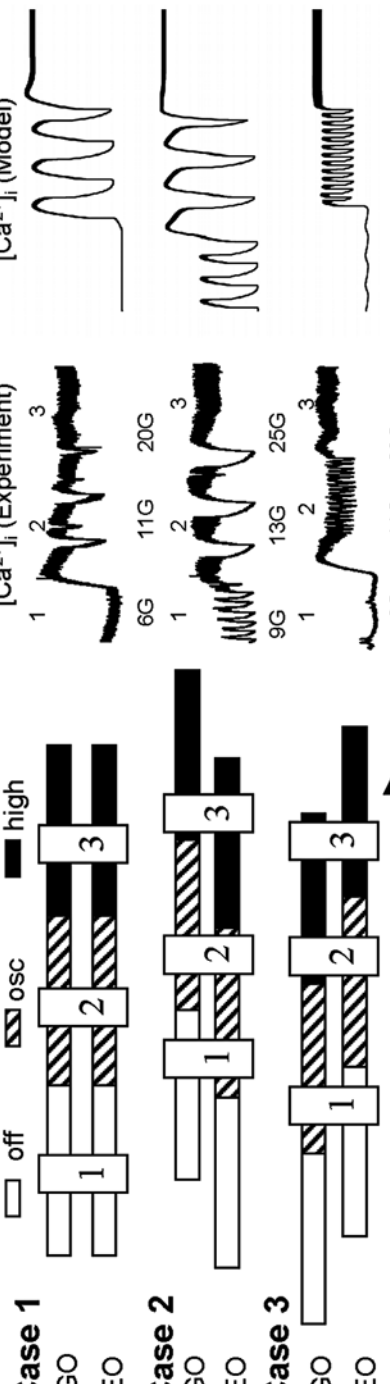

Fig. 12.5 Schematic diagram illustrating the central hypothesis of the dual oscillator model. In this hypothesis, there is an electrical subsystem that may be oscillatory (osc) or in a low (off) or high activity state. There is also a glycolytic subsystem that may be in a low or high stationary state or an oscillatory state. The glucose thresholds for the two subsystems need not be aligned, and different alignments can lead to different sequences of behaviors as the glucose concentration is increased. Reprinted with permission from [51, 85]

[82–84]. The increase in the glucose concentration in this regime has no effect on the amplitude of Ca^{2+} oscillations and has little effect on the oscillation frequency [85].

However, some islet responses have been observed to be transformed from fast to slow or compound oscillations when the glucose concentration was increased [85]. This dramatic increase in the oscillation period was accompanied by a large increase in the oscillation amplitude (Fig. 12.5, Case 2). We interpreted this as a switch from electrical to glycolytic oscillations and termed this transformation "regime change." The diagrammatic representation in Fig. 12.5 indicates that this occurs when the threshold for the GO is shifted to the right of that for the EO. This may occur if glucokinase is relatively active or K(ATP) conductance is relatively low.

At 9 mM glucose, the EO is on, but the GO is off, so fast Ca^{2+} oscillations predominate, due to fast bursting electrical activity. When glucose is increased to 13 mM, the lower threshold for glycolytic oscillations is crossed and the fast Ca^{2+} oscillations combine with glycolytic oscillations to produce much slower and larger amplitude compound oscillations.

A final example is Case 3. In this islet, subthreshold Ca^{2+} oscillations are produced in 6 mM glucose, which we believe are due to activation of the GO, while the EO is in a low activity (or silent) state. When glucose is increased to 11 mM, the lower threshold for electrical oscillations is crossed, initiating a fast oscillatory Ca^{2+} pattern. However, the upper threshold for glycolytic oscillations is also crossed, so the glycolytic oscillations stop. As a result, a fast oscillatory Ca^{2+} pattern is produced, with only a transient underlying slow component. This form of regime change is of particular interest since it suggests that the slow oscillations could occur without large-amplitude oscillations in Ca^{2+}. This would argue against any model in which the slow oscillations are dependent on Ca^{2+} feedback onto metabolism or ion channels.

In all three cases, when glucose is raised to 20 mM or higher, the system moves past the upper thresholds for both the GO and the EO, so there are neither electrical bursting oscillations nor glycolytic oscillations, and the islet generates a continuous-spiking pattern. The dual oscillator model accounts for each of these regime change behaviors, as shown in the right column of Fig. 12.5.

12.5 Functional Role for Compound Oscillations

Islets respond to increased glucose with increased amplitude of the insulin oscillations, while frequency remains relatively fixed [21]. This can be explained in part by the "amplifying pathway," in which an elevated glucose concentration amplifies the effect of Ca^{2+} on insulin secretion at a step distal to changes in Ca^{2+} [86]. A complementary mechanism, which we call the "metronome hypothesis," postulates a key role for compound oscillations in amplitude modulation of insulin secretion. In the dual oscillator model, the slow component of compound oscillations is provided by glycolytic oscillations. The period of this component sets the period of

the insulin oscillations, and computer simulations using a model glycolytic oscillator show that the period of glycolytic oscillations is only weakly dependent on glucose, except very close to threshold. The electrical bursting activity provides the fast component of the compound oscillations, and each electrical burst evokes insulin secretion. The plateau fraction of the bursting oscillations increases when the glucose concentration is increased, resulting in more insulin secretion. Since the electrical bursts occur only during the peak of a glycolytic oscillation (Fig. 12.4), and since the frequency of the glycolytic oscillations is only weakly sensitive to glucose, the effect of increasing glucose is to increase the amount of insulin secreted during each glycolytic peak, while having only a small effect on the frequency of the peaks. Thus, compound oscillations encode the stimulatory glucose level through amplitude modulation, as is the case in experimental studies. We thus suggest that the slow glycolytic component sets the timing of the insulin metronome, while the glucose-dependent plateau fraction of the fast electrical component determines the amplitude.

12.6 Islet Synchronization

Islet Ca^{2+} oscillations appear to be the driving mechanism behind pulsatile insulin. In one recent study, in vivo insulin oscillations were recorded in mice with periods of 3–5 minutes [5]. In vitro recordings of islets from the same mice showed similar periods as the in vivo insulin oscillations. The similarity of the frequencies further supports the hypothesis that the islet Ca^{2+} oscillations drive the whole-body insulin oscillations.

This then raises the question of how the oscillations synchronize from islet to islet within the intact pancreas. If the individual islet oscillators were out of phase and had widely discrepant frequencies, the net output would average out to a relatively flat insulin signal. It has been suggested that this synchronization is achieved through the actions of intrapancreatic ganglia [87–93]. The ganglia nerves form a connected network within the pancreas of rat, cat, rabbit, guinea pig, and mouse [90, 94–96], and are shown to be electrically excitable when autonomic nerve trunks are stimulated in the cat [90]. The fibers are primarily cholinergic [87], islets contain ample amounts of choline acetyltransferase and acetylcholinesterase [97], and β-cells express M_1- and M_3-type muscarinic receptors [98]. Finally, it has been shown that in vitro and in vivo vagal stimulations promote glucose-dependent insulin release from the pancreas [99–102]. It is thus plausible that cholinergic pulsing from the intrapancreatic ganglia to the subset of innervated islets entrains the islets, synchronizing their oscillations. If enough islets are synchronized in this manner, then the plasma insulin level will exhibit a coherent oscillation, as has been measured in many mammals, including man [103, 104].

The hypothesis that intrapancreatic ganglia act to synchronize endogenous islet oscillators is difficult to test in vivo, and indeed the hypothesis is largely untested. However, recent in vitro work has demonstrated the ability of a muscarinic agonist

to transiently synchronize a group of individual islets. In this study [91], three to six islets were included in an experimental chamber and intracellular Ca^{2+} levels in the islets within the chamber were monitored using the fluorescent dye fura-2/AM. The islets were uncoupled and in the presence of stimulatory glucose (11.1 mM) oscillated with different frequencies and were out of phase with one another. A single 15-second pulse of the muscarinic agonist carbachol was then applied to the bathing solution. In most cases, this brief pulse of agonist resulted in the transient synchronization of the islets (Fig. 12.6). The two panels of Fig. 12.6 show the synchronization for two trials, each containing three islets. The synchronization was transient, but in some cases lasted as long as experimental measurements were made (ca. 40 minutes). This transient synchronization was reproduced in computer simulations of the dual oscillator model, and a mechanism was postulated [91]. Thus, it appears that cholinergic stimulation can synchronize islets and could therefore be responsible for islet synchronization in vivo.

An alternate mechanism for islet synchronization has been suggested [4, 105–107]. According to this hypothesis, it is the interaction between pancreatic islets

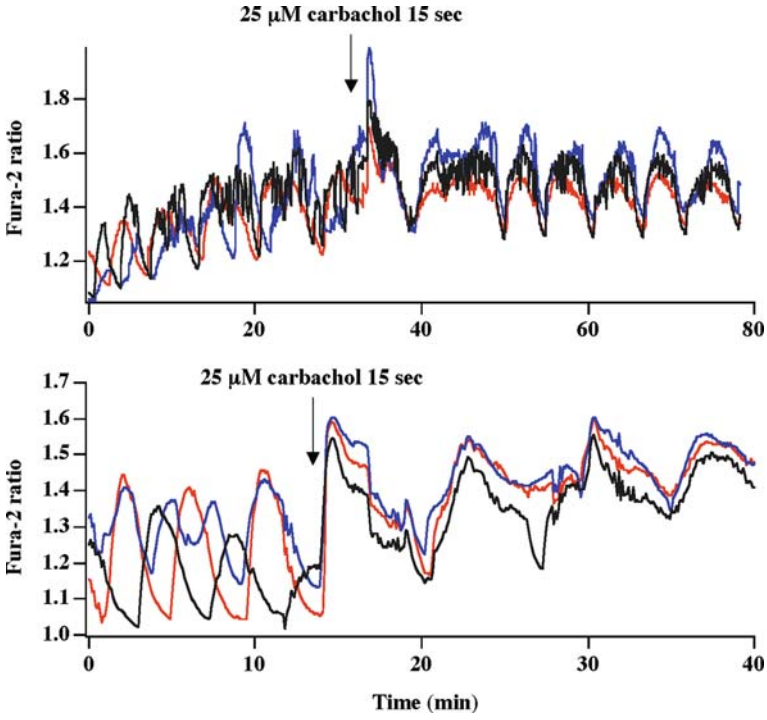

Fig. 12.6 A 15-second pulse of the muscarinic agonist carbachol (25 μM) synchronizes Ca^{2+} oscillations in islets maintained in 11.1 mM glucose. The two panels correspond to different groups of islets. Within each panel, different colors correspond to different islets. Reprinted with permission from [91]

and the liver that is responsible for islet synchronization in vivo. That is, the insulin secreted by islets acts on the liver, resulting in a reduction in the plasma glucose concentration. This change in the glucose level is then sensed by the entire islet population, providing global coupling among islets. It is plausible that this global coupling can, over time, lead to islet synchronization, but again the mechanism (which is very difficult to test) has not been tested experimentally. A recent mathematical modeling study investigated whether such a feedback system would lead to islet synchronization when the dynamics of the individual islets is described by the dual oscillator model and when the action of the liver is described by a simple equation that lowers the glucose level when the mean insulin level is elevated [105]. Figure 12.7A shows simulation results obtained with 20 heterogeneous model islets (islets have different endogenous oscillation frequencies in the model). The dashed curve is the mean level of the insulin secretion from the 20 islets, while the blue curve is this mean smoothed using a 1-minute moving average. The red curve is the extracellular glucose concentration, which is affected by the model "liver." For $t<20$ minutes, the glucose level is held constant. After 20 minutes, the glucose concentration is allowed to vary according to feedback from the liver in response to the mean insulin level. A clear small oscillation in the glucose concentration results (red curve). Simultaneously, the model islets become largely synchronized. This synchronization can be seen in the blue curve (smoothed insulin level), which exhibited small oscillations for $t<20$ minutes, but much larger oscillations for $t>20$ minutes. Thus, the dynamic islet–liver interaction leads to a coherent insulin oscillation. This effect would be more dramatic if more islets were used in the simulation, since then the oscillations for $t>20$ minutes would be larger relative to those for $t<20$ minutes when there are more islets that can synchronize. Figure 12.7B demonstrates that

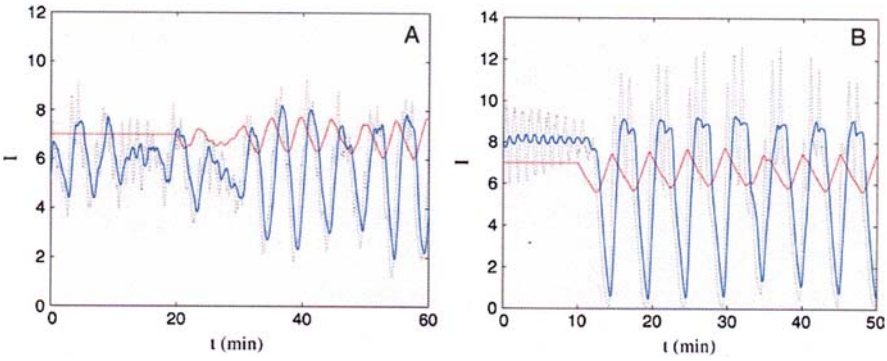

Fig. 12.7 Mathematical simulation showing that the interaction between a population of model islets and the liver can lead to islet synchronization and large insulin oscillations. Both panels show results for a population of 20 heterogeneous model islets. The glucose concentration (*red*) is held constant until $t=20$ minutes, after which it varies according to the mean insulin level (*dashed black curve*). A smoothed version of the mean insulin level (*blue*) is also shown. **(A)** Twenty oscillatory islets with different periods. **(B)** Twenty non-oscillatory islets that begin to oscillate once the "liver" is turned on. Reprinted with permission from [105]

even if the model islets are not oscillating initially, they can be induced to oscillate in phase once feedback from the liver is activated. Once again, interaction between the islets and the liver leads to a coherent insulin oscillation due to islet synchronization. A more recent modeling study, using simpler representations of islets and the liver, found results similar to those shown in Fig. 12.7A when insulin secretion was driven by the product of the glycolytic oscillator [108].

Which of the two mechanisms described above contributes to islet synchronization in vivo is not yet known. Indeed, it is possible that other mechanisms may serve this function. It is also possible that both mechanisms act together to synchronize the islets and that additional synchronizing factors such as ATP acting on purinergic receptors [109, 110] contribute to islet synchronization. Additional experiments must be performed to solve the mystery of islet synchronization in the intact pancreas.

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Chapter 13
Cyclic AMP Signaling in Pancreatic Islets

Brian Furman, Wee Kiat Ong, and Nigel J. Pyne

Abstract Cyclic 3'5'AMP (cAMP) is an important physiological amplifier of glucose-induced insulin secretion by the pancreatic islet β-cell, where it is formed by the activity of adenylyl cyclases, which are stimulated by glucose, through elevation in intracellular calcium concentrations, and by the incretin hormones (GLP-1 and GIP). cAMP is rapidly degraded in the pancreatic islet β-cell by various cyclic nucleotide phosphodiesterase (PDE) enzymes. Many steps involved in glucose-induced insulin secretion are modulated by cAMP, which is also important in regulating pancreatic islet β-cell differentiation, growth and survival. This chapter discusses the formation, destruction and actions of cAMP in the islets with particular emphasis on the β-cell.

Keywords Cyclic AMP · Adenylyl cyclase · Phosphodiesterase · Insulin secretion · Protein kinase A · Epac · GLP-1

13.1 Introduction

Interest in the role of cyclic 3'5' AMP (cAMP) in regulating insulin secretion dates back more than 40 years, since Turtle and Kipnis [1] showed increases in cAMP in isolated islets in response to glucagon. Increases in islet β-cell cyclic AMP occur in response to nutrients, especially glucose. Glucose has been widely shown to increase intracellular levels of cAMP in islets and various insulin-secreting cell lines [2–6]. Although cyclic AMP does not appear to be essential for glucose-induced insulin secretion [3, 7–9], it is established as an important intracellular amplifier of this process [10–12]. Several hormones exert their effects on insulin secretion through increased β-cell cAMP levels. These include glucose-dependent

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insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) which are collectively referred to as the incretins, and which are also secreted in response to nutrients [13–16]. GLP-1 and GIP serve to augment meal-related insulin secretion [17]. Their physiological importance is evident from observations that mice lacking receptors for both incretin hormones show marked glucose intolerance and impairment of insulin secretion [18]. This chapter focuses largely on cAMP in the β-cell. Much less is known about the role of cAMP in other islet cells, although there is some information on this in relation to glucagon and somatostatin secretion/synthesis and these aspects will be addressed briefly at the end of the chapter.

13.2 Control of cAMP Levels in the β-Cell

The level of cyclic AMP in the β-cell depends on the balance between its formation through the activity of adenylyl cyclases (ACs) and its destruction by cyclic nucleotide phosphodiesterases (CN-PDEs). This is summarized in Fig. 13.1 and discussed below.

13.2.1 Formation of Cyclic AMP in the β-Cell

Glucose-induced elevations in intracellular cAMP are probably secondary to changes in the concentration of calcium, which is itself elevated as a result of a number of mechanisms but primarily by Ca^{2+} influx through voltage-sensitive Ca^{2+} channels in response to membrane depolarization, following closure of ATP-sensitive potassium channels. Hormone-induced formation of cAMP results from stimulation of seven transmembrane G-protein-coupled receptors (GPCRs), leading to activation of the G_s protein and dissociation of the $G\alpha\beta\gamma$ heterotrimeric complex and sequential activation of adenylyl cyclases [19]. The β-cell expresses several GPCRs coupled to G_s, stimulation of which leads to elevation in the β-cell level of cAMP. These include receptors for GLP-1, GIP, PACAP as well as the receptor GPR119 (see below). On the other hand, reductions in cAMP occur in response to several agents that activate GPCRs coupled to G_i, for example adrenaline [20], PGE_2 [21] and NPY (Y_1) [22]. There is also evidence for the role of the pertussis toxin-insensitive G-protein G_z in the reduction of cAMP and inhibition of insulin secretion in response to prostaglandin E^1 [23].

GLP-1, through stimulation of its Class II GPCR, activates AC with consequent production of intracellular cAMP [24, 25]. Oxyntomodulin, which like GLP-1, is derived from the proglucagon gene, also binds to the GLP-1 receptor, increases cAMP levels and stimulates insulin secretion [26]. There is also evidence for coupling to G_i/G_o, and, in various, non-β-cell systems to other G-proteins ($G_q/_{11\alpha}$), although the physiological significance of this remains to be established. Sonoda et al. [27] identified an unusual role for β-arrestin-1 in coupling the GLP-1 receptor to

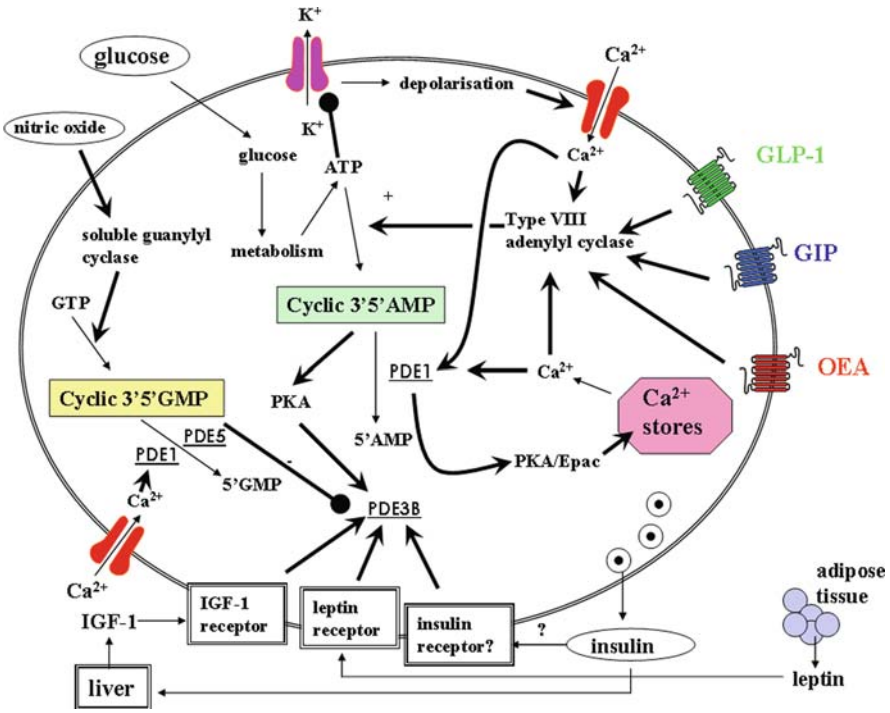

Fig. 13.1 Summary of the mechanisms for the formation and destruction of cAMP in the pancreatic islet β-cell. Glucose is transported into the β-cell using GLUT2 and is then metabolized generating ATP. This results in closure of the K_{ATP} channel, membrane depolarization and calcium influx through voltage-sensitive calcium channels. Calcium is also mobilized from intracellular stores by Ca^{2+} (calcium-induced calcium release – not shown). The increased cytosolic-free Ca^{2+} triggers exocytosis. These processes are amplified through increases in cAMP effected both through activation of adenylyl cyclases by glucose itself (through calcium-activated adenylyl cyclase – type VIII- AC VIII) and by the incretin hormones GLP-1 and GIP, acting through G-protein-coupled receptors in the β-cell membrane. Endogenous agonists for the G-protein-coupled receptor GPR119 include oleoylethanolamide (OEA). Activation of GLP-1 receptors acts synergistically with glucose in activating AC VIII and also activates other adenylyl cyclases, including soluble adenylyl cyclase (not shown). Activation of adenylyl cyclases increases the formation of cAMP which activates PKA and Epac which mediate the actions of cAMP in the cell. PKA/Epac facilitates calcium-induced calcium release which in turn may also activate AC VIII. The destruction of cAMP is effected through various phosphodiesterases (PDEs). Ca^{2+} activates PDE1 whereas PKA activates PDE3B, which is also activated by other signals generated through the IGF-1 and leptin receptors, as well as, possibly, the insulin receptor. On the other hand, PDE3B may be inhibited by increases in cGMP, allowing cross-talk between cGMP and cAMP signaling. Roles for other PDEs (PDE4, 8B and 10A) have been proposed (modified from [54])

adenylyl cyclase in INS-1 cells, thereby increasing cAMP and stimulating insulin secretion.

GIP produces its biological effects by interacting with its Class II G-protein-coupled receptor coupled to the production of cyclic AMP [28–30]. The pancreatic

islet β-cell GIP receptor is down-regulated by exposure to high concentrations of glucose, which prevents the GIP-induced elevation in intracellular cAMP [31]. This is hypothesized to explain the lack of response of diabetic patients to the peptide.

PACAP is expressed in nerve fibres and the pancreatic islets and is a potent stimulator of insulin secretion [32, 33] through activation of adenylyl cyclase [34]. There are several receptors for PACAP, with the PAC1 receptor (PAC1-R) and VPAC2 receptor (VPAC2-R) thought to be the most important in relation to insulin secretion [35].

GPR119 is a Class I GPCR, the expression of which is restricted largely to pancreatic islets, although lesser amounts of message are detected in the human gastrointestinal tract and in the rodent brain [36–38]. The potential endogenous ligands for this receptor so far identified are oleoyl lysophosphatidylcholine and oleoylethanolamide, although there is as yet no evidence that they are available in sufficient concentrations in the blood to stimulate the β-cell GRP119 receptor in vivo. The receptor is coupled through G_s to adenylyl cyclase, and its activation produces an increase in cAMP and stimulation of insulin secretion.

13.2.1.1 Adenylyl Cyclases in the Pancreatic Islet β-Cell

There are at least nine different membrane-bound isoforms of AC, described as AC I–AC IX and expressed in mammalian cells [39, 40]. An additional, soluble form is also expressed in certain mammalian cells [41]. RT-PCR studies, as well as immunohistochemical staining, using rat and human islets, rat β-cells, and clonal β-cell lines have shown expression of AC II [42] and III, IV, V, VI, VII and VIII [5, 43–45]. All isoforms of adenylyl cyclase, apart from ACIX, are activated by the diterpene forskolin, which produces marked increases in cAMP in numerous cell types [46, 47]. There are three calcium-activated ACs (AC1, ACIII and ACVIII), and the presence of calcium–calmodulin-activated ACVIII probably explains activation of cyclic AMP formation in response to glucose, which rapidly elevates $[Ca^{2+}]_i$. This AC isoform is synergistically activated by both $G_s\alpha$ and calcium/calmodulin [48]. Thus, the combination of glucose and GLP-1 increases cAMP accumulation in rat isolated primary β-cells or clonal β-cell lines more markedly than either alone, the effect being reduced if calcium entry through voltage-sensitive L-type channels is prevented using verapamil [45]. The expression of type VI (but not types II, III or V) adenylyl cyclase was increased along with the expression of the GLP-1 receptor rat pups fed a high-carbohydrate diet for 12 days [42]. These findings provide some circumstantial evidence that the type VI adenylyl cyclase may be associated with GLP-1 signaling. More recently, a role for soluble AC was proposed to explain the different kinetics of cAMP formation in response to glucose and GLP-1 in INS-1E cells. GLP-1 produced a rapid increase as a result of activation of transmembrane AC, whereas the increase in cAMP in response to glucose was delayed and was attributed to activation of the calcium, bicarbonate and ATP-sensitive soluble AC [6].

Paradoxically, acetylcholine, which increases insulin secretion through stimulation of muscarinic receptors coupled to phospholipase C/protein kinase C pathways,

also activated adenylyl cyclases and elevated cAMP content in islets from GK-diabetic rats [49]. The insulin secretory response to acetylcholine in these islets was blocked by inhibitors of adenylyl cyclase or PKA inhibitors. The abnormal nature of the islet in these rats may somehow has facilitated cross-talk resulting in activation of a calcium-sensitive adenylyl cyclase, or a PKC-sensitive adenylyl cyclase, e.g. ACII [40], in response to acetylcholine.

13.2.2 Destruction of cAMP in the Pancreatic Islet β-Cell -Cyclic Nucleotide Phosphodiesterases

Cyclic nucleotide phosphodiesterases (CN-PDEs) provide the only known means for the rapid inactivation of the cyclic nucleotides cAMP and cGMP in most cells. There are now known to be at least 100 PDE enzymes derived from 11 known gene families (PDE1-11). The enzymes show differences in their tissue distribution, substrate selectivities (cGMP vs cAMP), kinetics, regulation, and susceptibility to pharmacological inhibition. There are several excellent reviews [50–53], and the properties of those PDE enzymes present in pancreatic islets have been reviewed elsewhere [54, 55]. The key observations are summarized in this chapter, together with more recent findings.

Several PDE isoforms, including PDE1 [56–61], PDE3B [59–67], PDE4 [59, 60, 64] and PDE8B [68], contribute to the total β-cell PDE activity, and several of these isoforms regulate glucose-induced insulin secretion and other cAMP-mediated β-cell functions in islets and in cell lines [see 54, 55 for references]. There is much evidence from RT-PCR, immunostaining, siRNA and biochemical and functional studies using selective inhibitors that PDE3B plays a key role in both islets and insulin-secreting cell lines in terms of regulating insulin secretion [54, 55, 61, 63–66]. Additional evidence for the role of PDE3B in regulating β-cell cAMP and insulin secretion was obtained by over-expressing PDE3B in the INS-1 β-cell line and in islets and by using transgenic animals over-expressing PDE3B in the β-cell. These in vitro and in vivo studies clearly showed that glucose-induced, as well as GLP-1-induced, insulin secretion was impaired by PDE3B over-expression. Interestingly, both endogenous and over-expressed PDE3B was found to be located in insulin granules and the plasma membrane [67]. In vitro, the over-expression of PDE3B markedly reduced cAMP-induced exocytosis and animals over-expressing PDE3B in islets showed markedly impaired glucose tolerance [65–67]. In addition, activation of PDE3B appears to mediate the effect of IGF-1 [63] and leptin [69] in inhibiting insulin secretion.

The role of cGMP in regulating insulin secretion is not established, but several studies have shown that nitric oxide, acting through a soluble guanylyl cyclase and GMP formation, augments insulin secretion through several mechanisms shared with cAMP (see Section 13.3.1) [70–73]. These observations might be explained by cGMP-dependent inhibition of PDE3B and concomitant increases in $[cAMP]_i$.

Although evidence for the importance of PDE3B is widely supported there is also evidence, but no consensus, for roles for other PDEs. Roles for PDE1C and PDE4 have been suggested on the basis of the use of either selective inhibitors [59, 64] or siRNA [64]. Depletion of PDE8B using siRNA produced a marked enhancement of glucose-induced insulin secretion from INS-1E cells [64, 68] and rat islets [68]. A role for PDE10A has been proposed and selective inhibitors have been patented for the treatment of diabetes [74], but there is no consensus on the expression of this PDE in the β-cell, and in one study [64] selective knockdown of PDE10A failed to modify glucose-induced insulin secretion in INS-1 cells.

13.2.3 Dynamics of cAMP Formation and Destruction

Real-time measurements of changes in cAMP in β-cells or islets have been hugely facilitated by the development of new technologies, particularly the development of genetically encoded fluorescence resonance energy transfer (FRET)-based biosensors and the associated imaging techniques. These have either been transiently transfected into β-cell lines or primary β-cells [5, 75–78] or been incorporated in vivo by generating a transgenic mouse expressing a pancreatic β-cell-targeted cAMP reporter which was inducible in response to tetracycline [4]. In MIN6 β-cells, the use of the biosynthetic FRET-based cAMP sensor Epac1-camps, together with FURA-2 to detect $[Ca^{2+}]_i$, showed a close coupling of changes in cAMP and $[Ca^{2+}]_i$ [5]. Exendin-4 and forskolin induced pronounced FRET signals. Formation of cAMP in response to these agents was preceded by increases in $[Ca^{2+}]_i$ and was dependent upon extracellular calcium. Moreover, increases in $[Ca^{2+}]_i$ evoked by other agents (carbachol, K^+, and tolbutamide) also stimulated cAMP formation. Simultaneous imaging of $[Ca^{2+}]_i$ and cAMP during glucose stimulation (in the presence of TEA) revealed a tight coupling between oscillations in $[Ca^{2+}]_i$ and cAMP with peak cAMP concentrations being seen at the nadir of $[Ca^{2+}]_i$. The data are consistent with the possibility that Ca^{2+}-activated adenylyl cyclases (AC VIII or AC III) and PDEs (PDE1C?) contribute to the oscillatory changes in cAMP seen in these studies. How this concept fits with the widely accepted role of PDE3B in regulating the cAMP pool relevant to insulin secretion (Section 13.2.2) remains to be determined. Other experimental studies (Fig. 13.2) and mathematical modelling have supported these ideas [75]. Imaging of the islets from transgenic mice expressing a β-cell-targeted reporter showed a rapid, biphasic and concentration-dependent (5.5–35 mM) increase in cAMP in response to glucose. This preceded increases in $[Ca^{2+}]_i$ and was independent of extracellular $[Ca^{2+}]$ [4]. In INS-1 cells, GLP-1 produced marked oscillations in cAMP at low concentrations (0.3–1 nM) with higher concentrations (10 nM) producing more sustained elevations [77]. GLP-1 also produced marked Ca^{2+} spiking, which rapidly followed the increases in cAMP. This pattern of changes in cAMP and Ca^{2+} was mimicked by application of short pulses of the non-selective PDE inhibitor, IBMX. The rapidity of the cAMP-induced Ca^{2+} signal suggests a close proximity of the cAMP to the sites of calcium entry/release

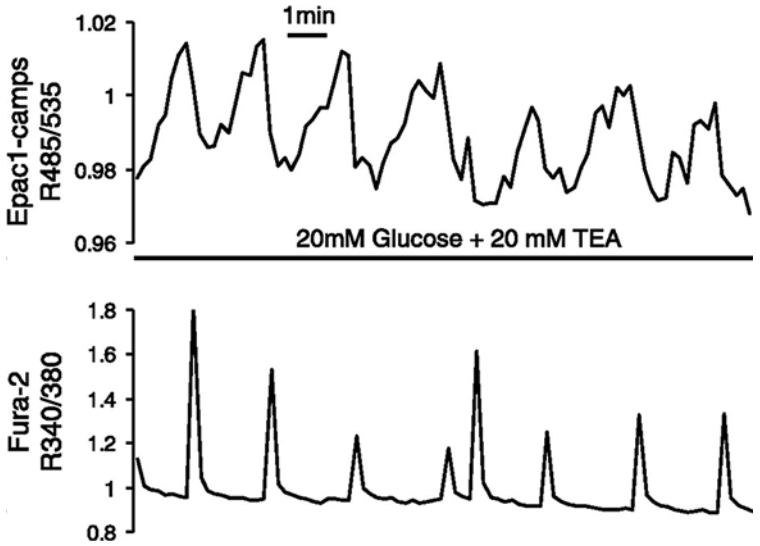

Fig. 13.2 Ca^{2+} and cAMP oscillations in glucose-stimulated MIN6 cells. Simultaneous imaging of cytosolic cAMP concentration ($[cAMP]_i$; *top trace*, $R_{485/535}$) and cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$; *bottom trace*, $R_{340/380}$) in a single MIN6 cell stimulated with 20 mM glucose and 20 mM tetraethylammonium chloride (TEA). Note that second messenger oscillations were out of phase, with each $[Ca^{2+}]_i$ spike coupled to a rapid and transient reduction in $[cAMP]_i$. (Reproduced from Fridlyand LE, et al [75])

(see next section). On the other hand, translocation of the catalytic subunit of PKA to the nucleus occurred relatively slowly and only in response to sustained increases in cAMP. Glucose also induced oscillations of intracellular cAMP levels in MIN6 and mouse primary β-cells. These oscillations correlated with pulsatile insulin secretion and both cAMP oscillations and pulsatile insulin release were reduced by inhibiting adenylyl cyclases [78]. Forskolin, glucagon and IBMX all augmented the frequency of glucose-induced oscillations in $[Ca^{2+}]_i$ in mouse pancreatic islets [79]

13.2.4 Intracellular Compartmentalization of cAMP Formation, Action and Degradation

It is now established that intracellular cAMP is not uniformly distributed in the cell and exists in different cellular locations to fulfil different functions. Localgeneration, hydrolysis and activity of cAMP are ensured by spatial distribution into compartments, or signaling complexes, of adenylyl cyclases, PDEs and effector proteins, as well as phosphatases that terminate the activity of various kinases (e.g. 80, 81). This spatial anchoring of signaling complexes is effected by a family of A-kinase anchoring proteins (AKAPs). Recent work has suggested the importance of AKAPs

in the insulin-secreting β-cell. Peptides that competitively inhibit the interaction between the regulatory subunit of PKA and the AKAP inhibited GLP-1-induced insulin secretion from rat islets without modifying its ability to elevate intracellular cAMP [9]. Expression of this inhibitory peptide in the clonal rat β-cell line, RINm5F, resulted in a redistribution of the PKA regulatory subunit and inhibited elevations in $[Ca^{2+}]_i$ and insulin secretion in response to a cAMP analogue. Expression of an AKAP (AKAP18) in clonal insulin-secreting cells (RINm5f) augmented GLP-1-induced insulin release, whereas expression of a mutant form in these cells was inhibitory [82]. These findings were supported by others [83] who used a cell-permeable peptide (TAT-AKAPis) to competitively inhibit PKA–AKAP interactions in INS-1 cells. This peptide disrupted PKA–AKAP interactions and inhibited both glucagon-induced augmentation of insulin secretion and phosphorylation of p44/p42 MAPKs and cAMP response element binding protein. While relatively little is known about the role of phosphatases in terminating phosphorylation-mediated actions of cAMP in the pancreatic islet β-cell [84], there is evidence that the AKAP AKAP79 (the human homologue of AKAP150) is important in targeting the serine–threonine phosphatase PP2B to PKA-sensitive target proteins [85].

13.3 Functions of Cyclic AMP in the Pancreatic Islet β-Cell

cAMP modulates a number of β-cell functions including insulin secretion, insulin synthesis, β-cell replication, and β-cell apoptosis. Actions of cAMP in general are mediated by at least two distinct mechanisms. The first of these is through protein kinase A (PKA)-mediated phosphorylation [86]. However, a second, and PKA-independent, effect of cAMP on insulin secretion [87–88] is mediated by the cyclic AMP-binding proteins known either as cAMP-regulated guanine nucleotide exchange factors (GEFs) or as exchange proteins activated by cAMP (Epacs) which target the small G-protein Rap1 [86]. Interestingly, most of the β-cell Rap1, at least in MIN6 cells, appears to be co-localized with insulin secretory granules [89]. When activated by cAMP, Epac, which exists as two isoforms (Epac1 and Epac2) exchanges GDP for GTP and activates downstream signaling. The pancreatic islet β-cell expresses both Epac1 and Epac2 [90]. Antisense oligodeoxynucleotides against Epac reduced the effect of a permeant cAMP analogue in augmenting glucose-induced insulin secretion in pancreatic islets [91]. Studies using selective inhibitors/activators of PKA, selective activators of Epac or the use of dominant-negative forms of Epac are revealing the roles of Epacs vs PKA in the β-cell. Novel cAMP analogues, such as 8-(4-chlorophenylthio)-2′-*O*-methyladenosine-3′-5′-cyclic monophosphate (8-pCPT-2′-*O*-Me-cAMP), and its much more cell-permeant acetoxy methyl ester [92] activate Epac but not PKA, having a 100-fold lower affinity for PKA relative to Epac [86]. Similarly, cAMP analogues such as N6-Bnz-cAMP selectively activate PKA relative to Epac. Both

Epac and PKA mediate the effects of cAMP on insulin secretion. However, at least in INS-1 cells, PKA-mediated effects account for the greater proportion of cAMP effects [92]. There is evidence for interaction between PKA-mediated and Epac-mediated effects in augmenting insulin secretion in native β-cells [93]. Some of the reported discrepancies may be explained by the poor cell permeability of some Epac-selective cAMP analogues [92].

The cyclic AMP-mediated effects of GIP and GLP-1 on insulin secretion involve both PKA [24] and PKA-independent actions. The latter are probably mediated through Epac, as evidenced by the comparative effects of the PKA inhibitor H89 and antisense oligodeoxynucleotides (ODNs) against Epac in reducing incretin-augmented insulin secretion [91, 94]. Interestingly, Epac-dependent effects of cAMP on insulin release are impaired in islets from mice lacking the SUR subunit of the K_{ATP} channel [94, 95].

13.3.1 Insulin Secretion

Malaisse's group was the first to systematically examine the actions of cAMP on insulin secretion [96, 97]. Elevations in cAMP in the β-cell augment glucose-induced insulin secretion at several sites in the secretory pathway.

13.3.1.1 Effects on the β-Cell ATP-Sensitive Potassium Channel

The β-cell ATP-sensitive potassium channel (K_{ATP} channel) plays a fundamental role in glucose-induced insulin secretion. Elevation of cAMP in the β-cell using GLP-1, forskolin, or the non-selective PDE inhibitor IBMX inhibits the β-cell K_{ATP} channel promoting depolarization of the cell [98–103]. This effect was reported to be mediated via PKA in INS-1 cells [101] through phosphorylation of the SUR1 subunit. On the other hand, Epac was found to inhibit this channel in both human β-cells and INS-1 cells, producing a leftward shift in the ATP-concentration–effect curve [102, 103]. The same study [103] suggested a PKA-mediated *activation* of the ATP-sensitive K channel.

13.3.1.2 Voltage-Sensitive Potassium Channels

Activation of voltage-sensitive potassium channels contribute to a restoration of the β-cell membrane potential and a termination of insulin secretion. GIP, acting through a PKA-dependent mechanism, reduced K currents through voltage-sensitive potassium channels in HEK cells transfected with the GIP receptor and Kv1.4 channels, as well as in human islets and INS-1 cells [104]. GLP-1 and the GLP-1 mimetic exendin-4 also inhibited voltage-dependent K currents effects again being PKA dependent as evidenced by the preventative effects of PKA inhibition [105, 106].

13.3.1.3 Elevations in Intracellular Calcium $[Ca^{2+}]_i$

Increases in $[Ca^{2+}]_i$ can be effected through two main mechanisms, namely influx through voltage-sensitive Ca^{2+} channels and mobilization of Ca^{2+} from intracellular stores and cAMP influences both these mechanisms in the β-cell.

Voltage-Sensitive Ca^{2+} Channels

Entry of Ca^{2+} through L-type voltage-sensitive calcium channels in response to membrane depolarization is an important trigger for exocytosis. Agents elevating cAMP as well as cAMP itself augment the opening of channel and increase calcium influx [99, 107–109] through PKA-dependent mechanisms. This is consistent with observations that forskolin and IBMX were shown to produce phosphorylation of the cardiac-type alpha 1 subunit of the voltage-sensitive calcium channel in a mouse β-cell line βTC3 [110].

Mobilization of Ca^{2+} from Intracellular Stores

Calcium-Induced Calcium Release

In addition to facilitating calcium entry, agents that elevate β-cell cAMP also promote calcium-induced Ca^{2+} release [111–116]. For example, the uncaging of calcium from a membrane-permeable caged calcium (NP EGTA) produced a large, transient increase in $[Ca^{2+}]_i$ but only in the presence of the GLP-1 mimetic exendin 4 or the adenylyl cyclase activator forskolin. This could be replicated by non-selective cAMP analogues or those that selectively activated either PKA or Epac. The effects of exendin-4 were relatively insensitive to the PKA inhibitor H89 but were inhibited by expression of a dominant-negative Epac2 [116], suggesting an important role of Epac2 in the sensitizing effect of cAMP on calcium-induced Ca^{2+} release. The importance of non-PKA-dependent effects of GLP-1 in elevating $[Ca^{2+}]_i$ was also reported previously [117].

The mechanism whereby cAMP promotes calcium-induced Ca^{2+} release may be through activation of the ryanodine channel in the ER [93, 112, 113] and/or through phosphorylation of the IP_3 receptor [118]. The interaction of cAMP, via PKA, with IP_3 receptors is supported by the finding that 2-aminoethoxydiphenyl borate, a cell-permeable IP_3-receptor antagonist, blocked the PKA-mediated cAMP amplification of calcium-induced Ca^{2+} release [119].

Generation of Ca^{2+}-Mobilizing Second Messengers

GLP-1 was shown to increase intracellular production of nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP-ribose (ADPR) through cAMP mechanisms mediated by both PKA and Epac [120]. The production of the second messengers, cyclic ADPR and NAADP, is catalyzed by ADPR cyclases. Both mobilize Ca^{2+} from intracellular stores and NAADP stimulates insulin secretion. The

relative role of cyclic ADPR and NAADP in producing cAMP-mediated increases in $[Ca^{2+}]_i$ remain to be determined.

13.3.1.4 Direct Effect on Exocytosis

Ammala et al. [107] and Gillis and Misler [121] were the first to demonstrate that cAMP produced direct effects on exocytosis. This effect was suggested to represent the most important effect of cAMP on insulin release [107]. Both GIP and GLP-1 promote PKA-dependent and PKA-independent exocytosis, independently of changes in calcium entry [87, 99, 122]. Moreover, photo release of caged cAMP produces a marked increase in granule exocytosis that is independent of changes in $[Ca^{2+}]_i$ [87, 99, 123, 124]. GLP-1 and cAMP augmented depolarization-induced exocytosis, and the effects of cAMP were mediated through both PKA-dependent and PKA-independent, Epac-mediated effects [95]. cAMP also enhanced exocytosis in single INS-1 cells, the effect being augmented by inhibition of PDE3 [65]. In permeabilized rat islets cAMP enhanced calcium-induced insulin secretion, independently of changes in $[Ca^{2+}]$i; this effect was largely dependent on Epac as it was mimicked by an Epac-selective, but not by a PKA selective, cAMP analogue and was unaffected by a PKA inhibitor [125]. Use of two-photon extracellular polar tracer (TEP) imaging and electron microscopy showed different roles of PKA or Epac in the enhancement by cAMP of calcium-evoked exocytosis of small compared with large, secretory vesicles [124]. Effects of cAMP on large vesicle exocytosis appeared to be PKA dependent, whereas effects on small vesicles were mediated via Epac.

There are different pools of insulin secretory granules in the β-cell. The first phase of glucose-induced insulin secretion is due to the release of granules docked at the membrane in a readily releasable pool and the second phase is dependent on the mobilization of granules to refill this readily releasable pool. The effects of cAMP, which augments both first and second phases of insulin secretion, are at least partly attributable to an expansion and refilling of the readily releasable pool [126–128]. Knockout of Epac2 specifically blocks the first phase of glucose-induced granule–plasma membrane fusions, suggesting the importance of cAMP signaling through Epac2 in this phase [89]. This supports earlier findings that the augmentation by cAMP of short depolarizations was Epac dependent, whereas the effect on longer depolarizations was largely PKA dependent and was more sensitive to cAMP [95]. The second phase of exocytosis appears to be mediated via both PKA and Epac [95, 127, 128], although a PKA dependency of the first phase of glucose-induced exocytosis has also been reported [123].

13.3.1.5 Activation of Protein Kinase C

Protein kinase C (PKC) is another second messenger contributing to the regulation of insulin secretion, and one study suggests that PKC may mediate some of the insulin secretory effects of agents that elevate cAMP. Thus, GLP-1 was shown to activate the translocation of PKCα and PKCϵ in INS-1 cells and its effects are

mimicked by forskolin. This activation was Ca^{2+} dependent, and it was hypothesized that it was effected through mobilization of Ca^{2+} as a result, for example, of PKA sensitization of the IP_3 channel and consequent Ca^{2+}-mediated activation of phospholipase C [129].

13.4 Role of cAMP in Insulin Synthesis and in β-Cell Differentiation, Proliferation, and Survival

The incretin GLP-1, acting to an important extent through cAMP effector mechanisms, increases insulin synthesis, promotes β-cell proliferation and inhibits β-cell apoptosis [25], although there is evidence for cAMP-independent effects [130]. Indeed much of the evidence for the importance of cAMP in these processes is derived from studies using GLP-1 and exendin-4. The finding that mice with a β-cell-specific deficiency in the α subunit of G_s showed reduced β-cell mass, reduced islet content of insulin, reduced β-cell proliferation, and increased β-cell apoptosis, and marked hyperglycaemia suggests the fundamental importance of responsiveness to incretin hormones [131] in β-cell homeostasis.

Glucose-mediated increases in insulin synthesis involve the phosphorylation of the transcription factor pancreatic duodenal homeobox-1 (PDX-1) and its translocation to the nucleus [132]. There is strong evidence for the importance of cAMP, acting through PKA-dependent mechanisms, in mediating the ability of GLP-1 to increase β-cell levels of PDX-1, stimulate its translocation to the nucleus and consequently activate the insulin gene promoter [133]. PDX-1 expression is itself required for the generation of cAMP in response to exendin-4 through controlling the expression of the GLP-1 receptor and the G_s protein a subunit [134].

CREB (cAMP response element binding protein) is the key transcriptional activator that mediates the effects of cAMP on gene regulation and its effects in regulating islet β-cell proliferation and survival. cAMP, through a PKA-dependent mechanism, and glucose act synergistically to regulate CREB activation in MIN6 or INS-1 cells [135, 136]. This appears to involve cAMP/PKA and glucose-induced modulation of the phosphorylation status of TORC2, a key co-activator of CREB, and the stimulation of its translocation to the nucleus [135, 136].

13.4.1 Immediate Early Response Genes

Cyclic AMP appears to mediate the effects of glucose in stimulating the β-cell expression of immediate early response genes such as *c-myc* [137] and *c-fos* [138], which probably play an important role in the effects of glucose in regulating the gene expression of metabolic enzymes, cell growth, and apoptosis. In Min6 insulin-secreting cells Glauser et al. [139] identified 592 targets and 1278 immediate early genes responding to co-stimulation with glucose and cAMP (chlorophenylthio-cAMP, a cell-permeant cAMP analogue) and suggested an important role for the transcription factor AP-1. Indeed, the AP-1-regulated gene sulfiredoxin was

identified among the targets that were sequentially induced in primary cells from rat islets. In the same context, cAMP also amplifies the effect of glucose in stimulating the MAPK/ERK pathway [6, 140–142]. The augmentation of glucose-induced activation of ERK in response to GLP-1 required both influx of Ca^{2+} through voltage-dependent calcium channels and was PKA dependent [143] and GIP activates this kinase pathway through cyclic AMP and PKA [144].

13.4.2 Protection Against β-Cell Apoptosis and Stimulation of β-Cell Proliferation

There is abundant evidence for suppression of β-cell apoptosis by agents that elevate cAMP, including GLP-1, GIP, exendin-4, ghrelin and obestatin [135, 145–151]. This appears to be PKA mediated [148, 149]. Paradoxically, some β-cell lines were made more susceptible to apoptosis following exposure to dibutyryl cyclic AMP [152] or the cyclic AMP-elevating agent forskolin [153]. The anti-apoptotic effects of cAMP are mediated, in part, by increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL [135, 146], and are PKA dependent [135, 146, 151]. The anti-apoptotic effects also involve caspase inhibition [147]. Inhibition of cytokine-mediated nitric oxide production by β-cells [154] may also be implicated.

In addition to preventing apoptosis of β-cells, the incretin hormones and other agents elevating cAMP promote β-cell proliferation through PKA-dependent mechanisms [134, 155, 156]. This effect appears to involve expression of cyclin D1 [155, 157] and cyclin A2 [134]. In this context, there may be an interaction of cAMP with Wnt signaling, which plays an important role in β-cell proliferation and survival with upregulation of cyclins D1 and D2 [158]. Thus, GLP-1 and exendin-4 activated Wnt signaling in INS-1 cells and in isolated islets [159]. Exendin-induced β-cell proliferation was inhibited by blocking β-catenin or the transcription factor TCF7L2, critical mediators of Wnt signaling [159].

An additional mechanism whereby cAMP modulates β-cell proliferation may be through regulation of the CREB antagonists cAMP response element modulator CREM-α and ICERI and the dual specificity phosphatase DUSP14, a negative regulator of the MAPK/ERK1/2 pathway. Thus, genes for these proteins were rapidly and strongly upregulated by GLP-1 in a β-cell line and in rat primary β-cells, an effect that was mimicked by forskolin and blocked by the PKA inhibitor H89 but not by an Epac inhibitor. shRNA-mediated knockdown of CREM-α or DUSP14, or expression of a dominant-negative DUSP14, augmented GLP-1-induced β-cell proliferation [156].

13.5 Possible Roles of cAMP in Other Islet Cell Types

Relatively little is known about the role of cAMP in other islet cells, although there is some information on its role in the glucagon-secreting and somatostatin-secreting

cells. Forskolin was shown to stimulate glucagon secretion from rat islets [160]. GLP-1 (and GIP) augmented depolarization-evoked exocytosis from rat α-cells; this effect was accompanied by elevations in intracellular cAMP, increases in Ca^{2+} currents and was mediated by PKA [161]. Exposure of an α-cell line (INRl-G9) expressing recombinant GLP-1 receptors to GLP-1 increased the formation of cAMP and elevated free cytosolic [Ca^{2+}] [162]. In the same cell line, an Epac-selective cAMP analogue stimulated the expression of the glucagon gene promoter and stimulated glucagon production, although not glucagon secretion [163]. Moreover, a dominant-negative Epac-2 attenuated forskolin-stimulated expression of the glucagon gene promoter in the InR1-G9 cells [163]. While these data indicate a stimulatory effect of GLP-1 on glucagon synthesis and secretion, GLP-1 is known to inhibit glucagon secretion, an action likely to contribute to its therapeutic effect in the treatment of diabetes [164]. The inhibition of glucagon secretion by GLP-1 is thus likely to be mediated by a paracrine action in the islets, for example, through stimulation of somatostatin secretion, which markedly inhibits glucagon release [165]. In this context, GLP-1, oxyntomodulin and glucagon were shown to potently stimulate somatostatin secretion from somatostatin-secreting cell lines (RIN T3; RIN 1048-38) and to stimulate the accumulation of cAMP [166, 167]. Increases in cAMP levels in response to forskolin, theophylline or dibutyryl cAMP were shown to be associated with increased somatostatin release from isolated islets [168].

Glucagon itself stimulates glucagon release by activating glucagon, rather than GLP-1, receptors, through cAMP-dependent mechanisms involving both PKA and Epac [169].

Adrenaline, or isoprenaline, acting through β-adrenoceptors, augmented depolarization-evoked glucagon secretion from rat primary α-cells [170]. This effect was mimicked by forskolin and was PKA dependent. As in the β-cell the PKA-dependent effects appear to involve more than one mechanism, including increased Ca^{2+} entry and augmentation of the effects of Ca^{2+}. Photo release of caged cAMP increased exocytosis even when intracellular [Ca^{2+}] was clamped [170]. These data were supported by observations using mouse primary α-cells, in which adrenaline-induced increases in α-cell $[Ca^{2+}]_i$ were mediated, in part, by elevations in cAMP and activation of PKA [171].

13.6 Conclusion

cAMP is clearly an important mediator/modulator of many β-cell functions from hormone secretion to proliferation, survival and synthetic functions and is also likely to be important in other islet cell types. Further work will elucidate the precise mechanisms whereby PKA and Epac, the known mediators of the effects of cAMP, exert their effects on these cellular processes. Novel ways of targeting cAMP mechanisms through small molecules, rather than peptides, may open up new treatments for diabetes mellitus. Small molecules targeting the GRP119 receptor are under

development [37]. A number of non-peptide agents that act both as direct agonists and allosteric modulators of the GLP-1 receptor are also being examined [172].

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Chapter 14
Exocytosis in Islet β-Cells

Haruo Kasai, Hiroyasu Hatakeyama, Mitsuyo Ohno, and Noriko Takahashi

Abstract The development of technologies that allow for live optical imaging of exocytosis from β-cells has greatly improved our understanding of insulin secretion. Two-photon imaging, in particular, has enabled researchers to visualize the exocytosis of large dense-core vesicles (LDCVs) containing insulin from β-cells in intact islets of Langerhans. These studies have revealed that high glucose levels induce two phases of insulin secretion and that this release is dependent upon cytosolic Ca^{2+} and cAMP. This technology has also made it possible to examine the spatial profile of insulin exocytosis in these tissues and compare that profile with those of other secretory glands. Such studies have led to the discovery of the massive exocytosis of synaptic-like microvesicles (SLMVs) in β-cells. These imaging studies have also helped clarify facets of insulin exocytosis that cannot be properly addressed using the currently available electrophysiological techniques. This chapter provides a concise introduction to the field of optical imaging for those researchers who wish to characterize exocytosis from β-cells in the islets of Langerhans.

Keywords Insulin secretion · Pancreatic islet · Sequential exocytosis · Two-photon microscopy

14.1 Introduction

Insulin is a growth hormone promoting cellular storage of carbohydrates, fats, and proteins. Insulin is the only hormone that reduces the blood glucose level, and it is selectively secreted from β-cells in the islets of Langerhans in response to

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elevated blood glucose and nutrient levels. The process of insulin secretion involves the formation of membrane-encased insulin granules, also referred to as large dense-core vesicles (LDCVs), which have diameters of 200–500 nm [1–3]. These granules are then transported to the plasma membrane, where the granule membrane fuses with the plasma membrane. This exocytotic fusion is characterized by the opening of a fusion pore connecting the two membranes. The granules are called LDCVs because in chemically fixed β-cells visualized via electron microscopy (EM), they appear to have a dense core surrounded by a halo (see Fig. 14.9). The observed dense core, however, is mostly an artifact of chemical fixation and staining, as it is not observed in quick-frozen β-cells [4] or adrenal chromaffin cells [5].

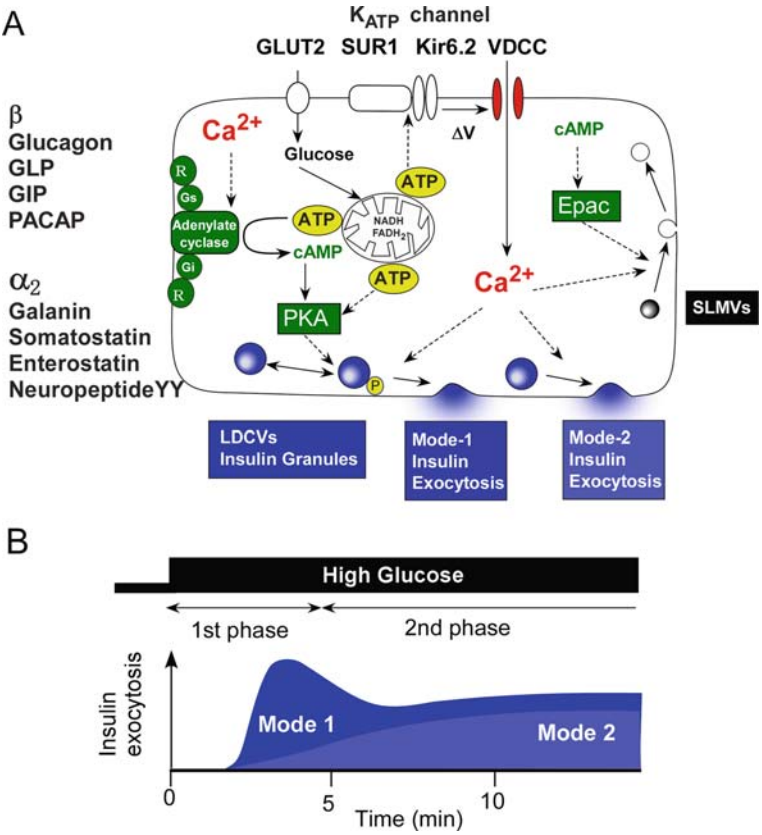

Fig. 14.1 Exocytosis in β-cells. **(A)** A simplified scheme for the exocytosis of LDCVs and SLMVs induced by glucose and hormones. Mode-1 insulin exocytosis requires the phosphorylation of targets by PKA, while Mode-2 exocytosis does not. See Section 14.4 for a more detailed explanation. (**B**) The two phases of exocytosis of LDCVs (insulin exocytosis) and the differential involvement of Mode-1 and Mode-2 insulin exocytosis in the two phases

It is generally accepted that high glucose levels increase cytosolic ATP, Ca^{2+}, and cAMP, which triggers exocytotic fusion of insulin granules (Fig 14.1A; Section 14.4.2). Such insulin exocytosis occurs in two phases (Fig. 14.1B) [6, 7]. The first phase of insulin secretion (2–5 minutes), which is particularly impaired in patients with type 2 diabetes mellitus, directly triggers glycogen synthesis in the liver and plays an important role in the control of the blood glucose level, while the second phase facilitates the utilization of glucose in a variety of tissues [8].

In addition to granules, β-cells also contain many synaptic-like microvesicles (SLMV, 50–100 nm) (Fig. 14.1) [9], which undergo Ca^{2+}-dependent exocytosis [10] in a manner that is similar to that observed in other cell types [11–14]. SLMVs in synaptic terminals, namely synaptic vesicles, are utilized for ultrafast secretion of neurotransmitters such as glutamate and γ-aminobutyric acid (GABA). The physiological role of SLMVs in non-neuronal cells, in contrast, may include housekeeping functions, such as membrane repair [15]. It is also possible that SLMVs may indirectly regulate the exocytosis of LDCVs [16]. As such, SLMV exocytosis may play a specific and unique role in different cell types, and thus the role of this process in islet cells needs to be elucidated in both physiological and pathological contexts (Section 14.5).

It has been 20-years since the key molecules involved in exocytosis were identified [17–20], and still, the molecular mechanisms of exocytosis are only partly understood. This is because a single exocytotic event involves multiple copies of a variety of different proteins, including the core fusion complex, soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs), putative Ca^{2+} sensors, synaptotagmins, and a myriad of membrane lipid molecules. β-cells express the full complement of proteins identified as playing a key role in neurotransmitter release, including the SNAREs, syntaxin-1, SNAP-25, and VAMP2; the SNARE-interacting proteins, Munc18-1, Munc13-1, complexin 1; and the GTPase Rab3A [21, 22]. In addition to these proteins, insulin secretion requires the actions of non-neuronal proteins, such as granuphilin, Noc2, and Rab27A, which are selectively enriched in endocrine cells [23, 24] and whose expression is regulated by microRNAs (Section 14.4.3).

Elucidation of the molecular mechanisms underlying insulin exocytosis therefore depends on a combination of modern biophysical and molecular biological analysis techniques. In this chapter, we first introduce methodologies to investigate exocytosis in β-cells, with emphasis placed on two-photon excitation imaging, which is the only imaging methodology available that allows for the examination of exocytosis in intact tissue preparations [2, 3, 25–32]. We then proceed to characterize insulin exocytosis from β-cells compared to exocytosis by other secretory cells, explaining the molecular bases of the two phases of glucose-induced insulin secretion, and we finally describe small vesicle exocytosis in β-cells. Throughout this process, we will address apparent discrepancies in data that have been obtained using different methodologies and discuss the rationale behind the notions of "docking," "priming," and the "readily releasable pool of vesicles."

14.2 Measurements of Exocytosis

14.2.1 Electrophysiological Approaches and Quantification

The classic methods of quantifying insulin secretion involve the measurement of the amount of insulin secreted from the islets using, for example, radioimmunoassays (RIAs) or enzyme-linked immunosorbent assays (ELISAs). More analytical approaches, however, are necessary to elucidate the mechanisms underlying this secretion. One such approach, membrane capacitance measurement, is based upon the assumption that the capacitance per membrane area is constant and thus employs the patch-clamp method to detect the increases in capacitance that result from the exocytosis-mediated increases in the membrane area (Fig. 14.2A) [33]. This approach is particularly useful when stepwise changes in capacitance can be detected, as this makes it possible to estimate the diameters of vesicles [33–35] and to characterize fusion pore properties with temporal resolution in the millisecond range [34, 36, 37]. When capacitance changes are not stepwise, however, exocytosis of LDCVs and SLMVs cannot be readily distinguished with this method [38]. Furthermore, concurrent exocytosis and endocytosis are also not distinguishable using this technique [30, 39, 40].

Electrochemical measurement of serotonin release from serotonin-loaded β-cells using carbon fiber electrodes (amperometric measurement) has also been

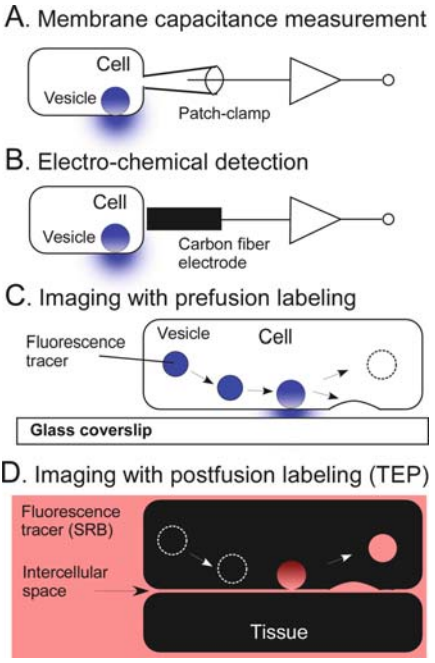

Fig. 14.2 Analytical methods used to study exocytosis and endocytosis. TEP represents two-photon extracellular polar-tracer imaging. Adapted from [62] with permission from Elsevier

employed to analyze the exocytosis of insulin granules (Fig. 14.2B) [38, 41]. Unlike capacitance measurement, amperometric measurement is unaffected by concurrent endocytosis. However, this approach generally assumes that serotonin is loaded selectively into insulin granules, which is a critical assumption that has not been validated experimentally. Differences between amperometric signals and capacitance increases have been reported in various secretory cells [42–45], including β-cells [38]. These differences likely reflect the delay associated with the diffusion of monoamines [45] and the involvement of SLMV exocytosis in the observed release [10, 38, 44]. Amperometric measurements also contain an artifact caused by the effects of UV irradiation on the carbon fiber electrode, which is particularly disruptive when rapid events are examined [46].

The release of fast neurotransmitters, such as ATP and GABA, can be detected via a biosensor method that uses chromaffin [47], INS-1 [48], and β-cells [49] exogenously expressing receptors for these transmitters. In these cells, the currents evoked by transmitter binding to the exogenously expressed receptors can be measured electrophysiologically as an indicator of transmitter release. This biosensor approach was used previously to characterize SLMV exocytosis based upon the assumption that GABA is selectively stored in SLMVs [49]; however, it has since been found that GABA is also stored in insulin granules [50], complicating the situation considerably. It is also likely that ATP is found in both types of vesicles, as ATP is an established neurotransmitter in synaptic vesicles. In support of this theory, the uncaging of caged Ca^{2+} in β-cells induced rapid ATP-mediated currents [46], which occur with a time constant that is similar to that of SLMV exocytosis measured with two-photon imaging [10] (Section 14.5). In short, electrophysiological methodologies are convenient tools for the quantification of exocytosis parameters, but they often lack the specificity necessary to provide a complete picture of the events involved.

14.2.2 TIRF Imaging and "Docking" of Granules

Live imaging is necessary to study dynamic structural processes like exocytosis. A popular live imaging methodology involves the staining of vesicles before fusion (Fig. 14.2C) and the visualization of exocytosis via total internal reflection (TIRF or evanescent-field) microscopy, which illuminates preparations less than 100 nm from the surface of a glass coverslip with high spatial resolution [51]. This approach is particularly well suited to investigations of the process of vesicle attachment or "docking" to the plasma membrane, which has often been considered to be a prerequisite for exocytosis [52, 53]. The first phase of insulin secretion has been reported in one study [54] to be mostly mediated by insulin granules "docked" to the plasma membrane; however, conflicting data have been presented in other studies [55, 56]. These latter studies demonstrate that fusion of granules with the plasma membrane can occur without apparent "docking" during insulin exocytosis in β-cells. Such "crash fusion" has also been reported in chromaffin cells [57–59]. Furthermore, EM

studies on β-cells have revealed that the apparent "docking" observed in TIRF imaging does not represent the actual attachment of vesicles to the plasma membrane [55] and that differences in the depth of penetration of TIRF microscopy might provide one explanation for the inconsistent detection of "docking" across laboratories [54–56]. In fact, experiments with granuphilin have even indicated that "docking" may actually delay the fusion of insulin granules [23, 55].

TIRF imaging detects only a small portion of individual LDCVs, as the diameters of LDCVs are larger than the depth of penetration of TIRF microscopy. Thus, in principle, fluorescence changes are not fully interpretable without additional information. For example, it is impossible to measure the distance between the vesicle and the plasma membrane without invoking a number of assumptions, for example, on the constancies in size, shape, and brightness of vesicle. TIRF imaging also cannot detect exocytotic events, such as compound exocytosis, that involve structures deeper than the evanescent illumination can penetrate (Section 14.2.3.2). In fact, TIRF microscopy can only be used to study vesicles associated with portions of the plasma membrane that are attached to the glass coverslip, which is an important consideration in light of the fact that these regions do not actually correspond to the sites of physiological exocytosis. If these points are carefully addressed, however, the high spatial resolution of TIRF microscopy can be useful in studies aiming to elucidate the molecular bases of exocytosis.

14.2.3 Two-Photon Imaging and the Spatial Organization of Exocytosis

14.2.3.1 TEP (Two-Photon Extracellular Polar-Tracer) Imaging

In general, imaging coupled with prefusion labeling (Fig. 14.2C) does not allow for estimation of vesicle diameters or tracking of vesicle fates after exocytosis and is subject to a selection bias for the subset of vesicles that are well labeled [60]. More seriously, methods that involve labeling vesicles with GFP-based probes can actually alter secretion kinetics [60]. Postfusion labeling, in contrast, does not suffer from the above-mentioned issues of selection bias and interference with secretion processes and provides an ideal method to study postfusion vesicle fates (Fig. 14.2D). We have found that such postfusion labeling experiments are ideally performed by immersing the secretory preparations in a solution containing fluid-phase polar tracers and visualizing the inside of the tissue using the two-photon excitation microscope [61] (Fig. 14.4A). We have screened a number of such polar tracers and have found sulforhodamine B (SRB) to be the best tracer available in terms of size, solubility, brightness, and cost effectiveness. The intercellular space of tissues is normally quite narrow at 20–40 nm, which is less than the diameter of most secretory vesicles (Fig. 14.2D), and thus allows for a high signal-to-noise ratio when employing imaging techniques. Also, TEP imaging overcomes many of the shortcomings discussed above for TIRF microscopy, as the intercellular space within tissue is very clean, unlike the space between a cell and a glass coverslip, and is

also the physiological site of exocytosis. Since staining with TEP imaging is non-selective, it will reveal all exocytotic events in the visual field [31] and thus can also be used to characterize abnormal secretion patterns in diseased or mutant animals with little selection bias.

Two-photon excitation can simultaneously excite multiple tracers with a single laser source because of the broader two-photon excitation spectra [61], making this technology ideal for simultaneous multi-color imaging. To date, it has been possible to perform up to four-color imaging using an 830-nm laser for excitation. Two-photon imaging thus provides the best method to correlate fluorescence resonance energy transfer (FRET)-based protein signals with cellular events, such as exocytosis and endocytosis. TEP imaging can also be combined with capacitance measurements [30], investigations involving photolysis of caged compounds coupled with electron microscopy [29, 30], and prefusion labeling of specific molecules [2, 32]. TEP imaging is also a good compliment for molecular biological techniques, as it allows for direct study of the tissues of mutant animals. We have succeeded in using TEP imaging to reproduce RIA findings regarding insulin secretion in three mutant mice: the *ashen* mouse, which lacks Rab27a [24], the collectrin transgenic mice [62], and the HNF-4alpha knockout mice [63]. The effects of knocking out CAPSs have also been similarly evaluated with both RIA and TEP imaging in another laboratory [64].

The major disadvantage of two-photon microscopy is the costly femtosecond laser and the difficulty involved in maintaining the laser and the microscope, which are unfamiliar technologies for most biologists and even many biophysicists. We hope that these difficulties will be overcome with time and that more laboratories will thus be able to enjoy the benefits of TEP imaging.

14.2.3.2 Spatial Organization of Exocytosis: Docking, Priming, and the Readily Releasable Pool of Vesicles

We have used TEP imaging to study a number of representative secretory cell types and have found that in each secretory cell type, the exocytotic machinery and the processes involved exhibit a unique pattern of spatial organization (Fig. 14.3). Full fusion of a vesicle with the plasma membrane is the dominant form of exocytosis in β-cells in the pancreatic islets [2, 3, 26, 31, 65, 66] (Fig. 14.3A), though other forms of exocytosis do exist in these cells (Section 14.3). In contrast, exocrine acinar cells frequently exhibit sequential exocytosis [25, 27, 28] (Fig. 14.3A) and adrenal chromaffin cells exhibit vacuolar sequential exocytosis [32] (Fig. 14.3A), where swelling of the granule contents facilitates sequential exocytosis. Sequential exocytosis is most suitable for those secretory cells that undergo massive exocytosis under the control of secretagogues. Sequential progression of exocytosis in these cells is indicative that some factors necessary for fusion diffuse from the plasma membrane into the vesicle membrane (Fig. 14.3B), an idea that has been confirmed via direct imaging (see Fig. 14.6). This may be one physiological function of the

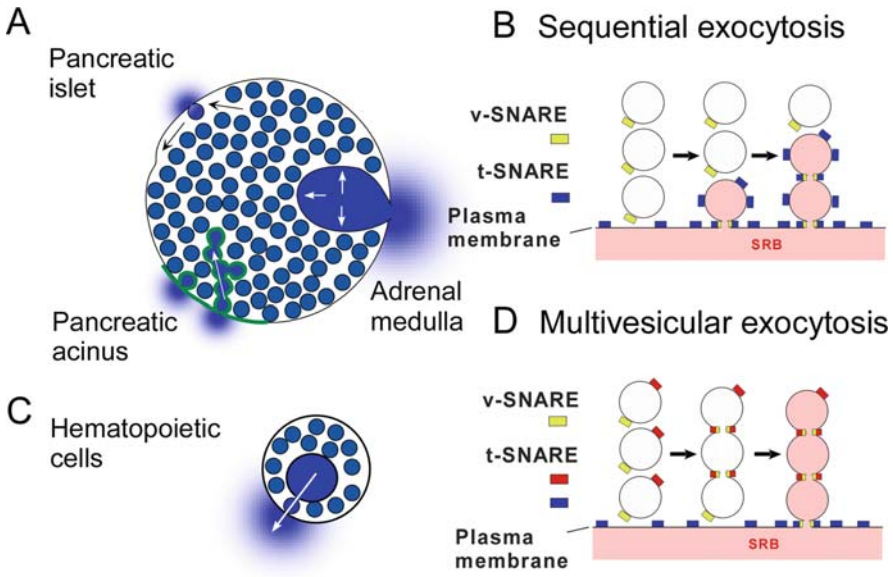

Fig. 14.3 Different forms of compound exocytosis. (**A**) Full fusion exocytosis, sequential exocytosis, and vacuolar sequential exocytosis in the pancreatic islet, the acinus, and the adrenal medulla, respectively. *Green outlines* in the pancreatic acinus indicate the actin coating. (**B**) Sequential exocytosis is supported by lateral diffusion of t-SNAREs from the plasma membrane. (**C**) Multivesicular exocytosis of eosinophils and basophils. (**D**) Multivesicular exocytosis may utilize two distinct t-SNAREs. Adapted from [61] with permission from Elsevier

observed tight "docking" of granules to the plasma membrane in sequential exocytosis, as this process has been shown to require stable attachment of the outermost granules to the plasma membrane, even after exocytosis [29, 32, 61].

There is another type of compound exocytosis, called multivesicular (or multigranular) compound exocytosis, where vesicles fuse with each other in the cytosol before fusing with the plasma membrane (Fig. 14.3C). Multivesicular exocytosis has been described in eosinophils [67, 68], where secretion can be explosive. This is in contrast to the sequential exocytosis described above, where secretion is under the specific control of regulatory agents (Fig. 14.3A, B). The term "compound exocytosis" has been used to describe sequential exocytosis in an early study of mast cells [69] but for multivesicular exocytosis in eosinophils [67, 68]. It should be noted, however, that these two forms of "compound exocytosis" most likely have distinct molecular mechanisms (Fig. 14.3B, D) and physiological functions.

In line with the "crash fusion" events observed via TIRF imaging of LDCVs [55, 56, 59], TEP imaging has revealed that SLMVs undergo massive exocytosis without the "docking" of vesicles to the plasma membrane prior to stimulation in PC12 cells [30] and β-cells [10]. Studies of sequential exocytosis have also indicated that prior "docking" of vesicles to the plasma membrane is unnecessary for exocytosis, because the vesicles in the deep cytosolic layer do not dock to the plasma membrane

but do undergo rapid exocytosis and participate in this sequential replenishment process [27, 32]. These findings thus suggest that a "priming" step may occur before the attachment of vesicles to the plasma membrane [70] and that the "readily releasable pool of vesicles" is not necessarily comprised of "docked" vesicles [56, 71].

14.3 Insulin Exocytosis

14.3.1 Single Insulin Granule Exocytosis

In TEP imaging, insulin exocytosis occurs as sudden appearance of a fluorescent spot close to the intercellular space in the islets (Fig. 14.4A– C). The increase in the fluorescence indicates the backfilling of granules with extracellular SRB (Fig. 14.4C), as these fluorescent spots are immunoreactive to insulin [26]. Two-photon extracellular tracer imaging-based quantification (TEPIQ) analysis has also indicated that the observed spots in islets have an estimated diameter that is consistent with that of insulin granules (Fig. 14.5A)[61]. TEPIQ has been used to estimate the diameters of exocytotic granules in various secretory cells based on the intensity of fluorescent spots relative to that of the extracellular medium, and these estimates have been consistent with the values obtained via EM measurements, supporting the accuracy of this method.

Fluorescence decays within several seconds during most release events (Fig. 14.4C), reflecting the full fusion of granules with the plasma membrane. In support of this, flattening of the Ω-shaped profile has been directly detected in *en face* events (Fig. 14.4C). Such exocytotic events are induced by an increase in the cytosolic Ca^{2+} concentration, which often occurs in an oscillatory manner (Fig. 14.4D). TEP imaging can be used to visualize most insulin exocytosis in a focal plane of the islet, as the amount of insulin secretion predicted based upon the number of glucose-induced exocytotic events observed via TEP imaging is in accord with the amount of insulin release measured by RIA [31]. The number of exocytotic events observed via TEP imaging per 800 μm^2 field correlates well with the reported number released by a single cell [31], which amounts to 6–12 LDCVs min^{-1} $cell^{-1}$. TEP imaging has also been used to show that the number of exocytotic events can be greatly potentiated, most prominently in the first phase, by treatment with forskolin, an activator of adenylate cyclase that increases the cytosolic concentration of cAMP (Fig. 14.4E). These features of exocytosis observed via TEP imaging are consistent with those observed via RIA [6] and TIRF imaging [56].

It is notable that exocytosis has been observed all over the plasma membrane of β-cells in TEP studies (Fig. 14.4B), though in accord with the findings of previous studies [72], there appears to be a slight trend toward exocytosis in the direction of blood vessels [26]. Given that no tight-junction structure is seen in islets [73] or endocrine cells in general, this indicates that the intercellular space of the gland is the major pathway for secretion of the hormone.

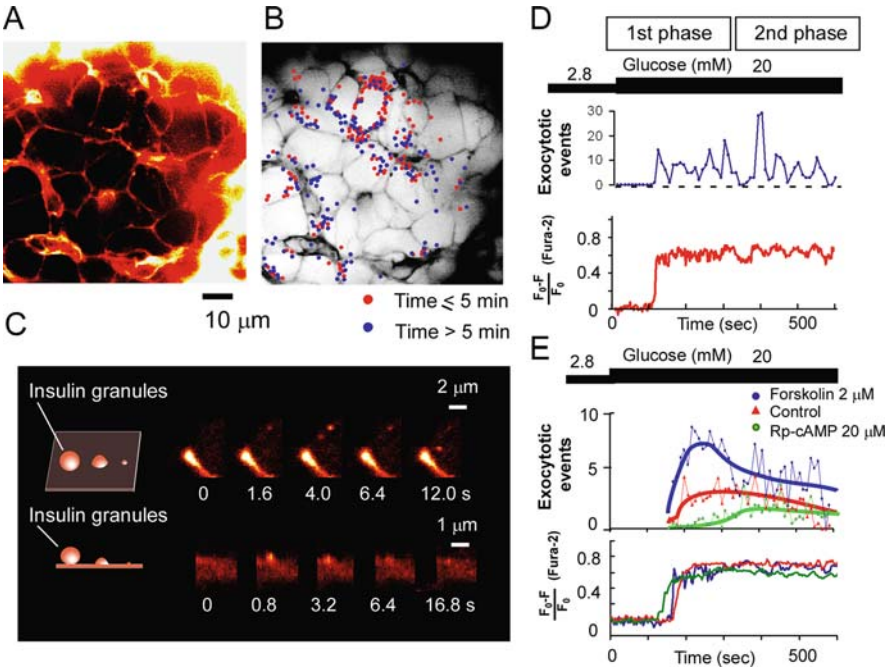

Fig. 14.4 Two-photon excitation imaging of exocytotic events in β-cells in mouse pancreatic islets. (**A**) A single islet immersed in a solution containing SRB. Large vessels can be seen at the *bottom left portion* of the panel. (**B**) Distribution of exocytotic events in an islet stimulated with 20 mM glucose. *Red* and *blue dots* represent sites at which exocytotic events were observed in the first and second phases of glucose-induced insulin secretion, respectively. The underlying *gray image* is the inverse image of the SRB fluorescence shown in (**A**). (**C**) Successive images of the glucose-induced abrupt appearance of SRB fluorescent spots. As illustrated by the schemes on the left, the *top images* were obtained from the interstitial space parallel to the imaging plane, while the *bottom images* show an *en face* view of exocytosis toward the vertically oriented interstitial space, and flattening of vesicles can be directly visualized. Glucose-induced exocytosis in a single islet (**D**) or averaged for several islets (**E**). The data in (**E**) were acquired from islets treated in the absence (*red*, $n = 5$) or the presence (*blue*, $n = 3$) of forskolin (2 μM) or Rp-cAMP (20 μM) (*green*, $n = 4$). Exocytotic events were measured within an arbitrary area (2000 μm^2) of islets. The bottom traces show the increase in the cytosolic Ca^{2+} concentration recorded from single islets. The cytosolic Ca^{2+} concentration is represented by $(F_0 - F)/F_0$, where F_0 and F stand for resting and post-stimulation fluorescence, respectively. Adapted from [26] with permission from AAAS

In our study, β-cells underwent full flattening with the plasma membrane in 92% of events [26]. This type of exocytosis, termed "full fusion exocytosis," is the simplest form of exocytosis and has been assumed to exist for a long time [74], but TEP imaging has provided the first definitive evidence of this process in mammalian secretory cells. Experiments with both SRB and FM1-43 have shown that full fusion occurs with two time constants, 1.5 and 15 seconds in β-cells (Supplementary Fig. S2 of [26]). The time course of decay was somewhat slower than in chromaffin cells,

where the mean lifetime of the Ω-shaped profile was about 0.25 seconds [32]. As discussed below, this may reflect the time required for the insulin crystal to dissolve. Consistent with the above data, a recent study using Zn-sensitive dyes demonstrated that most vesicles (60%) completely released insulin within 0.2–10 seconds of the onset of exocytosis in primary β-cells [66].

It has become evident that β-cells are rather exceptional in predominantly utilizing full fusion exocytosis with minor contributions by two other forms of exocytosis: kiss-and-run exocytosis (6%, see below) and sequential exocytosis (2%). Sequential or compound exocytosis has been observed in some studies of β-cells [75], particularly in cases where the islets were strongly stimulated, but this is far less common in these cells than in pancreatic acinar cells [25], adrenal chromaffin cells [32], and hematopoietic cells. We hypothesize that sequential exocytosis is suppressed in β-cells to prevent hypersecretion of insulin, which would result in hypoglycemic coma. One mechanism for the suppression of sequential exocytosis in these cells is that most vesicles undergo full fusion, and there is thus no chance for secondary exocytosis. There must, however, be an additional mechanism responsible for this blockade of hypersecretion (described in Section 14.3.3), as stimulation with a caged Ca^{2+}compound greatly prolonged the lifetime of the Ω-shaped profile without affecting the occurrence of sequential exocytosis [2].

14.3.2 Fusion Pore Kinetics and "Kiss-and-Run" Exocytosis

The fusion pore is the initial semi-stable aqueous pore that is formed during the fusion of two membranes. Time-resolved membrane capacitance measurement has been used to estimate the diameter of the pore to be about 0.3–2 nm, where it is stable for a period and can be reversibly closed [34, 36, 76]. Capacitance measurement, however, cannot estimate the diameters of pores larger than 2 nm for small vesicles or 6 nm for large vesicles [34, 36], possibly leading to underestimation of pore sizes.

To overcome this limitation, we used fluorescent polar tracers as nanometer-sized probes in TEP imaging experiments (Fig. 14.5B) [61]. In these experiments, we used two polar tracers, SRB (0.3–0.7 mM) and dextrans conjugated with fluorescein of different molecular weights (0.5–2 mM). Based on the molecular structures and light scattering, we estimated the hydrodynamic diameters of SRB and 10-kDa fluorescein dextran (FD) to be 1.4 nm and 6 nm, respectively [61], and found that large dense-core vesicles of adrenal chromaffin cells and PC12 cells were nearly simultaneously stained with the two compounds, with a time lag of less than 50 ms. This time lag is consistent with the 10–50 ms lifetime of the fusion pore observed in these cells in studies using capacitance measurements and amperometry [77].

In β-cells, there were significant time lags of 1–2 seconds between the SRB and 10-kDa FD signals, with a mean value of 1.8 seconds (Fig. 14.5B) [26, 31], suggesting that dilation of the fusion pore is exceptionally slow for insulin vesicles in β-cells. There are a number of observations that suggest that this is due to

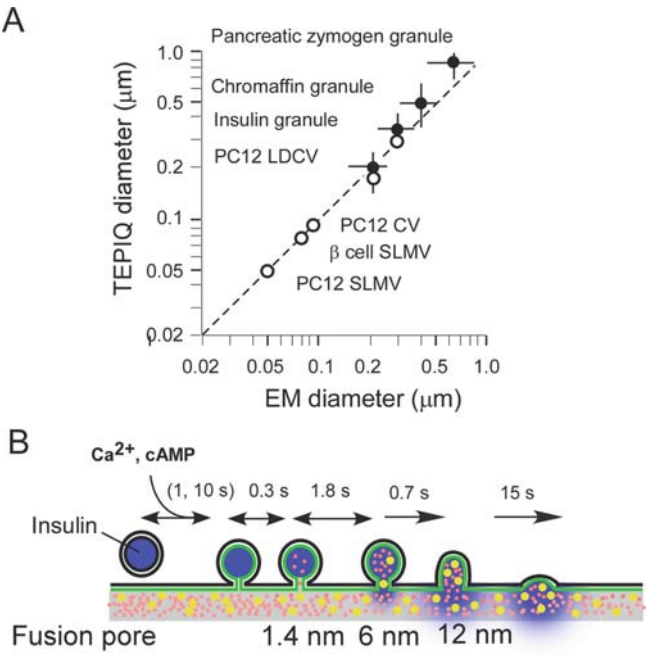

Fig. 14.5 Diameters of exocytotic vesicles and the fusion pore. (**A**) Diameters of exocytotic and endocytotic vesicles estimated via electron microscopy (EM) and TEPIQ analysis. The diameter of the zymogen vesicles in pancreatic acinar cells and those in large vesicles of chromaffin, insulin, and PC12 cells was estimated via TEPIQ analysis of ΔV[61]. Bars represent the SD. The diameters of clathrin vesicles (CV) and SLMVs (*open circles*) in PC12 cells and β-cells were estimated via TEPIQ analysis of $\Delta V/\Delta S$. (**B**) Gradual opening of fusion pores estimated by staining of polar tracers of different diameters: *Magenta points* represent SRB with a diameter of 1.4 nm and *yellow points* represent 10-kDa dextran fluorescein of a diameter of 6 nm. These conclusions are supported by the results of experiments with various fluorescent polar tracers [26]

crystallization of insulin in the vesicles, which prevents the dilation of vesicle contents and, consequently, the fusion pore. First, pore dilation was further slowed by the addition of zinc (3 mM), which is known to stabilize insulin crystals by binding to the insulin [78], to the extracellular solution [26]. Second, pore dilation is significantly faster in guinea pig islets, where crystallization of insulin is known to be less prevalent [78]. Finally, flattening of vesicles has been found to start after the fusion pores were dilated to more than 12 nm, as confirmed by experiments with 70-kDa FD [26] (Fig. 14.5B), which may be interpreted to mean that the flattening of vesicles occurs when the fusion pore allows permeation of the 36-kDa insulin hexamer. Thus, our study suggests that secretion of insulin takes several seconds, which is in line with the results obtained via Zn imaging [66]. In contrast, the decay of insulin-GFP release occurred within 1 second in TIRF imaging experiments [54–56], likely due to the lack of crystallization of insulin-GFP in these experiments.

Another interesting finding is that though the closure of the fusion pore can be reversible, transient openings are not larger than 6 nm in β-cells (Fig. 14.5B), which is similar to what has been reported in other cells [26, 30, 32]. Some granules at such transient pore sites also subsequently move away from the site of exocytosis (Movie 2 of [26]), suggesting that these granules were engaged in "kiss-and-run" secretion. Since insulin supposedly cannot be secreted through such a transient narrow fusion pore [26, 79], such kiss-and-run events are considered to be failures of insulin secretion. In fact, such kiss-and-run exocytosis represents only 7% of exocytotic events in β-cells, a number that is further reduced at higher cytosolic concentrations of cAMP [31]. It is notable that while experiments employing confocal imaging of islets [65] and Zn imaging of insulin release [66] also indicate that most insulin granules undergo full fusion exocytosis in β-cells, "kiss-and-run" exocytosis was detected more frequently in a TIRF imaging experiment [80]. This suggests that the process of exocytosis significantly differs at plasma membranes on glass cover slips and in cells expressing exogenous proteins.

14.3.3 Fusion Pore Compositions and Fusion Mechanisms

The slow dilation of the fusion pore of insulin vesicles has facilitated the examination of the molecular composition of such pores. This has been done by observing the time course of the staining of insulin vesicles with the lipidic dye FM1-43. Because the pore size of these vesicles remains too small for FM1-43 to pass through for a period of time preceding full fusion and because of the lipidic nature of FM1-43, if the fusion pore was proteinaceous, the aqueous pore would be the only pathway for FM1-43 staining (Fig. 14.5B) and the rate of staining of vesicles with FM1-43 would be similar to that of SRB. In contrast, if the fusion pore was lipidic, FM1-43 should stain insulin vesicles via lateral diffusion from the previously stained plasma membrane (Fig. 14.5B, green), and since these vesicles can be as small as 350 nm, this lateral diffusion could be quite rapid, depending on the geometry of the fusion pore.

When we performed these experiments, we found that FM1-43 stained the vesicles with an earlier onset and faster time course than did SRB [26, 31]. More precisely, FM1-43 had stained more than 64% of a given vesicle when the SRB signal started to appear. We used this information and estimated the diffusion constant of the FM1-43 molecule to be 3.3 $\mu m^2/s$ along the fusion pore [26], which is within the range of values expected for a pure lipid bilayer. This value is also greater than that observed in the plasma membrane (<1 $\mu m^2/s$), where lateral diffusion of membrane lipids is prevented by the actin-based membrane skeleton, which blocks diffusion via the "picket" mechanism [81]. These data indicate that the fusion pore is already lipidic when the pore size is about 1.4 nm and thus that the purely proteinaceous fusion pore model does not apply in this case. The data also indicate that any proteins that might be present do not substantially disturb the flow of a lipidic molecule like FM1-43. It is notable that the faster staining of vesicles by FM1-43 compared to SRB is seen only in insulin vesicles and not in the large dense-core

vesicles of PC12 cells [29] and adrenal chromaffin cells [32]. This, however, is most likely because the pore expands rapidly in these cells, thus allowing SRB to rapidly stain a vesicle, and does not necessarily indicate a difference in fusion pore composition across cell lines. The lipidic nature of the fusion pore has also been indicated in other studies employing entirely different experimental approaches [82, 83] (but see [84, 85]). Thus, our data suggest that SNARE proteins do not directly form the fusion pore in β-cells but rather that they trigger the formation of a lipidic fusion pore to initiate exocytosis.

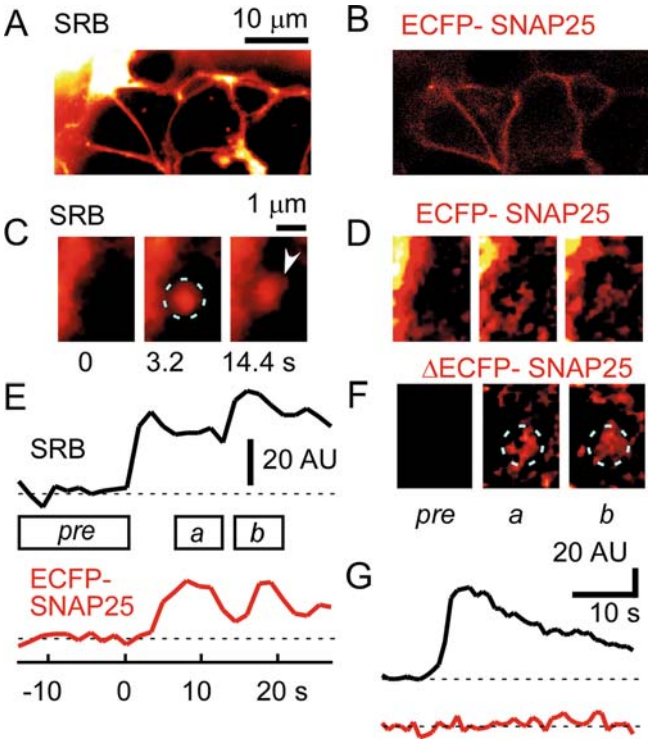

Fig. 14.6 Redistribution of SNAP-25 during sequential exocytosis. SRB (**A**) and ECFP-SNAP-25 fluorescence images (**B**) of an islet. The islet was transfected with an adenoviral vector encoding ECFP-SNAP-25 and then immersed in a solution containing the polar tracer SRB. Simultaneous measurement of SRB (**C**) and ECFP-SNAP-25 (**D**) fluorescence during a sequential exocytotic event. The number below each image in (**C**) represents the time after the onset of exocytosis. The blue dashed circle represents the region of interest. Each image in (**D**) was obtained by averaging 5–10 images in the three time periods shown in (**E**). (**E**) Time courses of fluorescence of SRB (*black*) and ECFP-SNAP-25 (*red*) in the region of interest shown in (**C**). *Open horizontal bars* represent time periods between –14.4 and 0 seconds after the onset of exocytosis (*pre*), between 6.4 and 12.8 seconds (a), and between 14.4 and 20.8 seconds (b), respectively. Dashed horizontal lines show baseline fluorescence levels. **F** Difference images obtained by subtracting image *pre* from three images in (**D**). **G** Time courses of fluorescence for solitary exocytotic events in a control cell. Adapted from [2] with permission from the Rockefeller University Press

14.3.4 Lateral Diffusion of SNARE Proteins

Sequential exocytosis has been observed in β-cells, though only as a small proportion of overall exocytosis (2%) and only with a long latency of 6.5 seconds [2]. We examined whether such sequential exocytosis was associated with lateral diffusion of SNAP-25 by expressing ECFP–SNAP-25 in the islets and performing TEP imaging together with ECFP imaging (Fig. 14.6A–D). We observed lateral diffusion of SNAP-25 in 6% of exocytotic events (Fig. 14.6E). Importantly, the lateral diffusion of SNAP-25 was still detected in 54% of vesicles undergoing sequential exocytosis in β-cells, but only in 5% of vesicles involved in solitary exocytotic events (Fig. 14.6G). Furthermore, when islets were treated with cyclodextrin to remove lipid rafts that might trap SNAP-25, sequential exocytosis was hastened and its proportion increased to 8.9% of total exocytosis, and lateral diffusion of SNAP-25 was detected in 15% of all events [2].

When we performed the same experiment in adrenal chromaffin cells, where sequential exocytosis has been found to occur, and occur rapidly, in 73% of exocytotic events [32], lateral diffusion of SNAP-25 was detected in 43% of exocytotic events. Furthermore, these sequential exocytosis events occurred with a short latency of only 1.2 seconds. Since the fluorescence signal from SNAP-25 was relatively small, it is likely that the actual diffusion occurred more frequently than we observed. Thus, lateral diffusion of SNAP-25 appears to occur in parallel with the sequential progression of exocytosis in both chromaffin cells and β-cells. Furthermore, the suppression of the lateral diffusion of SNAP-25 may account for the infrequency of sequential exocytosis in β-cells. Sequential exocytosis also involves other SNAREs, as redistribution of syntaxin-2 has been demonstrated via immunohistochemistry in pancreatic acinar cells [86].

14.4 Regulation of Insulin Exocytosis

14.4.1 Biphasic Insulin Exocytosis and Protein Kinase A

Insulin secretion in the islet is known to occur in two phases in response to glucose stimulation or increases in cytosolic Ca^{2+} concentrations (Fig. 14.4D). There are likely multiple mechanisms underlying the two phases of insulin secretion. Using TEP imaging, we have revealed that protein kinase A (PKA) is specifically involved in the first phase of insulin exocytosis [31], as a series of PKA inhibitors (PKI, Rp-cAMPS, H89, KT5720) all potently and specifically blocked the initiation of the first phase of insulin exocytosis from the islet. This is in contrast to previous reports based on RIA studies that PKA antagonists have no or only a small effect on insulin exocytosis [87–89]. This apparent contradiction may be due to the lack of time resolution of RIA and the fact that membrane-permeable inhibitors often do not penetrate into tissues when applied via superfusion [90]. In support of this second hypothesis, we have demonstrated certain PKA inhibitors (H89 and KT5720)

to be effective only in small cluster preparations, but not in the whole islets [31]. It is also notable that these inhibitors did not affect the increases in cytosolic Ca^{2+} induced by glucose [31]. It is therefore suggested that PKA plays a role in modulating insulin exocytosis, with this role being particularly important for the first phase of the process (Fig. 14.1).

The functions of Ca^{2+} and cAMP in insulin exocytosis have been intensively studied using both TEP imaging (Fig. 14.7D–G) [31] and amperometry to measure serotonin release in whole-cell clamped cells (Fig. 14.7A–C) [91]. We examined the exocytosis induced via flash photolysis of caged Ca^{2+} compounds [2, 31]. Rapid and large increases in cytosolic Ca^{2+} released via the uncaging of a caged Ca^{2+} compound induced the exocytosis of LDCVs with two time constants, 1 and 10 seconds (Fig. 14.7C). Interestingly, the first component (Mode-1 exocytosis) was strongly dependent upon cytosolic ATP levels and was more potently augmented in response to the addition of ATP-γS (Fig. 14.7C), suggesting that the effect is dependent upon ATP-mediated phosphorylation rather than the cleavage of ATP itself. The ATP effect was mediated by PKA, the activity of which was found to depend upon both ATP and cAMP levels in the cytosol [91] (Fig. 14.1A). We also used TEP imaging of intact islets of Langerhans to investigate whether PKA plays a similar role under glucose stimulation because whole-cell perfusion alters cytosolic environments.

14.4.2 Actions of Glucose and cAMP

The major effects of glucose on β-cells are thought to be mediated by mitochondrial generation of ATP, which results in the closure of K_{ATP} channels, the depolarization of the cell, and the activation of voltage-dependent Ca^{2+} channels (VDCC, Fig. 14.1A) [6, 7]. It is known, however, that glucose has stimulatory effects on insulin secretion beyond those mediated by the closure of K_{ATP} channels, an effect referred to as the K_{ATP}-independent action of glucose [92–94]. We thus examined the effect of glucose on LDCV exocytosis using TEP imaging in islets loaded with an AM ester of a caged Ca^{2+} compound (NP-EGTA) (Fig. 14.7D) and found that glucose stimulation produced a twofold increase in uncaging-induced exocytosis [31] (Fig. 14.7E, F). Interestingly, the glucose effect was completely eliminated by PKA inhibitors (Fig. 14.7G) [31]. Furthermore, forskolin, which activates PKA by increasing cytosolic concentrations of cAMP, potentiated exocytosis at high glucose concentrations, but not at low glucose concentrations (Fig. 14.7G), indicating that another factor, in addition to cAMP, is required for the PKA effect. One possible candidate for this "other factor" is cytosolic ATP, which is known to increase in response to glucose and which is known, as noted above, to potentiate insulin exocytosis in a manner that is dependent upon PKA [91, 95] (Fig. 14.7C). These experiments indicate that PKA is also involved in the glucose-sensing mechanism of islets and likely contributes to the K_{ATP}-independent action of glucose on insulin exocytosis [7, 92–94].

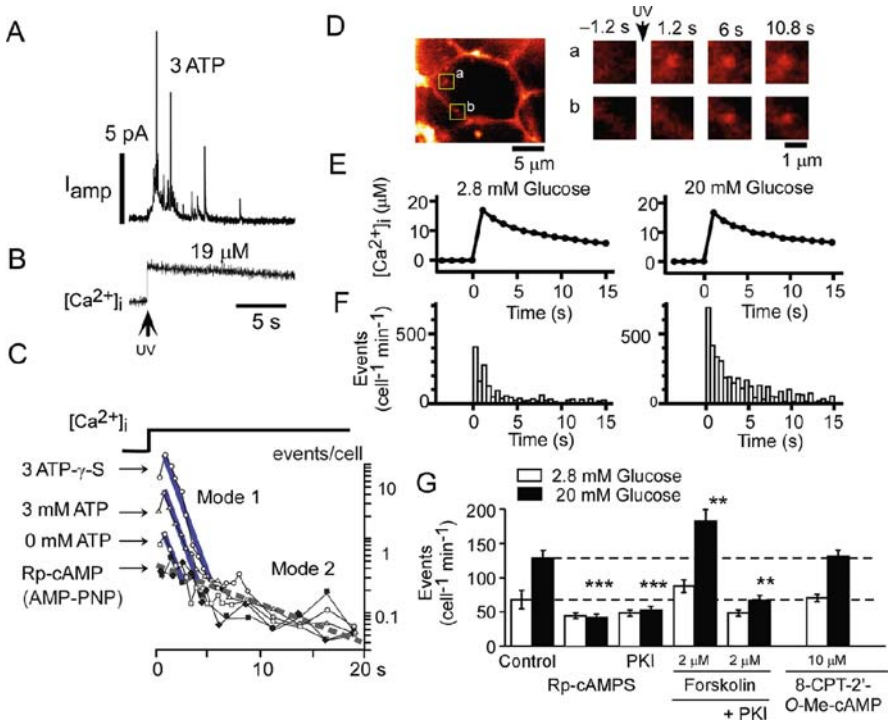

Fig. 14.7 Effects of cytosolic cAMP, ATP, and extracellular glucose on Ca^{2+}-induced exocytosis in β-cells. (**A**) Amperometric measurements of LDCV exocytosis from single β-cells in the presence of cytosolic ATP (3 mM). (**B**) $[Ca^{2+}]_i$ measured in the same cells. I_{amp}, amperometric current; UV, ultraviolet irradiation. (**C**) The regulation of insulin exocytosis by ATP is shown with semilogarithmic plots of amperometric latency histograms. Data shown are from cells perfused with 3 mM ATP[γS] (*open circles*), 3 mM ATP (*open triangles*), 0 mM ATP (*open squares*), or AMP-PNP (*closed diamonds*), as well as from those pretreated with Rp-cAMP before perfusion with 3 mM ATP (*closed squares*). Blue and dashed lines indicate the Mode-1 and Mode-2 components, respectively. (**D**) TEP imaging of LDCV exocytosis in a β-cell within an islet loaded with a caged Ca^{2+} compound, nitrophenyl-EGTA (NPE), and perfused with a solution containing SRB. Exocytosis was triggered by UV-induced photolysis of NPE at time 0. The *boxed regions* a and b in the micrograph on the *left* are sites of individual exocytotic events after UV irradiation and are shown at higher magnification on the *right*. (**E**) Increases in $[Ca^{2+}]_i$ induced by the uncaging of NPE at time 0 in islets loaded with the Ca^{2+} indicator fura-2FF and exposed to 2.8 or 20 mM glucose. The high-glucose solution was applied 1 minute before UV irradiation. Traces represent average time courses obtained from four to six islets. (**F**) Latency histograms of insulin exocytosis induced by the uncaging of NPE in islets exposed to 2.8 or 20 mM glucose. The bin width is 0.5 seconds, and the data represent averages from nine and six islets, respectively. (**G**) The pharmacology of Ca^{2+}-dependent insulin exocytosis in the presence of 2.8 and 20 mM glucose. Exocytotic events were counted during the first 15 s after the uncaging of NPE. Inhibitors were applied for 40 min, forskolin and 8-CPT-2'-*O*-Me-cAMP for 10 min, and 20 mM glucose for 1 minute before UV irradiation. Data are mean ± SEM. The actions of the various compounds were tested relative to the control values with the Dunnett test for 2.8 and 20 mM glucose, as indicated by dashed lines. ** and *** represent $P < 0.01$ and $P < 0.001$, respectively. (**A**–**C**) are adapted from [91] with permission from National Academy of Sciences, USA and (**D**–**G**) from [31] with permission from the Physiological Society

Although glucose stimulation does not always increase cytosolic concentrations of cAMP [92, 96–98], the mechanisms described above can explain the observed increase in insulin exocytosis in response to glucose, even in the absence of increases in cAMP [91, 94, 95]. In addition, recent studies employing cellular imaging of cAMP have revealed that cytosolic concentrations of cAMP may actually be increased by glucose in an oscillatory manner [99], providing one potential explanation for the oscillatory exocytosis of insulin observed during glucose stimulation [100] (Fig. 14.4D), as the effect of cAMP/PKA on insulin exocytosis occurs within a few seconds [10].

It is notable that the actions of cAMP on insulin release can be blocked by PKI (Fig. 14.7G) or H89 [56] but are not mimicked by 10 μM 8-CPT-2′ -*O*-Me-cAMP (Fig. 14.7G), which activates Epac [101] and enhances exocytosis of SLMVs (Fig. 14.10B) but does not activate PKA. This further indicates that the acute action of cAMP is mediated by PKA. It is possible, however, that Epac may regulate exocytosis of LDCVs under basal conditions [56]. Unlike the whole-cell clamp experiment with forskolin or ATP (Fig. 14.7C), the slow component of insulin exocytosis was also facilitated by forskolin in AM ester-loaded intact islets (Fig. 14.7F). This is probably due to the rapid recovery of Ca^{2+} concentrations in the intact AM ester-loaded cells and delayed Mode-1 exocytosis in these experiments.

Beyond glucose, there are many hormones that also influence cytosolic cAMP levels and insulin exocytosis (Fig. 14.1A), and these hormones thus play a crucial role in the regulation of blood glucose levels [7]. Furthermore, in addition to its direct effects on exocytosis, cAMP may enhance insulin exocytosis by potentiating VDCCs [102] and Ca^{2+} release from internal Ca^{2+} stores [103, 104]. We have also revealed that cAMP reduces the amount of kiss-and-run exocytosis [31] and further facilitates PKA-mediated insulin secretion. In contrast, transient opening of very small fusion pores, which cannot be detected via TEP imaging, was reportedly increased by forskolin in cell-attached capacitance measurement experiments [105], though insulin secretion is unlikely to be significantly affected by the opening of such small fusion pores.

14.4.3 Molecular Mechanisms of Insulin Exocytosis

In spite of the confirmed role of cytosolic Ca^{2+} and PKA in mediating exocytosis, their target molecules have not been identified. In β-cells, the absence of synaptotagmin I and II suggests a role for vesicular synaptotagmins VII [106, 107] and IX [108, 109] as the major Ca^{2+} sensors. One candidate target of cAMP-dependent phosphorylation by PKA is a plasma membrane SNARE, SNAP-25, as threonine-138 of this protein is phosphorylatable by PKA [110], and this protein has been implicated in the early phase of Ca^{2+}-triggered exocytosis of large dense-core vesicles in chromaffin cells [110]. Snapin, a protein that binds to SNARE complexes, is also a target of PKA in chromaffin cells [111], and while this protein is also expressed in β-cells, its role in the exocytosis of large dense-core vesicles is unknown.

Rab-interacting molecule-2 (RIM2) [112, 113] also contains a PKA phosphorylation site that regulates its binding to Munc13, which plays a priming role in vesicle exocytosis [114]. Furthermore, another RIM protein, RIM1, has been shown to regulate neurotransmitter release at synapses in a PKA-dependent manner [115].

The first phase of insulin exocytosis is markedly augmented by increased PKA in the islet (Figs. 14.1A and 14.4E) [31] and is also enhanced by cAMP/ Epac2/Rap1 signaling via mobilization of insulin granules toward the plasma membrane in isolated β-cells [56]. On the other hand, it has been reported that the second phase of insulin exocytosis is preferentially affected by CAPS [64], myosin Va [116], Cdc42 [117, 118], and CaV2.3 [119].

Many molecules regulating insulin secretion upstream of the fusion reaction have been identified. PKA promotes the translation of various proteins necessary for the biogenesis of insulin granules via phosphorylation of polypyrimidine tract-binding protein 1 (PTB1) [120]. Islet antigen-2 (IA-2) and IA-2b (or phogrin) are required for proper cargo loading and stabilization of LDCVs [121]. The mobility of insulin granules is then facilitated by Rap1[56] and myosin Va [122], and a small G protein Rab27 facilitates the transport of insulin granules to the plasma membrane [55], while its target granuphilin facilitates docking but prevents fusion by forming the fusion-incompetent syntaxin–Munc18 complex [23, 123]. In contrast, a target of Rab3, Noc2 facilitates insulin secretion via its interaction with Munc13 [124] or by inhibition of Gi/Go signaling [125], while another small G protein RalA plays a central role in the biphasic insulin secretion by regulating the mobilization of granules [126].

Interestingly, the protein affected in maturity-onset diabetes of the young (MODY3), hepatocyte nuclear factor-1a (HNF-1a), has been observed to reduce the expression of collectrin, which facilitates SNARE complex formation via its interaction with snapin [62], while MAPKp38 inhibits protein kinase D1 (PKD1), which regulates both insulin secretion and β-cell survival [127]. Finally, a metabolite of inositol phosphate $InsP_7$ has been reported to promote depolarization-induced capacitance increases in β-cells [128]. It should also be noted here that insulin secretion is also very sensitive to cholesterol contents in the plasma membrane [2, 129, 130].

Short noncoding RNAs (microRNAs) have been reported to directly or indirectly regulate the expression of proteins involved in secretion. A certain set of microRNAs, including miR375 and miR124a, are particularly enriched in β-cells. Furthermore, miR375 has been reported to suppress insulin secretion, possibly via the activity of myotrophin [131] and to maintain β-cell mass [132]. In addition, miR124a increases the expression of SNAREs and reduces Rab27A mRNA levels, thus suppressing glucose-induced insulin secretion [133]. Some of the effects of miR124a may be mediated by the transcription factor Foxa2, which inhibits a number of signaling pathways affecting insulin secretion in mature β-cells [134]. In addition, miR9 and miR96 increase the levels of granuphilin and reduce Noc2, thereby negatively regulating insulin exocytosis [135]. Elevation of the microRNAs may also contribute to the detrimental effects of palmitate on insulin exocytosis [136].

14.5 Exocytosis of Synaptic-Like Microvesicles (SLMVs)

14.5.1 Historical Perspective

The presence of SLMVs in β-cells was first indicated with the identification of synaptophysin-positive small vesicles in such cells [9]. Several years later, the exocytosis of small vesicles with a mean diameter of 80 nm was demonstrated via cell-attached capacitance measurements [35]. Additional evidence for the existence of SLMV exocytosis was provided by experiments employing whole-cell capacitance measurements that demonstrated a rapid increase in the plasma membrane area that was not associated with the exocytosis of insulin granules [38]. Building on these discoveries, SLMV exocytosis in β-cells was further characterized via TEP imaging combined with EM using the membrane tracer FM1-43 instead of a volume tracer (SRB). These experiments revealed rapid increases in fluorescence upon uncaging of caged Ca^{2+} (Fig. 14.8A–C), as well as similar increases after glucose stimulation [10]. These increases in FM1-43 fluorescence were diffuse (Fig. 14.8B) and rapid (Fig. 14.8D), unlike those associated with LDCV release (Figs. 14.4C and 14.7F). The diffuse fluorescence increases could also be detected using SRB, but the signals were far weaker than those obtained with FM1-43, suggesting that the fluorescence increases in these experiments were mediated by vesicles smaller than LDCVs. The diameter of vesicles was estimated via TEPIQ analysis to be about 80 nm (Fig. 14.5A) [10], which is in accord with the size predicted via capacitance measurements [8]. The TEP/EM experiments also revealed that after washout of the dyes, the fluorescence was mostly retained in the cells (Fig. 14.8E) and even moved into the cytosol (Fig. 14.8F), indicating that the vesicles were internalized.

The conclusions drawn from the imaging analyses above were tested in studies employing electron microscopy of islet cell clusters in which DAB was photoconverted by the fluorescence of an aldehyde-fixable analog of FM1-43, FM1-43FX [137–139]. As a control for this study, the constitutive endocytotic pathway was first labeled by immersing cells in FM1-43FX for 30 minutes without stimulation. This treatment produced many small DAB-positive vesicles as well as DAB-positive endosome- and lysosome-like structures (Fig. 14.9A), similar to those observed in studies of PC12 cells [30]. Notably, no staining of intracellular organelles was detected in cells exposed to FM1-43FX for 90 seconds before fixation (Fig. 14.9B). In contrast, many small DAB-positive vesicles were apparent in cells fixed within 15 seconds after stimulation via NPE photolysis during TEP imaging (Fig. 14.9C). In these samples, whereas some DAB-positive vesicles were still attached to the plasma membrane, many were scattered in the cytoplasm (Fig. 14.9C), which is consistent with the results obtained in the TEP imaging studies (Fig. 14.8F). The diameter of the stained vesicles in this series was about 70 nm, which is similar to the value estimated via both TEPIQ analysis and electrophysiology. The number of DAB-positive vesicles was 0.6 μm^{-2}, corresponding to a total of 4000 vesicles per cell or 13% of the original area of the plasma membrane. These results are consistent with the observed diffuse fluorescence increase of 20% and with the fact that most

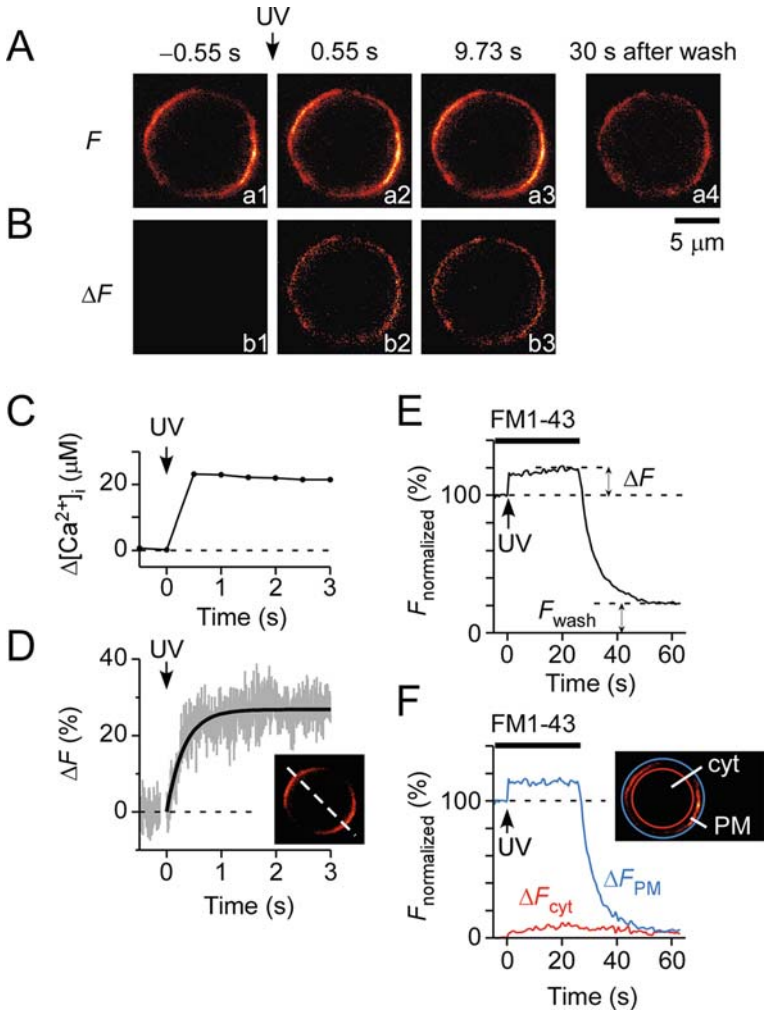

Fig. 14.8 TEP imaging of exocytosis and endocytosis using FM1-43 in isolated β-cells. (**A**) FM1-43 fluorescence (*F*) images of a cell loaded with NPE-AM are shown in frames a1–a4. Photolysis was induced at a time 0 between frames a1 and a2 (UV). The dye was washed out 30 seconds before frame a4. (**B**) Difference images (ΔF) obtained by subtracting the image at rest (a1) from frames a1 to a3 in (**A**) are shown in frames b1–b3. (**C**) Increase in $[Ca^{2+}]_i$ induced by photolysis of NPE in cells loaded with the Ca^{2+} indicator fura-2FF. (**D**) Time course of the change in FM1-43 fluorescence induced by photolysis of NPE during line scanning along the *dashed line* shown in the inset. Average values from six cells (*gray trace*) and the single-exponential fit (*black line*) are shown. (**E**) Time course of the change in FM1-43 fluorescence for the entire section of the cell shown in (**A**). The zero level of fluorescence was obtained before the application of FM1-43 to the cell. Fluorescence was normalized to that of the entire section of the cell before photolysis and is expressed as a percentage of the control value ($F_{normalized}$). (**F**) Time courses of the changes in fluorescence in the plasma membrane (PM) region (*blue*) and in the cytoplasmic (cyt) region (*red*) of the cell depicted in the inset. Adapted from [10] with permission from the Physiological Society

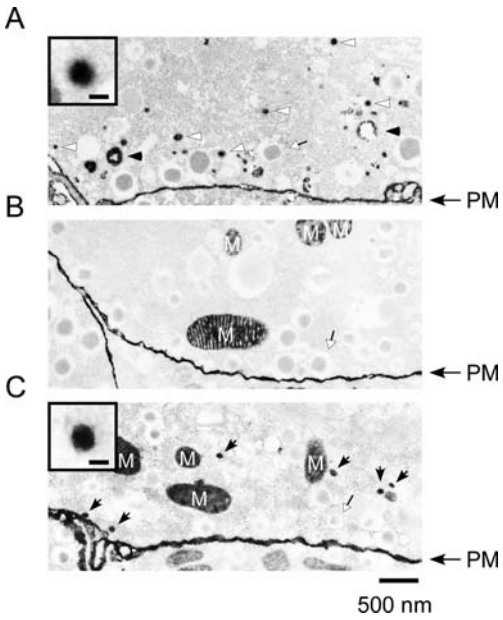

Fig. 14.9 Ultrastructural identification of endocytotic vesicles in β-cells. Endocytotic vesicles were examined via electron microscopy in cells loaded with FM1-43FX. Photoconversion of DAB was induced by the fluorescence of FM1-43FX remaining after extensive washout. Some FM1-43FX molecules remained in the plasma membrane (PM) despite washout, resulting in its staining with DAB (*long solid arrows*). (**A**) A cluster of cells immersed in a solution containing FM1-43FX for 30 minutes at rest. *Open arrows*, *open arrowheads*, and *closed arrowheads* indicate LVs, constitutive endocytotic vesicles, and lysosomes or endosomes, respectively. (**B**) A cluster of cells immersed in a solution containing FM1-43FX for 90 seconds without stimulation. M – mitochondria. (**C**) A cluster of cells immersed in a solution containing FM1-43FX for 1 minute before photolysis of NPE and fixed with glutaraldehyde within ∼15 seconds after photolysis. Short solid arrows indicate small endocytotic vesicles. The external scale bar (500 nm) applies to all panels with the exception of the insets in (**A**) and (**C**), which show magnified images of DAB-positive endocytotic vesicles and for which the associated scale bars represent 50 nm. Adapted from [10] with permission from the Physiological Society

of the membrane added to the cell surface during exocytosis events was recaptured by endocytosis events (Fig. 14.8E).

It is very interesting to note that images of LDCV exocytosis are rarely captured via EM, even though exocytosis of LDCVs is frequently detected by TEP imaging. This apparent discrepancy can be explained by our estimation of the rate of insulin exocytotic events as 20 $cell^{-1}$ within 15 seconds after photolysis of NPE [31], which means that the predicted number of LDCVs undergoing exocytosis in the thin sections required for electron microscopy is 0.009 μm^{-2}[10]. This value is only 1.5% of the corresponding value for SLMVs (0.6 μm^{-2}), which means that it is very challenging to detect insulin exocytosis in β-cells via EM [140]. The fact that SLMV exocytosis could be readily identified via EM (Fig. 14.9C) supports our conclusion

that the frequency of SLMV exocytosis is far greater than that of LDCV exocytosis in β-cells.

Abundant Ca^{2+}-dependent exocytosis of SLMVs has been observed in every type of cell examined to date. In PC12 cells, massive exocytosis of SLMVs has been experimentally associated with a rapid increase in membrane capacitance [30, 40, 44]. Similar rapid capacitance increases have been reported in other cell types, including mast cells [42], fibroblasts [141, 142], pancreatic acinar cells [143] and adrenal chromaffin cells [44, 45]. Thus, mammalian cells may commonly possess numerous SLMVs that can undergo rapid Ca^{2+}-dependent exocytosis [12, 15, 144].

14.5.2 Regulation by cAMP

We examined the effect of cAMP on the Ca^{2+}-dependent exocytosis of SLMVs in β-cells. Forskolin increased SLMV exocytosis induced by photolysis of NPE by 39%, which was detected as an increase in the diffuse FM1-43 fluorescence signal (Fig. 14.10A). The potentiation of SLMV exocytosis by cAMP was not blocked by antagonists of PKA, including PKI and Rp-cAMPS (Fig. 14.10B). A portion of the PKA-independent cAMP signaling is mediated by guanine nucleotide exchange factors that are directly activated by cAMP (Epac) and are also specifically activated by 10 μM 8-CPT-2′ -*O*-Me-cAMP [101]. The finding that 10 μM 8-CPT-2′-*O*-Me-cAMP mimicked the effect of forskolin on SLMV exocytosis (Fig. 14.10B) suggests that the potentiation of Ca^{2+}-dependent exocytosis of SLMVs by cAMP is dependent upon Epac, but not PKA.

The regulation of SLMV exocytosis by cAMP is strikingly different from that of LDCV exocytosis [31]. First, Ca^{2+}-dependent exocytosis of LDCVs was significantly enhanced by forskolin at a high glucose concentration (20 mM), but not at a low glucose concentration (2.8 mM) (Fig. 14.7G), whereas SLMV exocytosis was potentiated by forskolin at a low glucose concentration (Fig. 14.10B). Second, the effect of forskolin was inhibited by PKI (Fig. 14.7G), while 10 μM 8-CPT-2′-*O*-Me-cAMP did not increase the extent of insulin exocytosis (Fig. 14.7G) [31]. These observations indicate that the acute effects of Epac and PKA are specific to Ca^{2+}-dependent exocytosis of SLMVs and LDCVs, respectively, in β-cells. The fact that rapid exocytosis of SLMVs is selectively modulated by Epac is consistent with reports that the observed rapid capacitance increase was found to be resistant to Rp-cAMPS and PKI [145], and that 8-CPT-2′-*O*-Me-cAMP potentiated the rapid component of such capacitance increases [146]. Similar effects of Epac on increases in the membrane capacitance were reported in melanotroph [147].

The potentiation of SLMV exocytosis by cAMP was faster than that of LDCV exocytosis [10]. The latency to augmentation of LDCV exocytosis (∼5 s) may reflect the time required for protein phosphorylation and activation by activated PKA to take place. In contrast, the action of Epac may be faster because it requires only nucleotide exchange, which occurs within a fraction of a second [148, 149]. Epac has also been proposed to regulate exocytosis via direct binding to Rim2

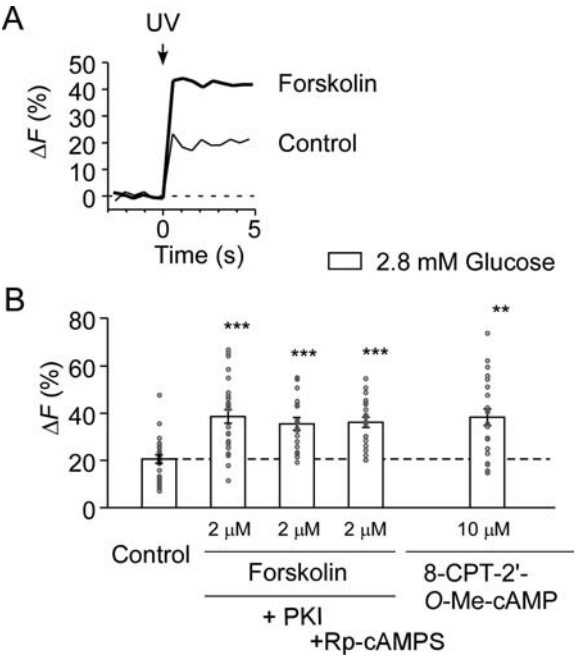

Fig. 14.10 Pharmacology of Ca^{2+}-dependent exocytosis of SLMVs in β-cells. (**A**) Time courses of FM1-43 fluorescence after photolysis of NPE in representative β-cells either maintained under control conditions or pretreated with forskolin (2 μM) for 10 minutes. (**B**) Cells were exposed (or not) to PKI (5 μM) or Rp-cAMPS (200 μM) for 30 minutes before incubation for 10 minutes with forskolin (2 μM) or 8-CPT-2′-*O*-Me-cAMP (10 μM), as indicated. The cells were then stimulated by NPE photolysis, and the diffuse increase in FM1-43 fluorescence was measured. *Circles* represent data obtained from individual cells, with columns and error bars indicating mean ± SEM. The *dashed line* shows the control level (NPE photolysis alone). Statistical analyses were performed using the Kruskal–Wallis test ($P < 0.001$), followed by the Steel test for comparison with control values. ** and *** represent $P < 0.01$ and $P < 0.001$, respectively. Adapted from [10] with permission from the Physiological Society

[150]. Rim proteins are putative effectors of Rab3 and are thought to serve as Rab3-dependent regulators of synaptic vesicle fusion [151], a role that they may also play in the exocytosis of SLMVs in β-cells. Activation of rap by Epac might also be involved in this process [56, 101].

14.5.3 Functional Role of SLMV Exocytosis in β-Cells

Glucose stimulation also induces massive exocytosis of SLMVs in β-cells [10]. If we assume that the diameters of SLMVs and β-cells are 80 nm and 12 μm, respectively, an increase in FM1-43 fluorescence of 3% $cell^{-1}$ min^{-1} represents exocytosis of 675 SLMVs $cell^{-1}$ min^{-1}, a rate that is more than 100-fold greater

than that previously reported for LDCV exocytosis (6.4 LDCVs cell^{-1} min^{-1}) [31]. Exocytosis of SLMVs in β-cells thus likely plays a significant physiological role. Although SLMVs in β-cells contain GABA [9], fewer than 100 GABA-induced quantal currents were detected after photolysis of a caged Ca^{2+} compound in β-cells expressing recombinant $GABA_A$ receptors [49], whereas exocytosis of ∼4500 SLMVs was detected via TEPIQ analysis (a 20% increase in membrane area) and, similarly, 4000 endocytotic vesicles were detected via electron microscopy in response to this stimulus. These observations suggest that GABA is present in, at most, only ∼1% of SLMVs in β-cells, which is similar to the situation observed with acetylcholine in PC12 cells, where only a small proportion of SLMVs contain detectable acetylcholine [30, 44]. It is therefore unlikely that the physiological role of SLMV exocytosis lies in the secretion of vesicle contents [10].

The trafficking of proteins and lipids between the plasma membrane and endosomes is another possible function of SLMV exo-endocytosis. SLMVs likely contribute more efficiently to membrane trafficking than they do to secretion because of their large surface-to-volume ratio. Membrane area added to the plasma membrane via SLMV exocytosis is ∼3% cell^{-1} min^{-1} during glucose stimulation, a rate that is about five times greater than that for LDCV exocytosis (0.54% cell^{-1} min^{-1}). Furthermore, SLMVs may play a similar membrane trafficking role in "non-secretory" cells that nonetheless exhibit a substantial amount of SLMV exocytosis [12, 15, 144].

The finding that the actions of both Ca^{2+} and cAMP were faster for SLMVs than for LDCVs indicates that SLMV exocytosis-mediated trafficking of molecules precedes LDCV exocytosis in response to a stimulatory event. Exocytosis of SLMVs may thus precondition the plasma membrane for LDCV exocytosis and endocytosis. An example of a molecule that may be trafficked in this manner is a sialylated form of the cell adhesion molecule NCAM (PSA-NCAM), which is expressed specifically in β-cells and is mobilized to the cell surface in an activity-dependent manner [152]. Moreover, the surface expression of PSA-NCAM is correlated with glucose-stimulated insulin secretion [152]. In pancreatic islets, NCAM is thought to contribute to the maintenance of cell–cell interactions and is required for normal turnover of secretory granules [153, 154]. As such, increased surface expression of PSA-NCAM might therefore facilitate contact between β-cells and other islet cells in order to preserve islet integrity in the face of secretion of reactive substances stored in insulin granules, such as insulin, Zn, protons, ATP, GABA, carboxypeptidase E, and islet amyloid polypeptide [155–157]. Impairment of such SLMV-mediated preconditioning might thus result in islet dysfunction.

14.6 Perspectives

Various methodologies have been employed to analyze the various types of exocytosis from β-cells, and considerable progress has recently been made in elucidating the mechanistic underpinnings of these processes. Our knowledge of exocytosis,

however, still remains incomplete because none of the available methodologies is ideal for investigating all facets of these processes. Genetic approaches can correlate molecules with phenotypes, but the knowledge gap between molecular and cellular phenomena is immense, and imaging and reconstruction approaches, though very powerful and full of potential, are still unable to completely bridge this gap. Thus, these techniques should be further developed to gain more direct insight into the complex cellular processes involved. We need to fully characterize the molecular basis of the fusion reaction itself and its preparatory reactions, without relying on the arbitrary assumptions used in the age of electrophysiology. In this vein, it should also be kept in mind that the quantitative characteristics of exocytosis differ considerably between neurons and secretory cells [13], and thus the molecular mechanisms must also differ. We also need to identify and characterize the functions of and physiological roles played by SLMVs in both secretory and "nonsecretory" cells.

We hope that the studies described in this review provide a thorough introduction to the power of two-photon imaging in investigating exocytosis and endocytosis in secretory tissues. TEP imaging is the only method currently available that allows for the investigation of intact secretory tissues, and it can be used to address almost every important parameter related to exocytosis, including vesicle diameter, fusion readiness, fusion pore properties, lifespan of the Ω-shaped profile, swelling of vesicles, compound exocytosis, endocytosis, the spatial organization of exocytosis, and the proteins involved in exocytosis, such as SNARE proteins and the actin cytoskeleton. TEP imaging is also well suited to the study of mutant animals and even human specimens, because intact tissue preparations may be examined. Another benefit of TEP imaging is its compatibility with electron microscopy via the photoconversion of DAB using FM1-43. The powerful biophysical tool of caged compounds can also be utilized to quantify the processes of exocytosis. In the future, the power of this technology will only increase as it will be possible to perform TEP imaging simultaneously with other methods, such as prefusion labeling, to track the entire life cycle of vesicles, both before and after exocytosis, and to reveal the molecular mechanisms underlying exocytosis. Thus, we predict that two-photon imaging will play an increasingly important role in the full characterization of exocytosis and endocytosis in β-cells, as well as other cell types.

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Chapter 15
The Novel Roles of Glucagon-Like Peptide-1, Angiotensin II, and Vitamin D in Islet Function

Po Sing Leung and Qianni Cheng

Abstract Pancreatic islets secrete multiple factors that act as endocrine, paracrine, and/or autocrine pathways in regulating pancreatic endocrine function. As such, the islets perform critical biological activities in synthesizing metabolic peptide hormones, notably insulin and regulating body glucose homeostasis. These functions are controlled by various conditions and signaling molecules, particularly nutrients like glucose levels. However, more and more clinically relevant regulators, including molecules which stimulate islet β-cell metabolism, regulate β-cell [Ca^{2+}] homeostasis and related channels or adjust β-cell membrane, and nuclear receptors activity continue to be discovered and characterized. Of great interest in this context, glucagon-like peptide-1 can improve glycemic control by regulating insulin secretion and islet cell mass; vitamin D can regulate islet physiology directly by binding its receptors; in addition, the peptide hormone angiotensin II has been implicated in islet function and exhibits effects on islet cell secretion as well as cell mass. In this chapter, these three novel regulators in islet function and thus its clinical relevance to type 2 diabetes mellitus will undergo critical appraisal. Since all of these molecules have biological interactions with pancreatic islets, potential relationships may exist among them and they will also be discussed.

Keywords Angiotensin II · Diabetes · Glucagon-like peptide-1 · Islet function · Vitamin D

15.1 Introduction

The endocrine component of the pancreas consists principally of the islets of Langerhans, which are distributed throughout the pancreatic tissue and constitute around 2% by volume of the gland. Pancreatic islets secrete various hormones and

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generate paracrine and autocrine signaling. The islets are made up of five cell types, namely insulin-secreting β-cells, glucagon-secreting α-cells, somatostatin-secreting δ-cells, pancreatic polypeptide-secreting PP cells, and ghrelin-secreting epsilon cells [1]. The endocrine pancreas is much more highly vascularized than the exocrine pancreas and has its own vascular support component called insulo-acinar portal system.

The islets perform vital functions, most notably synthesizing metabolic hormones, such as insulin, and mediating glucose homeostasis in the body. These functions are controlled by various factors and signals. Typically, insulin secretion is influenced by glucose levels, the presence of particular nutrients, neurotransmitters, and hormones. Triggering and amplifying pathways are involved in the regulation of islet function [2]. However, additional clinically relevant regulators continue to be discovered and characterized. Such regulators represent putative targets for antidiabetic agents. Regulators that are of particular clinical interest include molecules which stimulate β-cell metabolism, regulate β-cell $[Ca^{2+}]$ and related channels or regulate β-cell membrane and nuclear receptors activity [2, 3]. Abnormality of these regulators will lead to pancreatic islet dysfunction. Usually, the increase of islet apoptosis rendering islets failing to regulate blood glucose levels and, in many animal or human studies with T2DM, islet β-cell mass is decreased by almost 60% in human or even 80% in some rodents [4, 5].

There is a keen interest in researching antidiabetic agents that affect metabolic pathways and thus an increase in β-cell mass improves islet function. For example, gastrointestinal peptide hormones known as incretins can improve glycemic control by regulating insulin secretion via binding stimulatory G proteins (G_s) associated with adenylate cyclase signaling pathways [6]. In addition, some nuclear receptors, such as the vitamin D receptor (VDR), have, when bound by their ligands, direct regulatory effects on islet physiology. Furthermore, the peptide hormone angiotensin II, known for its blood pressure maintenance and body fluid balance, has been implicated in a local islet renin-angiotensin system (RAS). In this chapter, these three novel regulators and their effects on islet function thus type 2 diabetes mellitus will undergo critical appraisal. Since all of these molecules have biological interactions with pancreatic islets, potential relationships may exist among them and they will also be discussed.

15.2 Glucagon-Like Peptide-1 in Islet Function

Incretins are synthesized by intestinal cells and released into the blood. They regulate the insulin response to the nutrients in ingested food. Hence, incretins can be considered ingestion triggered hormones [7]. Indeed incretin-induced insulin secretion constitutes 50–70% of the total insulin secretion stimulated by oral glucose intake [8]. Two peptide hormones with incretin effects have been described in humans: glucose-dependent insulinotropic polypeptide or gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). GIP and GLP-1 belong to the glucagon peptide superfamily and share close amino acid homology.

GIP is a 42 amino acid hormone synthesized in and secreted by K cells in response to nutrients in the proximal small intestine. Blood levels of GIP are increased by intake of glucose and/or fat [9]. The insulinotropic effects of GIP are mediated by GIP-specific receptors (GIPRs), which belong to the class II glucagon-like subfamily of receptors, also known as the G-protein coupled receptors (GPCRs) [10]. GIPRs are predominantly expressed in pancreatic β-cells [11] and increase intracellular cAMP and Ca^{2+} levels upon stimulation [7]. GIP can influence fat metabolism in adipocytes and modulate fatty acid synthesis [12]. It can also improve β-cell proliferation [13]. GIPR expression is decreased in diabetic animals and control of GIPR activity could help regulate glucose homeostasis [14, 15]. Although many studies have demonstrated that a dearth of GIP or GIPRs leads to the development of glucose intake stimulated glucose intolerance [16, 17], GIP activity alone does not fully reflect incretin effects in vivo [18]. Indeed GIP has yet to be proven to be a therapeutically effective diabetes treatment agent [19].

The more recently described incretin GLP-1 has also been shown to induce glucose-dependent insulin secretion [20]. GLP-1 is released from intestinal endocrine L cells in response to various nutrient, neural, and endocrine factors [21]. In both rodents and humans, a rapid release of GLP-1 into the blood can be triggered by oral intake of nutrients. There is an early phase (within 10–15 min) [22] of GLP-1 secretion triggered by glucose stimulation of sweet taste receptors in L and K/L cells [23]. Neuroendocrine factors can also affect GLP-1 secretion [21]. Several signaling pathway molecules, such as calcium, protein kinase A (PKA), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK), mediate the stimulation of GLP-1. Some studies have found that glucose-induced GLP-1 release is affected by the closure of K_{ATP} channels [24] and stimulated by glucose via mechanisms involving sodium-dependent glucose transporters (SGLTs) [25].

In circulation, GLP-1 is represented by active forms able to stimulate insulin secretion [26] as well as inactive forms. GLP-1(1-27) and GLP-1(1-36) are inactive forms, while GLP-1(7-37) and GLP-1(7-36) are active forms produced from full-length precursors by prohormone convertases 1 and 3 (PC1/3) [21, 27]. Active form GLP-1 levels decrease rapidly in response to renal clearance and degradation by dipeptidyl peptidase IV (DPP IV) [28] as well as neutral endopeptidase (NEP) [29]. The GLP-1 receptor (GLP-1R) was first identified in rat pancreatic islet cells [30] and later found in human islets [31]. Like GIPR, GLP-1R is a GPCR [32] that couples with G_s, thus activating adenylate cyclase, and is linked to downstream pathways, including some involving PKA and cAMP-regulated guanine nucleotide exchange factors of the Epac family [33].

Various effects of GLP-1 have been studied, including stimulation of glucose-dependent insulin secretion, induction of insulin biosynthesis, inhibition of glucagon secretion, gastric emptying, and food intake [34]. GLP-1's expression extends beyond the gut. Indeed, it is also synthesized in the brainstem and distributed along networks in central nervous system (CNS). In situ autoradiography hybridization has revealed GLP-1Rs in numerous CNS locations [35]. GLP-1Rs are also known to exist in the gastrointestinal tract, kidney, heart, lungs, and adipose

[34]. GLP-1 treatment (intravenous or intra-cerebral ventricular delivery) has been reported to increase heart rate and blood pressure in a GLP-1R-dependent manner in rats [36]. GLP-1 may also modulate bone resorption [37] and suppress plasma levels of triglycerides and nonesterified fatty acids [38].

GLP-1 is a very important regulator of glucose homeostasis and metabolism. Activation of GLP-1Rs can stimulate insulin secretion, chronically increase pancreatic β-cell proliferation, and inhibit apoptosis. Although no direct relationship between the GLP-1 level and the pathogenesis of T2DM has been found [39, 40], GLP-1 has several important effects on pancreatic islet function as follow:

1. *Insulin secretion and biosynthesis*. GLP-1 stimulates glucose-induced insulin secretion in humans and laboratory animals via GLP-1Rs [16], and mice without GLP-1Rs have abnormal glucose homeostasis [41]. In humans, circulating

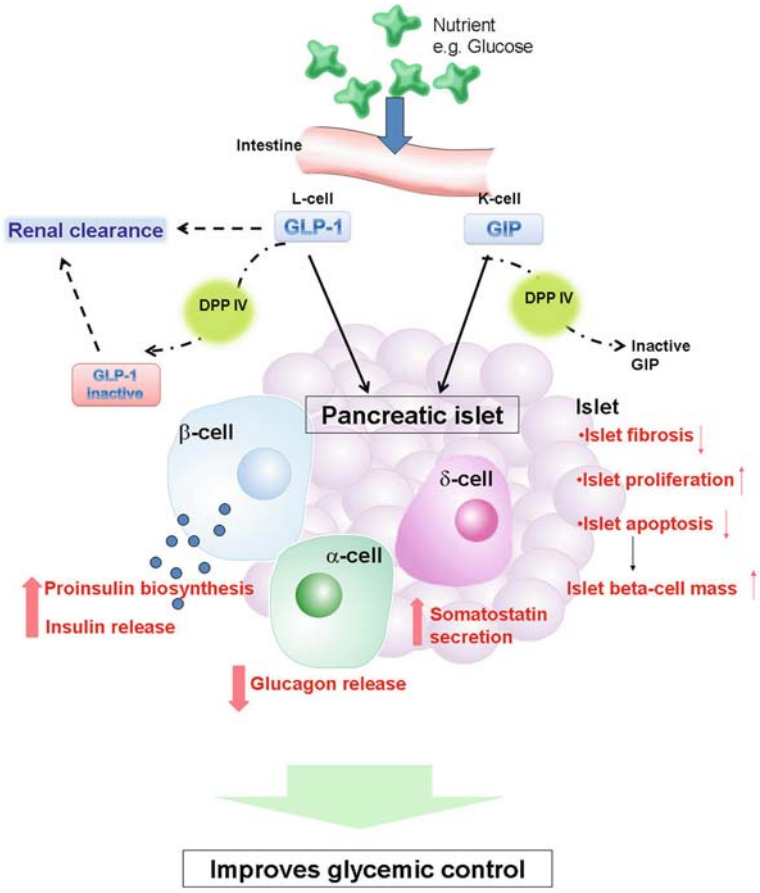

Fig. 15.1 Summary of physiological function of the incretins GLP-1 and GIP on islet cells. GLP-1 is secreted by intestine L cells, while GIP is released by K cells. Both are degraded by the enzyme DPP IV

GLP-1 levels are closely related to insulin secretion, and GLP-1 is inactive during periods of low blood glucose concentration [42, 8]. GLP-1 is critical to control fasting plasma glucose level [43] and glucose clearance. GLP-1 can induce insulin biosynthesis by stimulating insulin gene transcription [12].

2. *Glucagon secretion*. GLP-1 can suppress pancreatic glucagon release mediated by insulin and somatostatin secretion [8]. GLP-1 has been shown to inhibit hepatic production of glucose stimulated by glucagon and this inhibition effect is glucose dependent, suggesting that GLP-1 effects on glucagon release are conditional [44, 45].
3. *Islet proliferation and apoptosis*. GLP-1 (or its analog exendin-4) increased β-cell mass in diabetic or impaired glucose tolerance animals [46]. This trophic effect may be due to an increase in β-cell proliferation, a decrease in apoptosis, or both [47]. Figure 15.1 summarizes some important physiological roles of incretins (GLP-1 and GIP) in pancreatic islet cell (β, α, and δ-cells) secretion and islet cell mass.

15.3 Angiotensin II in Islet Function

The RAS is well known for regulating blood pressure as well as electrolyte and fluid homeostasis [48]. This system also plays novel roles in phathophysiology of diabetes as it has been reported to get involved in glucose homeostasis as well as insulin secretion and biosynthesis. Being a main active peptide in the RAS, angiotensin II can regulate insulin signaling, thus leading to insulin resistance probably via an enhanced NADPH oxidase activation which in turn an increase in ROS production [49]. These changes may interfere with insulin singnaling pathways via phosphatidylinositol 3-kinase (PI3K) and concomitantly followed with protein kinase B (Akt) pathway [50], thus affecting the balance of RhoA/Rho-kinase activation and inhibiting insulin actions [51, 52]. The circulating RAS is composed of several components including the precursor angiotensinogen, renin, angiotensin-converting enzyme (ACE), angiotensin II peptide and its receptors [53]. Renin converts angiotensinogen into angiotensin I (1–10), which is then converted into angiotensin II (1–8) by ACE. Alternate enzymes to renin and ACE can also generate a variety of biologically active angiotensin peptides such as angiotensin III, angiotensin IV, and angiotensin (1–7), which interact with respective receptors expressed on target tissues to exhibit specific activities and functions [54]. Apart from this classical RAS, local RASs have been recognized in multiple organs and systems in the past decade including, but are not limited to, the heart vasculature, kidney, adipose tissue, nervous system, reproductive system, and gastrointestinal system [55]. These local RASs play paracrine, autocrine, and/or intracrine roles in the regulation of functions specific for tissues and organs. Among them is the discovery of the local RAS in the pancreas which has various physiological effects, to name but a few, cell proliferation and apoptosis, reactive oxygen species (ROS) production, inflammation, fibrosis, and hormonal secretion [53, 54, 56–58].

Importantly, the endocrine pancreas contains all components of an intrinsic RAS [58, 59, 60]. For example, pro-renin is expressed in pancreatic islets, mainly in reticular fibers within islets and to a lesser extent in β-cells [56]. Low pro-renin mRNA levels in β-cells indicate that pancreatic islets may take up the pro-renin synthesized in the interstitium of islets [61, 62]. Though not detected with earlier methods [63], angiotensinogen expression has recently been demonstrated in rat pancreas, predominantly in glucagon-secreting α-cells [64]. While glucagon is secreted through regulated pathways in α-cells, angiotensinogen is secreted through a constitutive pathway in α-cells [65]. Pancreatic ACEs are expressed mainly in the microvasculature and periphery of islets [62]. On the other hand, angiotensin II type 1 and type 2 (AT1 and AT2) receptors are both present, though differentially distributed, in pancreatic islets [58, 60]. AT1 receptors are located mainly in cells at the islet center and co-localized with insulin-secreting β-cells. AT1 receptors expressed by β-cells in vitro can be upregulated by exposure to high glucose levels [66]. Meanwhile, AT2 receptors are found mainly co-localized with somatostatin-secreting δ-cells in the outer areas of the islets. Angiotensin II has higher local islet expression than at the circulation level. Early attempts to document angiotensin II immunoreactivity were identified in the pancreatic islets [67]. Recently, solid evidence has been substantiated and support unambiguously for the existence of a local islet RAS in the pancreas [68].

There is a growing body of clinical evidence implicating the RAS in islet function. In this aspect, angiotensin II suppressed glucose-dependent insulin release in islets [69, 70]. In concordance, islet blood flow was decreased by local angiotensin II and this effect could be attenuated with a RAS blocker [71, 72]. AT1 receptor expression in β-cells is upregulated in obesity-induced animal model of T2DM, i.e., db/db mice, and giving these mice with the AT1 blocker losartan decreased their blood glucose levels while improving their glucose tolerance and increasing their insulin secretion and pro-insulin biosynthesis [73]. Similar effects were also found in other T2DM animal model, such as the Zucker diabetic fatty rat [61, 74].

Continued islet functionality depends on maintenance of β-cell mass, which is determined by a balance between apoptosis and proliferation. Islet β-cell areas were reduced in db/db mice, but this deficit could be reversed by treatment with the AT1 blocker valsartan [75]. The decreased β-cell area in db/db mice may due to an imbalance between islet cell apoptosis and proliferation, islet oxidative stress, and/or inflammation-related islet fibrosis [76]. On the other hand, AT2 receptors appear not to play a direct role in glucose-induced insulin secretion, though they can stimulate somatostatin expression in a dose-dependent manner [77].

In summary, islet RAS, notably by activation of the AT1 receptors, regulates pancreatic islet insulin and somatostatin secretion, influences islet function, and thus regulates the body's glucose homeostasis. The AT1 receptor is expressed at abnormally high levels under diabetic conditions [73], and AT1 receptor antagonism can enhance insulin secretion, pro-insulin biosynthesis, islet blood flow, β-cell mass, and glucose homeostasis [68, 71, 73]. Figure 15.2 is a summary of the biosynthetic cascade of the RAS and the AT1 receptor-mediated islet cell function and structure of the pancreas.

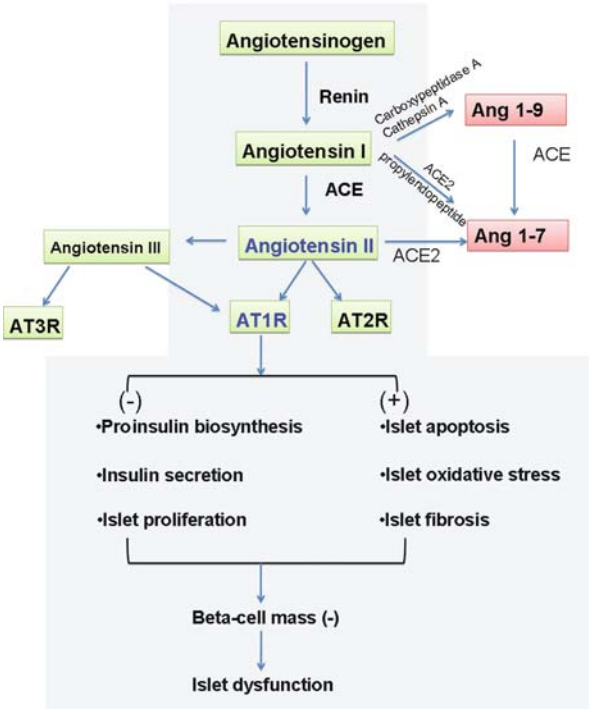

Fig. 15.2 RAS metabolism and the relationship between RAS and islet function. The major islet RAS components and functions are contained in the shaded region. (+): increase or activate; (−): decrease or inhibit

15.4 Vitamin D in Islet Function

Vitamin D is in actuality an essential steroid hormone (daily requirement 400–600 IU). The two major forms of vitamin D are cholecalciferol (vitamin D_3), produced in skin, and ergocalciferol (vitamin D_2), produced in plant tissues. Hence, it can be absorbed from foods of animal or plant origin. The best food sources include fatty fish and their liver oils, eggs, and fortified foods [78]. However, the major source of vitamin D for humans is our own skin, which synthesizes vitamin D under the influence of ultraviolet light [79].

The active form of vitamin D (1,25-dihydroxyvitamin D [$1,25(OH)_2D_3$]) is produced from its precursor by hydroxylation (Fig. 15.3). The precursor 7-dehydrocholesterol is converted to cholecalciferol, which is then transported to the liver and converted to 25-hydroxyvitamin D [$25(OH)D_3$], the major circulating form of vitamin D. $25(OH)D_3$ is converted to $1,25(OH)_2D_3$ in the kidney by 1-α-hydroxylase [80]. $1,25(OH)_2D_3$ binds to vitamin D receptors (VDRs) and activates various transcription factors. VDR usually binds to the retinoic X receptor (RXR) as a heterodimer and thus stimulates the RXR nuclear pathway [81]. The presence of

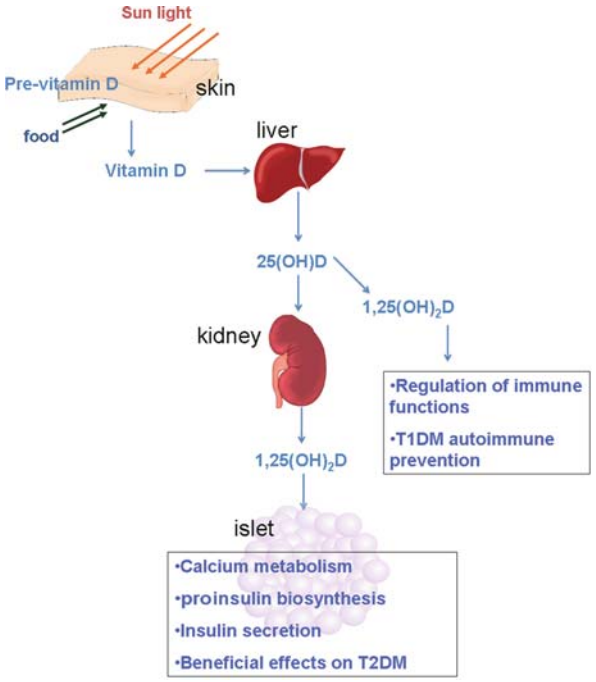

Fig. 15.3 Metabolism and physiological functions of vitamin D that are related to diabetes. Activated vitamin D can enhance islet calcium metabolism and increase insulin synthesis and secretion, thus exerting beneficial effects on T2DM. Vitamin D also regulates the immune system, which is particularly relevant to the autoimmune pathology of T1DM

the classical vitamin D response element and other response sites in genes important for β-cell and immune system physiology suggests that intervention in the vitamin D pathway may help treat diabetes [82–84].

The major function of vitamin D is to regulate mineral metabolism and bone growth. It prevents hypocalcemia muscular tetany and supports bone mineralization by increasing plasma calcium and phosphorus levels [85], stimulating renal reabsorption of calcium [86], and promoting bone growth and remodeling by osteoblasts and osteocalsts [87]. Growing evidence suggests that vitamin D homeostasis is also important for non-calcemic functions, including effects on insulin secretion and action [88]. These effects may be mediated by the binding of β-cell VDRs by $1,25(OH)_2D_3$ or the activation of $25(OH)D_3$ by 1-α-hydroxylase expressed in β-cells. Interestingly, VDR gene polymorphisms have been linked with both T1DM risk and T2DM pathogenesis, although in different populations [81]. Vitamin D may also regulate insulin secretion through its effects on β-cell calcium homeostasis [80]. These influence the activity and expression of insulin receptors in various types of cells; there are some potential VDRE sequence in insulin receptor promoter [89] by which it triggers the so-called genomic pathway-dependent nuclear VDR. On the other hand, $1,25(OH)_2D_3$ also increases insulin secretion through non-genomic

pathways or rapid response (RR) via binding with membrane VDR (mVDR) in the caveolae of the cell membrane; by doing so, it activates several second messenger systems including, but are not limited to phospholipase C (PKC), protein kinase C, G-protein receptors. The opening of voltage-gated calcium channel and rapid calcium influx together with glucose levels stimulates insulin secretion [90, 91]. Some of the RR can trigger second messengers which may be involved in the interaction between genomic and non-genomic actions of vitamin D as to regulate gene expression [91].

There is a purported link between vitamin D deficiency and risk of type 1 diabetes mellitus (T1DM). Indeed, exposure to vitamin D supplementation during fetal life or early childhood may decrease the risk of developing T1DM [92, 93]. Furthermore, given that T1DM has an autoimmune etiology, vitamin D's influence on the immune system is important to consider. The vitamin D producing enzyme 1-α-hydroxylase is present on immune cells, and $1,25(OH)_2D_3$ has been found to affect immune cells and T cell cytokines [94–96]. Moreover, $1,25(OH)_2D_3$ can prevent damage to β-cells by various inflammation factors, such as IL-1ß and IFN-γ [97], and induce anti-apoptotic protein expression [98].

There is a well-established relationship between vitamin D deficiency and T2DM [99]. Vitamin D deficiency was shown to inhibit insulin release in animal models almost three decades ago [100]. Vitamin D protection of β-cells is thought to be mediated by vitamin D binding of the calcium-binding protein calbindin-$D_{28\,K}$ [101, 102]. The local expression of VDR and 1-α-hydroxylase in islets suggests that vitamin D plays important roles in islet functions [79, 103]. Vitamin D deficiency is associated with impaired insulin secretion and glucose tolerance [100, 104, 105]. High-concentration $1,25(OH)_2D_3$ administration increases insulin synthesis and release in isolated neonatal islets from normal animals [106]. Deficiency of vitamin D or hypovitaminosis D is associated with increased risk of T2DM, cardiovascular disease, and other metabolic syndromes [99, 107].

In summary, though the full mechanistic picture of vitamin D's role in islet physiology has yet to be clarified, the evidence has revealed some important relationships of vitamin D and islet dysfunction thus T1DM and T2DM as shown in Fig. 15.3. In this context, the influence of vitamin D on cytosolic calcium levels, which affect insulin secretion and other β-cell signaling pathways, is particularly salient [108, 109]. The role of vitamin D in supporting β-cell survival via modulation of pro-apoptotic cytokines is also noteworthy [88].

15.5 GLP-1-Angiotensin II-T2DM Axis

Through their receptors on pancreatic islets, incretins like GLP-1 can directly enhance islet functions (see Section 15.2). In particular, GLP-1 stimulation can increase insulin gene expression and insulin biosynthesis [110], stimulate Ca^{2+} metabolism in β-cells, and modulate signaling pathways (e.g., PKA) that influence insulin release [33]. GLP-1 can also increase islet mass by favoring β-cell proliferation and reducing islet cell apoptosis [111]. GLP-1 activity is dramatically reduced

in diabetic patients [112], and as shown in Fig. 15.4 the beneficial effects of GLP-1 on islet physiology can be blocked by the DPP IV enzyme [28, 113]. Hence, the incretin benefit may be realized in diabetic patients by virtue of pharmacological inhibition of DPP IV or administration of GLP-1 analogs such as exendin-4.

GLP-1-based therapies including incretin mimetics and incretin enhancers. Incretin mimetics are GLP-1 analogs that are not rapidly degraded by DPP IV. Meanwhile, incretin enhancers inhibit DPP IV, enabling endogenous incretin molecules to persist. The most well-known incretin mimetic is exendin-4, which is much more potent than GLP-1 and has slower renal elimination [114]. Exendin-4 may decrease fasting plasma glucose and postprandial glucose excursions, especially when combined with other antidiabetic agents like metformin and sulfonylureas [115]. Other incretin mimetics under investigation include liraglutide [116] and cjc-1131 [117]. More work is needed to develop GLP-1 analogs without bothersome side effects that can be administered orally [115]. Not only GLP-1 analog but also treatment with native GLP-1 by continuous infusion was found to improve pancreatic islet proliferation and differentiation [118]. Meanwhile, numerous DPP IV inhibitors have been studied in animals and humans, including vildagliptin, sitagliptin, saxagliptin, and P32/98 [119]. Investigators examining DPP IV inhibitors are focusing on decreasing side effects as well as enhancing efficacy and prolonging duration of action [120–122].

Growing evidence suggests that incretins can affect cardiovascular diseases. GLP-1 infusion inhibited the development of salt-induced hypertension in rats [123]. The GLP-1 mimetic exendin-4 alleviated high systolic blood pressure, apparently through angiotensin II-induced phosphorylation of ERK1/2 [124]. The results demonstrated an anti-hypertensive effect of GLP-1 and protective actions on the kidney which expresses receptors for both GLP-1 and angiotensin II.

A convergence of actions of GLP-1 and angiotensin II on pancreatic islets is consistent with the possibility that an islet GLP-1–angiotensin II axis may also exist. Previous studies in our laboratory have demonstrated beneficial effects of chronic GLP-1 agonism (with the DPP IV inhibitor LAF237) and AT1 receptor antagonism (with valsartan) on islet functions and glycemic control in db/db obese diabetic mice [75]. Furthermore, a combined treatment with DPP IV inhibitor and AT1R blocker synergistically stimulated insulin release and enhanced maintenance of islet architecture and of β-cell mass, which is critical for maintenance of islet function [125, 126]. The tissue maintenance effects can be attributed to a reduction of ROS formation and fibrosis and a reversal of the apoptosis and/or proliferation balance [75]. The combined DPP IV inhibitor and AT1R blocker treatment also decreased islet expression of nitrotyrosine, a marker of cell damage and inflammation. This is a critical observation given that conditions that disrupt islet function, such as fibrosis, are associated with inflammation [127] and oxidative stress [128]. Angiotensin II can exacerbate islet dysfunction by stimulating superoxide production generation, inflammation, and apoptosis [129]. On the other hand, blockade of AT1R can decrease oxidative stress and apoptosis [76].

There is clinical evidence of an inverse relationship between GLP-1 concentration and inflammation [130]. Combined treatment with a GLP-1 analog (exendin-4)

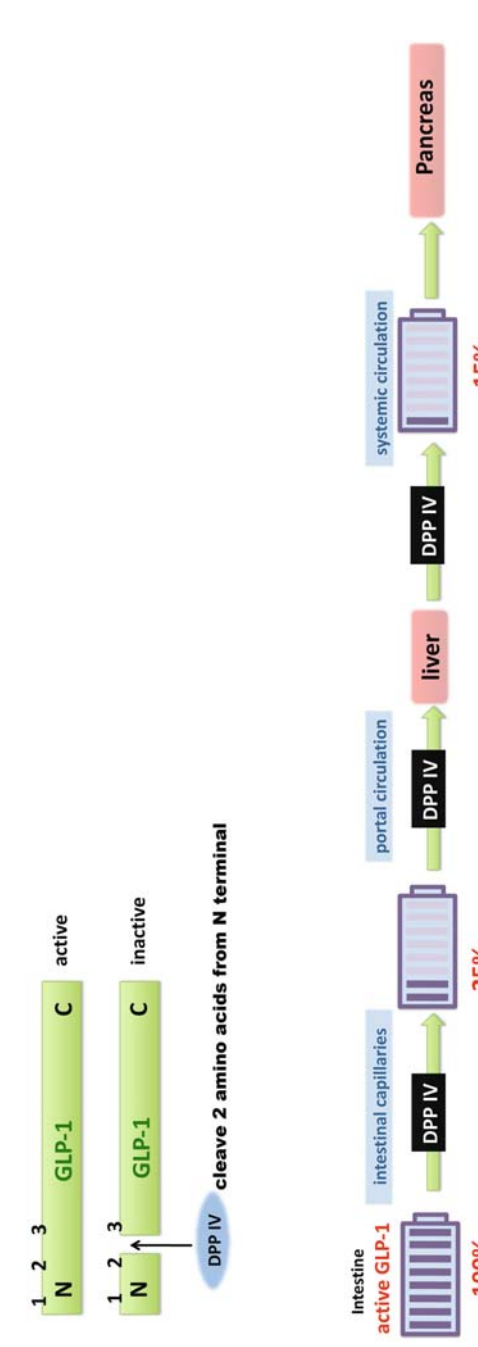

Fig. 15.4 Degradation of GLP-1 by the enzyme DPP IV. (**A**) DPP IV degrades active GLP-1 into its inactive form by cleavage of two amino acids from the N-terminal. (**B**) Progressive degradation of active GLP-1 from the intestine (source tissue), through the blood vessels and liver, to the pancreas (target site). Only ~15% of the GLP-1 produced in the intestine reaches the pancreas in its active form

and an ATIR blocker acted synergistically to increase insulin release from isolated islets, indicating that there is an acute interaction of GLP-1 receptor activation and AT1 receptor blocking on islet insulin secretion [75]. This synergistic effect could be explained as follows. Exendin-4 stimulates GLP-1Rs, which activates the PKA signaling pathway, and can thereby increase insulin secretion and decrease UCP-over-expression in diabetic β-cells [131, 132]. Meanwhile, AT1R blockade may inhibit islet oxidative stress via suppression of UCP2 expression, reduction of NADPH oxidase activity [76], and reduction of expression of the oxidant nitrotyrosine [133]. UCP2 serves as the common downstream target in this hypothesis. UCP2 is a well-known regulator of the diabetic condition and inhibitor of glucose-induced insulin secretion, and UCP2 knockout mice have increased β-cell mass and improved islet function [134]. Activated GLP-1Rs may also suppress islet nitric-oxide synthase [135], which could regulate UCP2 in adipocytes [136]. These studies indicate that ROS-induced and UCP-mediated islet dysfunction may be the mechanism by which GLP-1 interacts with RAS, hence constituting a GLP-1–angiotensin II axis. Figure 15.5 is a schematic representation of the potential interaction between GLP-1 and angiotensin II/AT1R in the regulation of pancreatic islet cell function.

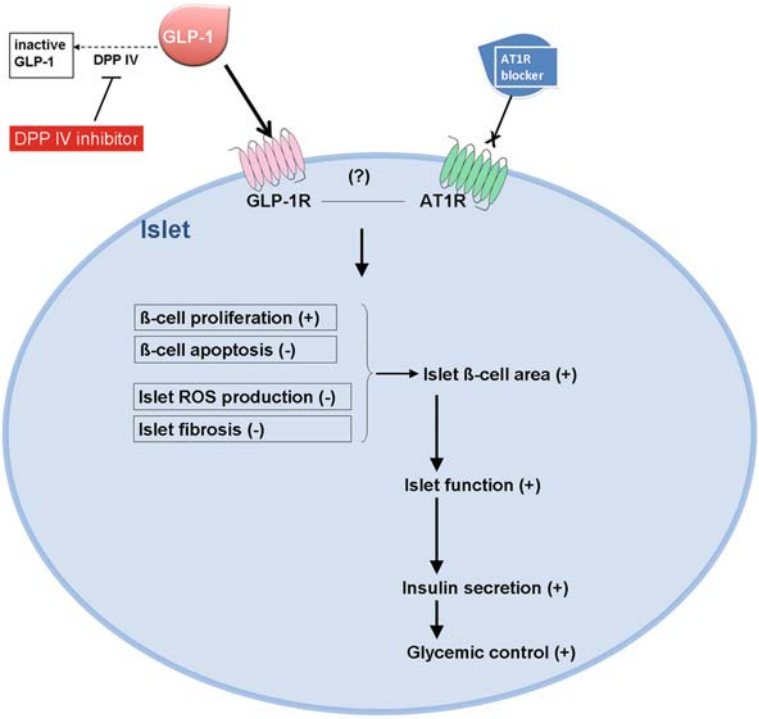

Fig. 15.5 Schematic depiction of the interaction between GLP-1 activity and AT1 blockade on islets. (?): potential interaction between GLP-1R and AT1R, (+): activate or increase, (−): inhibit or decrease

15.6 Vitamin D-Angiotensin II-T2DM Axis

Clinical studies have suggested a possible relationship between vitamin D and blood pressure related to circulating renin activity [137, 138]. VDR-null mice have abnormally high levels of renin in their kidneys and of angiotensin II in their plasma [139]. Kidney function is compromised in VDR-null mice, while plasma sodium and potassium homeostasis remain normal [140]. VDR-null mice have high blood pressure, cardiac hypertrophy, and exhibit abnormal drinking behavior [141]. These symptoms can be alleviated by treatment with an ACE inhibitor or an angiotensin II receptor blocker, indicating that these phenotypic features of the VDR-null mouse can be attributed to the RAS. In consistent with these findings, mice lacking 1-α-hydroxylase, the enzyme that produces active vitamin D, also demonstrated upregulated renin expression in the kidney, and $1,25(OH)_2D_3$ treatment could suppress renin expression [141]. Interestingly, although vitamin D normally has effects on calcium and parathyroid hormone (PTH) metabolism, its negative-regulation effect on renin expression does not depend on calcium metabolism [137, 142]; the effects of PTH on vitamin D-mediated renin regulation have yet to be resolved [143]. The suppressive effect of vitamin D on renin expression may be due to a direct negative regulation of the renin gene promoter by vitamin D. In cultured As4.1 cells, $1,25(OH)_2D_3$ treatment dramatically reduced renin-I gene promoter activity, thus suppressing renin gene transcription mediated by VDR actions [139]. This suppression effect suggests that vitamin D, or an analog, could be used to treat RAS-related diseases. Indeed, some low-calcemic vitamin D analogs have even been used in clinical applications [144, 145].

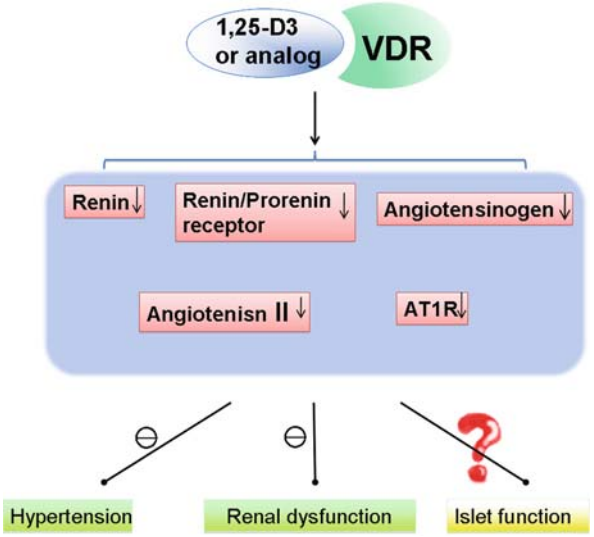

Fig. 15.6 Regulation of the RAS activity by vitamin D as shown in circulating (regulation of blood pressure) and in kidney (prevention of kidney diseases) RAS. The effects of vitamin D on islet RAS and thus islet function have yet to be investigated. ○: decrease or prevent

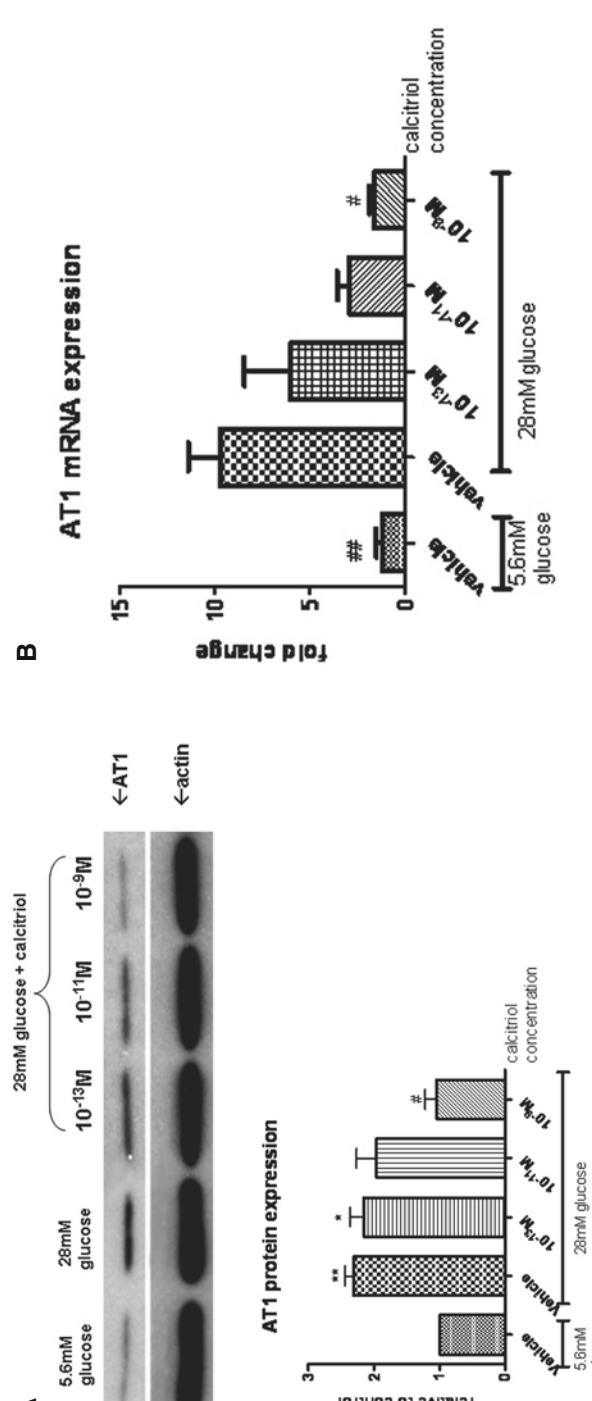

Fig. 15.7 AT1 receptor protein (**A**) and mRNA (**B**) expression in isolated islets with or without calcitriol (active form of vitamin D) treatment. AT1 expression is increased in the presence of 28 mM glucose. The vitamin D analog calcitriol reverse this effect at a concentration of 10^{-9} M. $*p < 0.05$, $**p < 0.01$ vs. 5.6 mM G + vehicle, $\#p < 0.05$, vs. 28 mM G + vehicle

The abnormally high expression of local RAS components in the islets of diabetic animals [73] could promote apoptosis and oxidative stress and thereby disrupt islet function [76]. Controlling islet RAS could improve islet function by increasing β-cell mass, balancing islet cell proliferation and apoptosis, and decreasing fibrosis. If RAS–vitamin D interactions observed in the circulatory system and in the kidney also occur in the pancreas (Fig. 15.6), then pathology related to excessive RAS activity in islets could potentially be treated with vitamin D or a vitamin D analog. Additional research is needed to clarify the effects of vitamin D on RAS, especially as additional information about RAS components and signaling is uncovered.

Ongoing work in our laboratory is examining whether vitamin D treatment can modulate pancreatic RAS expression and activity. Vitamin D analog (calcitriol) treatment can inhibit abnormally high expression of ACE mRNA (data not shown) and of AT1 mRNA and protein (Fig. 15.7, unpublished data) in isolated islets under a high glucose condition (28 mM). The effective concentration of vitamin D analog (10^{-9} M) is higher than the circulating or physiological concentration of vitamin D, indicating the importance of a local vitamin D endocrine system. The effect may be achieved via the islet VDR, located in nuclei as well as on the membrane and in the cytoplasm of β-cells. VDR expression may be dose dependently regulated by vitamin D activation (unpublished data). In light of the importance of islet AT1 in islet function, it is reasonable to hypothesis that vitamin D may improve islet function by down-regulating islet RAS excess in the diabetic condition, probably through maintenance of β-cell mass and reduction of fibrosis and inflammation.

15.7 Summary

Preservation of pancreatic islet cell function and islet cell mass is becoming central goals of diabetes therapies. Incretins such as GLP-1, the hormone vitamin D, and the islet locally produced angiotensin II have common effects on islet function and structure by regulating the balance of cell proliferation and apoptosis as well as reducing islet oxidative stress. The synergistic actions of GLP-1 and an AT1R blocker on islet function suggest that vitamin D could regulate abnormally expressed islet RAS components, and thus there may be GLP-1—angiotensin II and vitamin D–angiotensin II interactions that can be targeted to maintain normal islet function. All three, if these factors are biologically active in pancreatic islets and all of them contribute to the maintenance of islet function, indicate that the potential GLP-1–angiotensin II–vitamin D axis exists and that it may be involved in T2DM pathogenesis. Of note, the short-term effect of GLP-1 as well as the relative long-term effect of vitamin D-induced genomic signaling, and the effect of angiotensin II indicates a potential triumvirate action of these factors in the regulation of islet function and structure thus T2DM. According to this model, GLP-1 and vitamin D may regulate the activities and function of local islet angiotensin II and thereby they may be targeted to prevent or control T2DM (Fig. 15.8).

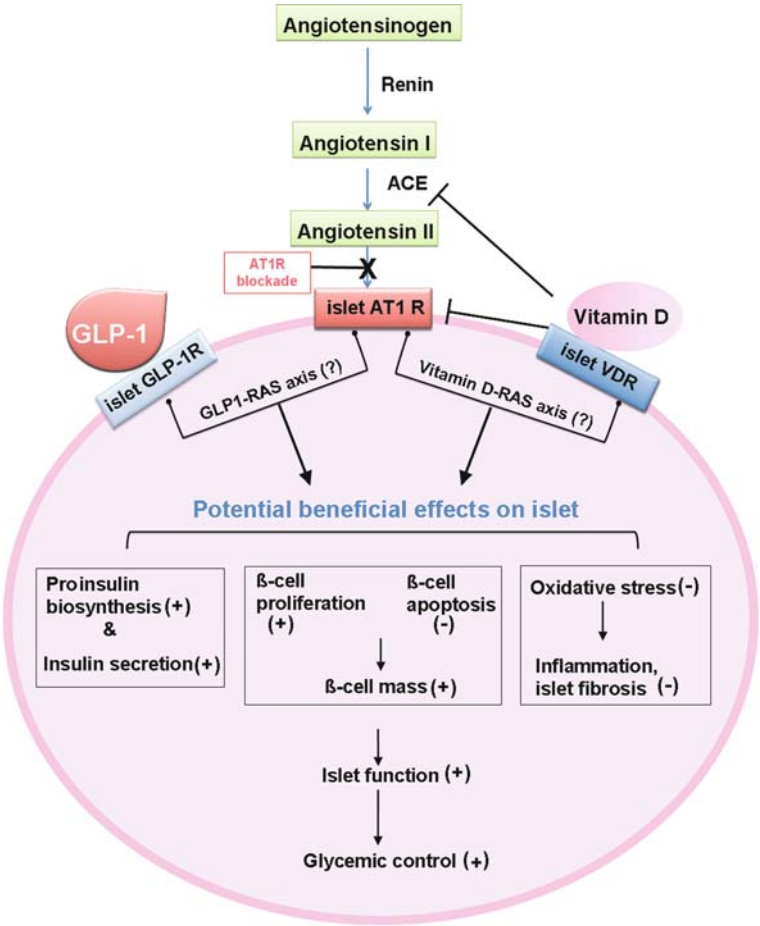

Fig. 15.8 Summary of potential interaction among GLP-1, vitamin D, and angiotensin II in the regulation of islet cell function and islet cell mass. All three regulators have specific receptors on pancreatic islets. The potential GLP-1–angiotensin II–vitamin D axis may exist in this model. The beneficial effects of intervening in these proposed axes of interaction on islet function remain to be intensively investigated. (?): need further studies; (+): increase or activate; (−): decrease or inhibit

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Chapter 16
Proteomics and Islet Research

Meftun Ahmed

Abstract The complementary disciplines of genomics and proteomics offer better insights into the molecular mechanisms of diseases. While genomics hunts for defining our static genetic substrate, proteomics explores the structure and function of proteins expressed by a cell or tissue type under specified conditions. In the past decade, proteomics has been revolutionized by the application of techniques such as two-dimensional gel electrophoresis (2DGE), mass spectrometry (MS), and protein arrays. These techniques have tremendous potential for biomarker development, target validation, diagnosis, prognosis, and optimization of treatment in medical care, especially in the field of islet and diabetes research. This chapter will highlight the contributions of proteomic technologies toward the dissection of complex network of signaling molecules regulating islet function, the identification of potential biomarkers, and the understanding of mechanisms involved in the pathogenesis of diabetes.

Keywords Proteomics · Islets · Two-dimensional gel electrophoresis · Mass spectrometry · Proteome · Glucolipotoxicity

16.1 Introduction

Pancreatic islets, the fascinating little magic box, because of their vital performance in blood glucose regulation have long been central focus of diabetes research. The essential illusion of these magical islets is the β-cell, a 'mysterious maiden' with bags full of insulin. Search for the understanding of the β-cells has given rise new ideas, imagination, and creativity in the worldwide scientific community, but till now not a single phenomenon of the β-cell has been fully understood. Every new

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discovery tells a tale about the previous one – a little more, but the story seems a never ending one. In this promising journey of biomedical discovery, the completion of the human genome project has facilitated the entry of the biomedical researchers into a new dimension – the post-genomic era. This era is marked by an explosion of terms containing the suffix 'omics,' like the word genomics, transcriptomics, and metabolomics. One of the very stylish and trendy labels carrying the appellation 'omics' is *proteomics,* which attracted the attention of contemporary scientists and offered to fill the void left by the human genome project to gain an in-depth understanding of future disease prevention and innovation of novel drug targets. The contributions of proteomic technologies toward the insights of the pathophysiology of the pancreatic islet function will be discussed in following sections.

16.2 Proteome and Proteomics

All cells in the human body have essentially the same genetic information, and the genes possess only the information which is sequentially encoded to construct the final products – the proteins. These proteins are dynamic in nature and considered as the molecular engineers for a cell; their composition in a cell may vary at its different stages of development whereas the genes remain as the static component of a cell. A classic example is the caterpillar and its mature form, the butterfly; they have the same genetic makeup whereas their protein composition is quite different and it is the protein which is responsible for different shapes and forms of the organism. The renaissance of proteomics is due to the fact that proteins are expressed in quantities and physical forms that cannot be predicted from DNA and mRNA analysis [1, 2]. In addition, the diseased cells often produce proteins that healthy cells do not have and vice versa. Hence, scientists are aiming toward creating a complete catalogue of all the human proteins with an intention to uncover their interactions with one another [1, 3]. Their definitive goal is to discover biomarkers and to devise better drugs with fewer side effects. Significant progress has already made in biomarker discovery where several groups have announced that using proteomic techniques it is highly possible to make an accurate early diagnosis for cancers including ovarian, breast and prostate cancer [4–6].

In general, proteomics includes cataloging all the proteins present in a cell or tissue type at a specific time under specific conditions, quantitation, and functional characterization of these proteins to elucidate their relationships (protein–protein interaction networks) and functional roles and ultimately outlining their precise three-dimensional structures in order to find where the drugs might turn their activity on or off – the 'Achilles heels' [7–9]. The term proteome was coined as a linguistic equivalent to the concept of genome and first used in 1994 at the "Siena 2D Electrophoresis" meeting (9–11). It denotes the entire PROTEin complement to a genOME, expressed by a cell or tissue type, at a specific time in the development of the organism under specific conditions [12, 13]. While humans are estimated to have approximately 20,000–25,000 genes, alternate RNA splicing and posttranslational

modification may led to encoding as many as 250,000–1 million individual proteins or peptides. For example, more than 22 different isoforms of α-1-antitrypsin exist in human plasma [14]. In addition, the proteome undergoes dynamic changes as it continuously responds to autocrine, paracrine, and endocrine factors as well as exposure to any pathogen, changes in external environment, and during time course of disease and drug treatment. Various gene products, including microRNA [15], as well as epigenetic factors [16] also influence the expression levels of genes and their transcripts. As a consequence, the proteome is far more complex than the genome. Thus, the scale of protein discovery task is challenging and very large indeed. And multiple specialists from different fields must collaborate to provide a range of sophisticated tools to analyze nature's tremendous complexity. However, proteomics is still in an early stage and at the time when mRNA expression arrays are spreading like cell phones in industry and in academic institutions, systems for large-scale protein analysis are still novelties. The commonly available proteomic technologies to date are summarized in Table 16.1.

Table 16.1 Currently used proteomic technologies

Proteome profiling	**Emerging technologies**
1D gel electrophoresis	SILAC
2D gel electrophoresis	Imaging mass spectrometry (IMS)
2D-DIGE	Molecular scanner
MS-based methods	iTRAQ
SELDI-TOF	ICAT
MALDI-TOF	HysTag
CE-ESI-MS	Label-free LC-MS/MS quantitation
LC-MS	Protein chips:
Protein identification	*Spotted array-based tools:*
Mass spectrometry	Forward-phase arrays (FPA), e.g., antibody arrays, protein arrays
Mud-PIT or shotgun proteomics	Reverse-phase arrays (RPA)
2-D LC-MS/MS	*Microfluidic-based tools*
Protein function	
Yeast two hybrid	
Phase display	
Surface plasmon resonance analysis	
Immunoaffinity	
Structural proteomics	
X-ray crystallography	
NMR spectroscopy	
Electron tomography	
Immunoelectron microscopy	

2D-DIGE, two-dimensional differential in-gel electrophoresis; CE-ESI-MS, capillary electrophoresis electrospray ionization mass spectrometry; ICAT, isotope-coded affinity tags; iTRAQ, isobaric tagging for relative and absolute quantitation; LC-MS liquid chromatography mass spectrometry; Mud-PIT, multidimensional protein identification technology; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; NMR, nuclear magnetic resonance; SELDI-TOF, surface-enhanced laser desorption/ionization time of flight; SILAC, stable isotope labeling by amino acids in cell culture.

Over the years, proteomics has expanded to include profiling, quantitative, functional, and structural proteomics based on a broad range of technologies. *Protein profiling* involves identifying and making a list of the proteins present in a biological sample [17]. *Quantitative proteomics* discovers molecular physiology at the protein level and allows comparisons between samples by measuring relative changes in protein expression in response to external stimuli [18, 19]. *Functional proteomics* attempts to identify proteins in a cell, tissue, or organism that undergo changes in abundance, localization, or modification in response to a specific biological condition and discover their functions based on the presence of specific functional groups or based on their involvement in protein–ligand interactions [17, 20]. Similarly, pathways can be characterized as a cascade of specific protein interactions required to activate cellular functions. Functional proteomics thus focuses on understanding part of the wiring diagram of a cell. *Structural proteomics* attempts to determine the three-dimensional structure of proteins, the structure of protein complexes, and small molecule protein complexes. X-ray crystallography and NMR are its main approaches [21, 22].

In the plethora of proteomic technologies, two-dimensional gel electrophoresis (2DGE) remains as a cornerstone of protein profiling [23, 24]. The 2DGE separates proteins according to two independent parameters, isoelectric point (p*I*) in the first dimension and molecular mass (*M*r) in the second dimension by coupling isoelectric focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [25, 26]. Theoretically, 2DGE is capable of resolving up to 10,000 proteins simultaneously, with approximately 2,000 proteins being routine and detecting and quantifying protein amounts of less than 1 ng per spot [23, 24]. Despite the well-known limitations of the 2DGE approach, e.g., poor solubility of membrane proteins, limited dynamic range, difficulties in displaying and identifying low-abundant proteins, lack of reproducibility and automation, 2DGE will remain as a powerful and versatile tool for display and quantification of a majority of proteins in biological samples. The detailed technology, challenges as well as the application, potential and future of high-resolution 2DGE have been elegantly reviewed in several papers [23, 24, 27, 28]. However, gel-free high-throughput protein profiling techniques have leapt prominence and now become preferred method of choice including multidimensional protein identification technology (Mud-PIT) [29], molecular scanner [30], stable isotope labeling by amino acids in cell culture (SILAC) [31, 32], isotope-coded affinity tag (ICAT) [2], isobaric tagging for relative and absolute quantitation (iTRAQ) [33], protein microarrays [34–37], and HysTag reagent [38]. It should be noted that the use of these emerging techniques is limited to certain specialized and privileged laboratories. Also, the choice of a given proteomic approach depends on the type of biological question asked, since each proteomic technology is characterized by specific applications, technical advantages, and limitations. A typical gel-based proteomic work flow is schematically illustrated in Fig. 16.1. Peptide mass fingerprinting (PMF) and tandem mass spectrometry (peptide fragmentation to generate partial sequence, MS/MS) are commonly used for protein identification on two-dimensional proteomic patterns [39–42]. The recent progress in the sensitivity of mass spectrometry analysis has

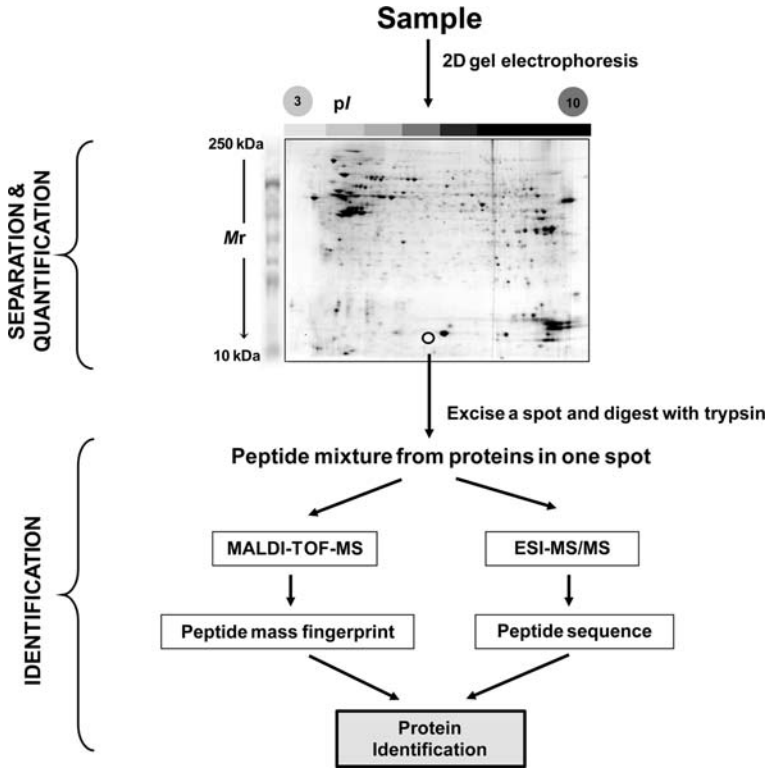


Fig. 16.1 A two-dimensional gel-based proteomic workflow. There are two principal steps. The *first* is separation and quantification of proteins in a sample using 2D gels. In the first dimension, proteins are separated in a pH gradient according to their molecular charge, known as isoelectric focusing. In the second dimension, the proteins are separated orthogonally by electrophoresis based on their molecular mass. The end result is a 2D gel with thousands of spots where individual spot represents a protein/peptide or a mixture. The *second* is identification of the separated proteins, typically using mass spectrometry (MS) techniques and bioinformatics. A protein spot can be excised from the 2D gel, digested with a protease and the peptides extracted. These peptides can then be analyzed using MS techniques such as matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) and electrospray ionization tandem MS (ESI-MS/MS)

significantly increased the applicability of proteomic technologies [43] as protein identification and profiling tool as well as determining protein interactions and the type and location of posttranslational modifications [41, 44, 45]. Surface-enhanced laser desorption/ionization time of flight (SELDI-TOF) is a suitable technique for high-throughput proteomics analysis of complex mixtures of proteins where proteins are retained on solid-phase chromatographic surfaces with specific properties and are subsequently ionized and detected by TOF MS [46–48]. However, this system is limited for profiling low molecular weight proteins (<20 kDa) [47]. In another protein profiling strategy, commonly referred as 'bottom-up' *shotgun proteomics* (multidimensional LC-MS/MS or Mud-PIT), complex protein mixtures are digested

into peptides, followed by chromatographic separation of peptides prior to analysis by tandem mass spectrometry, and computer algorithms then map the peptides onto proteins to determine the original content of the mixture [49].

Quantifying changes in protein abundance between samples is a key goal of proteomics. Promising novel methods for high-throughput quantitation involve '*label-free*' approaches. Several studies have demonstrated that LC-MS peptide ions spectral peak intensities are directly proportional to the protein abundances in complex samples [50]. Another label-free method, termed spectral counting, compares the number of MS/MS spectra assigned to each protein [50, 51]. With controls for normalization between runs, label-free quantitation offers a simpler approach for analysis. Spectral sampling also enables ranking different proteins by their relative abundances, providing information that other methods cannot achieve [52].

In addition to the protein profiling and comparative proteomics, functional study of target proteins is essential in any successful proteomic study. Functional proteomic approaches are based on interactions of proteins or specific activities of proteins. Phage display is a powerful proteomic tool used to express proteins or domains of proteins [53, 54]. The system has played a pivotal role in mapping epitopes of monoclonal and polyclonal antibodies, defining amino acid substrate sequences, and identifying peptide ligands for drug research. Yeast two-hybrid system detects binary protein interactions by activating expression of a reporter gene upon direct binding between the two tested proteins [55, 56]. SELDI-TOF MS has also been used to characterize protein–protein interaction [47]. Recently, for studying the functions and interactions of proteins, protein microarrays have been developed in analogy to DNA microarrays which can also be applied for comparative studies of expression of large sets of proteins [57]. There are two major types of protein microarrays – forward (FPA) and reverse-phase array (RPA) [58, 59]. In forward protein arrays, thousands of recombinant antibodies carrying the desired specificities are arrayed on glass slides, which make it very well suited for high-throughput screening of biological samples for specific disease markers [60, 61]. The BD Clontech™ Ab Microarray 500 represents a significant step in that direction. With this array, over 500 specific proteins can be assayed to detect and compare expression level of both cytosolic and membrane-bound proteins representing a broad range of biological functions, including signal transduction, cell cycle regulation, gene transcription, and apoptosis. In contrast to using chips with immobilized antibodies to detect specific proteins, protein chips carrying the proteome of a specific organism or cell type can be made by cloning and purification of these proteins [62]. This protein microarray can then be screened on the basis of the ability of the chip to bind specific ligands or interact with specific proteins. The human ProtoArray® protein microarray (Invitrogen™) contains more than 8,000 full-length human proteins purified under native conditions. This high-content discovery tool provides highly sensitive and reproducible results enabling rapid and easy profiling of thousands of biochemical interaction. In reverse-phase microarrays, tissues [63], cell lysates [64], and serum samples [65] are spotted on the surface and probed with specific antibodies per analyte for a multiplex read-out. Thus, this analysis evaluates the expression level of defined target proteins in

multiple samples. Both forward- and reverse-phase protein microarrays are novel technologies in proteomics and offer great promise for use in clinical applications.

16.3 Application of Proteomics in Islet Research

The accomplishment of human genome sequences has conferred the islet scientists with immense errands to assess the relative levels of expression of these gene products including the proteins and their posttranslational modifications in pancreatic islets. In the post-genomic era, to clarify the molecular mechanism of islet function in both normal and disease states, it is important to understand the entire gene products which regulate the phenotypes of islet cells and their ability to differentiate and secrete specific hormones. An important advantage of global protein expression profiling compared with individual gene or protein regulation studies is the ability to monitor changes in several functional groups simultaneously. It should be kept in mind that proteomics per se is not a hypothesis-driven experimental approach, but rather a hypothesis generating '*fishing-expedition*' where one explores the proteins that are not a priori expected to be associated with any pathophysiological conditions, which allows discovering novel proteins and signaling networks opening new research avenues. Since its introduction in 1994, the proteomic booms continue and got considerable attention of the islet researchers as well. Improvements of the core technologies, especially advancement of protein identification by mass spectrometry and bioinformatics tools, have recently encouraged the application of proteomics to unlock the secret of islet pathophysiology. It is indeed interesting to note that the most widely used protein separation technique, the 2DGE, has been employed in 1982 for insulin granule protein profiling [66]. In those early days more than 150 protein/peptide spots were detected in a 2DG of insulin secretory granule and some of the high molecular weight spots were presumed as glycoprotein. Lack of high-throughput protein identification method did not permit annotation of the granule proteins but provided an opportunity to study the functional properties of the insulin secretory granule and to dissect the molecular events of exocytosis. A similar proteomic approach has been utilized to explore the glucose-responsive granule proteins in ^{35}S-methionine-labeled rat islet and insulinoma cells and the study showed that biosynthesis of 25 granule proteins were stimulated 15–30-fold by glucose [67]. In a subsequent subproteomic study, almost after 25 years, Brunner et al. [68], separated the INS-1E granule proteins by 1-dimensional SDS-PAGE and identified 130 different proteins by LC-MS/MS.

16.3.1 Protein Profiling of Pancreatic Islets

A high-quality 2DGE reference map of the isolated pancreatic islets is essential for a 2DG-based comparative proteomics study and for generation of hypothesis. In the holy grail of protein profiling of pancreatic islets, Sanchez et al.

[69] did a pioneering work where they mapped 63 spots corresponding to 44 mouse islet protein entries. This protein map is available in the Swiss-2D database (http://us.expasy.org/ch2d/). Nicolls et al. [70] identified 88 proteins in total from mouse islets of which 18 were already identified by Sanchez and coworkers. Continued attempts in '*shooting at stars*' generated another mouse islet 2DGE reference map where 124 spots corresponded to 77 distinct proteins [71]. A reference map of rat insulinoma-derived clonal INS-1E β-cell proteins has also been constructed (Fig 16.2). This 2D map contains 686 valid spots, among which 118 spots corresponding to 63 different proteins have been successfully identified by MALDI-TOF MS and a combination of liquid chromatography and electrospray tandem MS (LC-ESI-MS/MS). Using 2DGE and MALDI-TOF MS the first protein map and database of human islets have been generated in 2005 where 130 spots corresponding to 66 different protein entries were successfully identified [72]. A high level of reproducibility was reported among the gels, and a total of 744

Fig. 16.2 2-D PAGE image of INS-1E proteins. Proteins (200 μg) were loaded onto an IPG strip (pH 3–10 NL) and subsequently separated by mass on a gradient (8–16%) SDS-PAGE gel. The gel was stained with colloidal Coomassie blue and the filtered image was generated by PDQuest software. Experimental masses and p*I*s are indicated. The gene names mark the location of the corresponding proteins on the gel. Aco2, aconitate hydratase, mitochondrial; Actb, β-actin; Ak2, adenylate kinase isoenzyme 2, mitochondrial; Alb, serum albumin; Aldoa, fructose-bisphosphate aldolase A; Anx2, annexin A2; Anxa4, annexin A4; Anxa5, annexin A5; Arhgdia, Rho GDP-dissociation inhibitor 1 (Rho-GDI α); Atp5a1, ATP synthase subunit α, mitochondrial; Atp6v1a, V-type proton ATPase catalytic subunit A; Calr, calreticulin; Cfl1, cofilin-1; Eef1a1, elongation factor 1-α 1; Eef1a2, elongation factor 1-α 2; Eno1, α-enolase; Gapd, glyceraldehyde-3-phosphate dehydrogenase; Gnb2, guanine nucleotide-binding protein $G_i/G_s/G_t$ subunit β-2; Grp58, protein disulfide isomerase A3; Grp75, stress-70 protein, mitochondrial (75 kDa glucose-regulated protein); Grp78, 78 kDa glucose-regulated protein; Hadha, trifunctional enzyme subunit α, mitochondrial; Hadhsc, hydroxyacyl coenzyme A dehydrogenase, mitochondrial; Hnrpa2b1, heterogeneous nuclear ribonucleoproteins A2/B1; Hnrpk, heterogeneous nuclear ribonucleoprotein K; Hnrpl, heterogenous nuclear ribonucleoprotein L; Hsc70, heat shock cognate 71 kDa protein (Hspa8); Hsp40, DnaJ homolog subfamily B member 1 (heat shock 40 kDa protein 1); Hsp60, 60 kDa heat shock protein, mitochondrial; Idh3a, isocitrate dehydrogenase [NAD] subunit α, mitochondrial; Ihd2, isocitrate dehydrogenase [NADP], mitochondrial; Krt8, keratin, type II cytoskeletal 8; Mdh1, malate dehydrogenase, cytoplasmic; Mdh2, malate dehydrogenase, mitochondrial; Nme2, nucleoside diphosphate kinase B; Orp150, 150 kDa oxygen-regulated protein (hypoxia up-regulated protein 1); Pdia1, protein disulfide isomerase; Pdia6, protein disulfide isomerase A6; Pebp, phosphatidylethanolamine-binding protein 1; Pfn1, profilin-1; Pgk1, phosphoglycerate kinase 1; Pgrmc1, membrane-associated progesterone receptor component 1; Phgdh, D-3-phosphoglycerate dehydrogenase; Pkm2, pyruvate kinase isozymes M1/M2; Ppia, peptidyl-prolyl *cis–trans* isomerase A (cyclophilin A); Prdx1, peroxiredoxin-1 (thioredoxin peroxidase 2); Rpsa, 40S ribosomal protein SA; Sod1, superoxide dismutase [Cu-Zn]; Stip1, stress-induced phosphoprotein 1; Tkt, transketolase; Tpm5, tropomyosin α-3 chain; Tra1, endoplasmin; Tuba, tubulin α; Tubb5, tubulin β-5 chain; Txndc4, thioredoxin domain-containing protein 4; Ubc, polyubiquitin; Uchl1, ubiquitin carboxyl-terminal hydrolase isozyme L1; Vcp, transitional endoplasmic reticulum ATPase; Vdac, voltage-dependent anion-selective channel protein; Ywhae, 14-3-3 protein ϵ; Ywhaz, 14-3-3 protein ζ/δ (protein kinase C inhibitor protein 1)

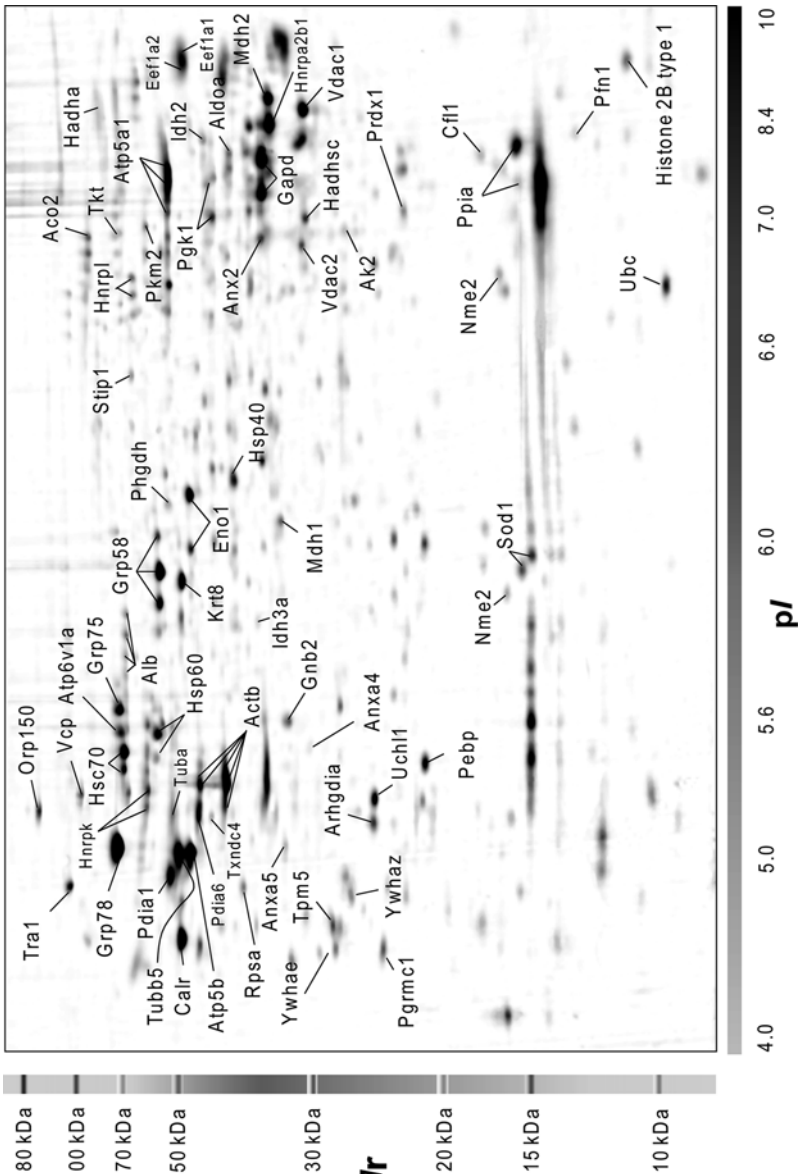


Fig. 16.2 (continued)

protein spots were detected [72]. All the protein profiling studies [69–72] using 2DGE categorized the identified proteins according to cellular location and function. Any attempt to compare these studies renders déjà vu since a number of prevailing proteins were repeatedly reported and most proteins fell into the cytosolic category followed by mitochondrial and endoplasmic reticulum (reviewed by [73]). In aforementioned studies a large part of the proteins have either chaperone (e.g., protein disulfide isomerase, PDI; calreticulin; 78 kDa glucose-regulated protein, GRP78; 58 kDa glucose-regulated protein, GRP58; endoplasmin) or metabolic (e.g., α enolase, transketolase, pyruvate kinase, and hydroxyacyl-CoA dehydrogenase, SCHAD) functions. However, every laboratory blessed with the successful application of 2DGE has its own protocol for protein extraction, isoelectric focusing, and SDS-PAGE. Therefore, a reference map produced by one group cannot necessarily be useful for any other group interested in comparative islet proteomics. Moreover, since introduction in SWISS-2D database, the islet proteome map has not been updated assigning identification of more protein spots. Therefore, the technical hurdle remains for the laborious protein identification procedure even if one follows a similar protocol.

Recent advances in mass spectrometry techniques allowed use of strong cation exchange fractionation coupled with reversed phase LC-MS/MS and characterization of 2,612 proteins in the mouse islet proteome [74]. Using nano-UPLC coupled to ESI-MS/MS more than thousand proteins have been identified in mouse islet (unpublished data). A 2D LC-MS/MS study of the human islets characterized 3,365 proteins covering multiple signaling pathways in human islets including integrin signaling and MAP kinase, NF-κβ, and JAK/STAT pathways [75]. Combined genomic and proteomic techniques have been employed for profiling of glucagon secreting α-cells [76]. While a total of 5,945 gene products were detected in α-cells by the gene chips alone, only 1,651 proteins were identified with high confidence using shotgun proteomics and rigorous database searching. Seven hundred sixty-two cross-mapped gene product pairs (both the gene and the corresponding protein) were jointly detected by both platforms. Conversely, 126 gene products were detected exclusively by proteomics, being somehow missed by the gene chip platform [76]. In recent years the growing number of islet proteomic data necessitates development of bioinformatics tools for easy data handling and data mining to assign subcellular location, functional properties, molecular networks, and known potential posttranslational modifications. It is becoming essential to create a common platform for islet proteomic users integrating molecular, cellular, phenotypic, and clinical information with experimental genetic and proteomics data.

An important feature of proteomics is that protein isoforms generated by posttranslational modifications can be separated by 2DGE. Among the hundreds of different types of protein modifications, reversible protein phosphorylation is a key regulatory mechanism of cellular signaling processes [77–79]. To detect global phosphoproteome profiles of islets, the advantages of the fluorescent dye Pro-Q Diamond, which is suitable for the fluorescent detection of phosphoserine-, phosphothreonine-, and phosphotyrosine-containing proteins on 2D gels directly [80], have been exploited and 90 different phosphorylated proteins were detected

on the 2D map (unpublished data). However, vanishingly small amounts of phosphorylated proteins in cells and lack of robotic picker in our laboratory precluded spot cutting and identification of most of the spots. Only a few, including ATP synthase α chain, elongation factor 1-α, actin, γ-aminobutyric-acid receptor α-3 subunit, and α-2-HS-glycoprotein could be successfully identified. Further isolation and purification of phosphoproteins and increasing the loading amount by pooling islet samples will possibly increase the chances for better identification for comparative studies to elucidate how posttranslational modifications regulate insulin secretion. LC-MS/MS analysis for posttranslational modifications of mouse islet proteome identified relatively abundant secretion-regulatory proteins including chromogranin A and secretogranin-2 [74]. Then again, it is just the very minute tip of the phosphoproteome iceberg.

16.3.2 Comparative and Quantitative Islet Proteomics

The ability of the islet of Langerhans to respond with proper insulin release when the ambient glucose concentration is changed is of fundamental importance for glucose homeostasis [81]. In diabetes mellitus this ability is impaired with reduction in both first- and second-phase insulin secretion [82, 83] which leads to postprandial hyperglycemia. In the search for islet-derived factors responsible for the deranged insulin secretion, isolated islets have typically been cultured under different conditions, and it is well documented that elevated glucose concentrations (11 mM) during culture are essential for maintaining islet β-cell functions [84]. Individual islets from the NOD and *ob*/*ob* mouse, which are animal models of type 1 and type 2 diabetes, respectively [85, 86], have demonstrated improved glucose-stimulated insulin secretion (GSIS) after exposure to high glucose in culture medium [87, 88]. Such beneficial effects on GSIS have been correlated to changes in expression of individual proteins like glucokinase, glucose transporter 2, and uncoupling protein 2 [89–91]. However, molecular details of the phenotypic shift in response to elevated glucose are to a large extent unknown. Since GSIS is a multifactorial event, approaches capable of determining multiple proteins simultaneously are essential for the elucidation of molecular mechanisms responsible for changes in GSIS. 2DGE and MS have been employed to characterize changes in global islet protein expressions related to exposing islets to high glucose [71]. In this proteomic study, the prohormone convertase 2 and cytokeratin 8 appeared as distinct spots on 2D gels of islets exposed to high glucose, but the proteins were barely visible on gels of freshly isolated islets [71]. The observed glucose-induced changes in global protein expression pattern suggested that enhanced insulin synthesis, restoration of insulin content and granule pools, and increased chaperone activity and antioxidants are important mechanisms underlying the augmented secretory effect of glucose in mouse islets. In comparison to other discrete hypothesis-driven studies, this report, for first time, showed orchestrated changes of multiple islet proteins that may contribute to the enhanced GSIS observed in these islets [71]. From this

proteomic study, it is unclear how glucose-induced increase in cytokeratin interacts with kinesin–microtubule system and contributes, if any, in enhanced glucose responsiveness. However, it has been conjectured that kinesin-dependent interaction of cytokeratin with microtubules is mediated by the insulin granules where cytokeratins can interact with various lipids of the insulin granules, which are anchored to microtubules through kinesin interaction. In support of this view, oligonucleotide microarray studies showed an increase in cytokeratin 19 gene in pancreatic β-cells exposed to high (25 mM) glucose compared to low glucose (5.5 mM) for 24 h [92]. Increase in cytokeratins level in different cultured cells has also been reported [93, 94]. This type II cytoskeletal 8 protein (KRT8) has been detected on 2D maps of glucose-responding mouse islets, INS-1E cells [95], and human islets [72]. In search for glucose-responsive proteins, a 65 kDa protein has been detected on 2D map of mouse islets [96] and glucose-induced synthesis of this protein was blocked by D-mannoheptulose, a specific blocker of glucose phosphorylation and metabolism. However, isolation and characterization of this protein has not been performed. Among the 2,000 different islet protein spots, 1.5% was reported to be regulated by glucose in physiological concentration range [97]. In another study, depolarization induced Ca^{2+} influx and insulin release was found to be highly correlated with phosphorylation of a 60 kDa protein [98]. Identification of this phosphoprotein revealed an intermediate filament protein of the keratin class in hamster insulinoma cells and in pancreatic islets [99]. This cytokeratin protein exists in both phosphorylated and unphosphorylated state and corresponds to the gel position of KRT8 detected by Ahmed et al. [71, 72, 95]. The gel position of the unidentified glucose-responsive 65 kDa protein also matches with the KRT8. In support of the suggestion that cytokeratin may be involved in the regulation of insulin release, cytokeratins 7, 8, 18, and 19 were localized to adult endocrine pancreas and insulinoma cells by immunohistochemistry and immunoblot analysis [99–102], and it has been well documented that disturbances in cytoskeleton of the pancreatic β-cells drastically reduced their insulin secretory function and lifetime [103].

Comparative proteomics of glucose-responsive and glucose-nonresponsive MIN-6 cells using 2D-differential in-gel electrophoresis (DIGE) [104] also contributed to the understanding of the proteins involved in GSIS. Similar to the findings of Ahmed et al. [71], they showed that glucose-nonresponsive cells have lower ER chaperone proteins (e.g., PDI, GRP78, endoplasmin, endoplasmic reticulum protein 29) and decreased antioxidative enzymes (e.g., carbonyl reductase 3, peroxiredoxin 4, and superoxide dismutase 1) suggesting proper protein folding and protection against oxidative stress are required for glucose-stimulated insulin release from pancreatic β-cells. To dissect the molecular events associated with β-cell dysfunction and development of diabetes, Lu et al. [105] characterized global islet protein and gene expression changes in diabetic MKR mice and compared with nondiabetic control mice. Using iTRAQ, 159 proteins were found to be differentially expressed in MKR; marked up-regulation of protein biosynthesis and endoplasmic reticulum stress pathways and parallel down-regulation in insulin processing/secretion, energy utilization, and metabolism were observed. One hundred fifty-four of the differentially expressed proteins were able to be mapped to probe IDs

on the microarray. In this study about 45.2% of the differentiated proteins showed concordant changes (i.e., changes in the same direction) in mRNA, 0.6% were discordant (i.e., having higher protein expression but lower mRNA expression), and notably 54.2% showed changes in the proteome but not in the transcriptome. Similar approaches have been used for better understanding of the cellular and molecular functions of the signaling pathway of insulin synthesis and release in human β-cells [106]. Of the 97 differentially expressed proteins involved in improved insulin release, the changes in protein and mRNA expression for 49 proteins (50.5%) were in the same direction, while they moved oppositely for 14 proteins (14.4%). Thirty-four of the 97 differentially expressed proteins were identified by protein expression but not by mRNA expression. The proteomic and genomic data indeed supplement each other and suggest a posttranscriptional and/or posttranslational regulation of a substantial number of differentially expressed proteins is involved in islet function.

Imaging mass spectrometry (IMS) has been applied to identify differential expression of peptides in thin tissue section of pancreas of control and *ob*/*ob* mice [107]. Improvement and successful application of the IMS may lead to the discovery of new disorder-specific peptide biomarkers with potential applications in disease diagnosis. Protein expression profiling in fetal rat islets after protein restriction during gestation expanded our knowledge in the pathogenesis of type 1 and type 2 diabetes [108].

16.3.3 Glucolipotoxicity and Islet Proteomics

Whereas glucose is the most important physiological stimulus for insulin secretion, chronic hyperglycemia causes desensitization and impairment of insulin release in response to glucose [109–112]. Similarly, a high-fat intake, particularly if rich in saturated fatty acids, is associated with impaired insulin sensitivity and secretion and development of type 2 diabetes [113]. It is commonly accepted that acute exposure (1–3 h) of pancreatic islets to free fatty acid leads to stimulation of GSIS both in vitro [114–117] and in vivo [118–120]. However, the impact of long-term (>6 h) FFA exposure remains controversial [118, 121, 122]. The discrepancies may depend on the circulating free fatty acid levels and also on the percentage of unsaturation of the fatty acids [123, 124]. It has been proposed that an increased FFA concentration alone is insufficient to induce β-cell failure and that an elevation of FFAs combined with high glucose is required to result in β-cell malfunction [125, 126], possibly as a result of accumulation of harmful lipid metabolites, e.g., ceramide in the cytoplasm [127, 128]. This in turn is believed to interfere with the ability of the β-cells to respond to glucose with enhanced insulin secretion. Although the concept of glucolipotoxicity has become very popular and often debated, the underlying causes as well as functional consequences remain poorly defined. The main dietary fatty acids palmitate and oleate modulate the immediate early response genes, *c-fos* and *nur-77*, and a number of late genes of fatty acid metabolism including acetyl

CoA carboxylase and fatty acid synthase [129]. By analyzing global gene expression profiles in chronic fatty acid-treated MIN6 cells, it was found that the major groups of genes regulated by fatty acids are metabolic enzymes, transcription factors, and genes controlling distal secretory processes [130]. However, in another study long-term lipid infusion in normal rats showed little influence on broad spectrum of islet-associated genes [131]. A series of selected 'candidate genes' have also been studied recently [132]. The insulin (Ins1) and Glut2 transcript levels were significantly down-regulated in the presence of both palmitate and oleate. Transcription of the mitochondrial acyl-CoA transporter carnitine palmitoyltransferase I (CPT I) was up-regulated almost 4-fold. In contrast to previous findings [133–135], the uncoupling protein UCP-2 was up-regulated 2-fold in the presence of high glucose but no additional effect by FFAs was detected [132]. Therefore, it has been suggested that the failure of glucose to stimulate insulin secretion from FFA-pretreated islets is conceivably not due to increased uncoupling and reduced ATP generation [132]. However, conflicting opinion also exists since Western blot analysis indicates that high glucose and fatty acid synergistically impaired the production of ATP in β-cells through reduction of ATP synthase β-subunit protein expression [136]. Interestingly we have found that the expression of ATP synthase subunit α (1.21-fold) and ATP synthase subunit β (1.16-fold) was significantly increased ($p < 0.05$) in islets isolated from high-fat-fed mice (unpublished data). In this proteomic study, compared to control mice, islets from high-fat-fed mice showed differential expression of 1,008 proteins. In accordance with the previous findings of fatty acid-induced inhibition of insulin gene transcription [132], insulin-degrading enzymes [137] were highly over-expressed in islets isolated from high-fat-fed mice whereas both insulin 1 precursor and glucagon precursor were down-regulated. Top 10 down-regulated proteins in high-fat-diet islets include ARF (ADP ribosylation factor) GTPase-activating protein GIT1, flavin adenine dinucleotide (FAD) synthetase, CPT I, laminin subunit β2 precursor, γ-aminobutyric acid receptor subunit α-3, vesicle transport protein SEC20, reticulon 1, early endosome antigen 1, β-1,4-mannosyl-glycoprotein 4-β-N-acetylglucosaminyltransferase and tudor domain-containing protein 5. The largely down-regulated proteins include kelch-like protein 8, leucine-rich repeat containing protein 8D, transcription factor E3, ras-related protein Rab 11B, Na^+–K^+ ATPase subunit α2 precursor, putative ATP-dependent RNA helicase DHX33, SCHAD, F-actin capping protein subunit β, arylacetamide deacetylase, and type I inositol 3,4 bisphosphate 4 phosphatase. The vast amount of lipotoxicity proteomic data contains many novel proteins and opens new avenues for islet researchers. A recent SELDI-TOF analysis of INS-1E cells exposed to 0.5 mM palmitate for 48 h in the presence of high glucose (20 mM) identified calmodulin as palmitate-regulated protein (Sol EM, personal communication).

In a pioneering glucotoxicity proteomic study, Collins et al. [97] used 2DGE of ^{35}S-methionine-labeled islet proteins that were exposed in vivo or in vitro to either low or high glucose. Approximately 2,000 protein spots were detected on 2D gels and 1.5 and 1.6% detectable proteins showed differential expression in response to prolonged glucose load in vitro and in vivo model, respectively. Lack of mass spectrometry did not allow protein identification of those glucose-responsive proteins.

Schuit et al. [138], purified rat β-cells and performed 2DGE of ^{35}S-methionine-labeled proteins synthesized over 4 h at 10 mM glucose after 10 days culture in low (6 mM) or high (20 mM) glucose. They distinguished two patterns of β-cell proteome change between 6 and 20 mM glucose. In one pattern two spots corresponding to proinsulin were increased almost 9-fold in the presence of high glucose. Similar to this finding, on the 2DG map of INS-1E cells, proinsulin appeared as two spots. However, while one spot showed almost 2-fold up-regulation in the presence of high glucose (25 mM) the other spot was 5-fold down-regulated by high glucose compared to exposure to low glucose (5.5 mM, unpublished data). The other pattern described by Schuit et al. [138] showed suppression of translation of multiple spots close to pH 7 on 2D gels when the β-cells were exposed to 20 mM glucose. However, the identities of these protein spots were not determined. SELDI-TOF analysis of the different mitochondrial samples from INS-1E cells incubated for 5 days at 5.5, 11, 20, and 27 mM glucose showed 34 differentially expressed peaks among the samples [139]. Such changes in expression of proteins were correlated with impairment of GSIS. Nevertheless, no identification of the differentially expressed peptides has been carried out. Comparison of INS1-E mitochondrial 2DG proteome revealed 75 spots showing 2-fold or more significant changes ($p < 0.05$) in relative abundance in the presence of 20 mM glucose compared to the cells exposed to 5.5 mM glucose. Thirty-three protein spots appear only on the mitochondrial map of the INS-1E cells exposed to 5.5 mM glucose. Mitochondrial protein spots down-regulated in glucotoxic conditions include ATP synthase α chain and δ chain, stress-70 protein, mitochondrial (75 kDa glucose-regulated protein; GRP 75; HSPA9), malate dehydrogenase, aconitase, trifunctional enzyme β subunit and NADH-cytochrome b5 reductase, and voltage-dependent anion-selective channel protein (VDAC) 2. There were up-regulation of protein spots corresponding to heat shock protein 60, mitochondrial (HSP60) and 10 kDa heat shock protein, mitochondrial (HSP10). Typical to 2D map single protein appeared in multiple spots and several proteins co-migrated. For example, on the mitochondrial 2D map five different spots corresponding to VDAC1 appeared at same molecular weight but having different p*I*. Three spots showed over-expression in response to high glucose and two other spots were down-regulated. Changes in expression of a single isoform (spots) of a protein on 2DG do not necessarily signify alteration in total protein amount. Therefore, caution should be undertaken before concluding expression level of a protein on 2DG without validating the data with Western blot or other methods. In addition to the mitochondrial proteins, other differentially expressed proteins in glucotoxic condition includes proinsulin, calreticulin, protein disulfide isomerase A6 (PDIA6), PKC substrate 60.1 kDa protein, hypoxia up-regulated protein 1 (ORP150), endoplasmin, heat shock cognate 71 kDa protein (HSPA8), heterogeneous nuclear ribonucleoproteins D0 and A2/B1, lamin B1, histones H2B, H3.3, and H4 and elongation factor 1-α-1. With label-free LC-MS/MS approach 353 proteins were found to be differentially expressed in INS-1E cells exposed to 25 mM glucose compared to the cells cultured in the presence of 5.5 mM glucose (unpublished data). Ingenuity pathways analysis (IPA) revealed strong association of differentially expressed proteins with energy production, lipid

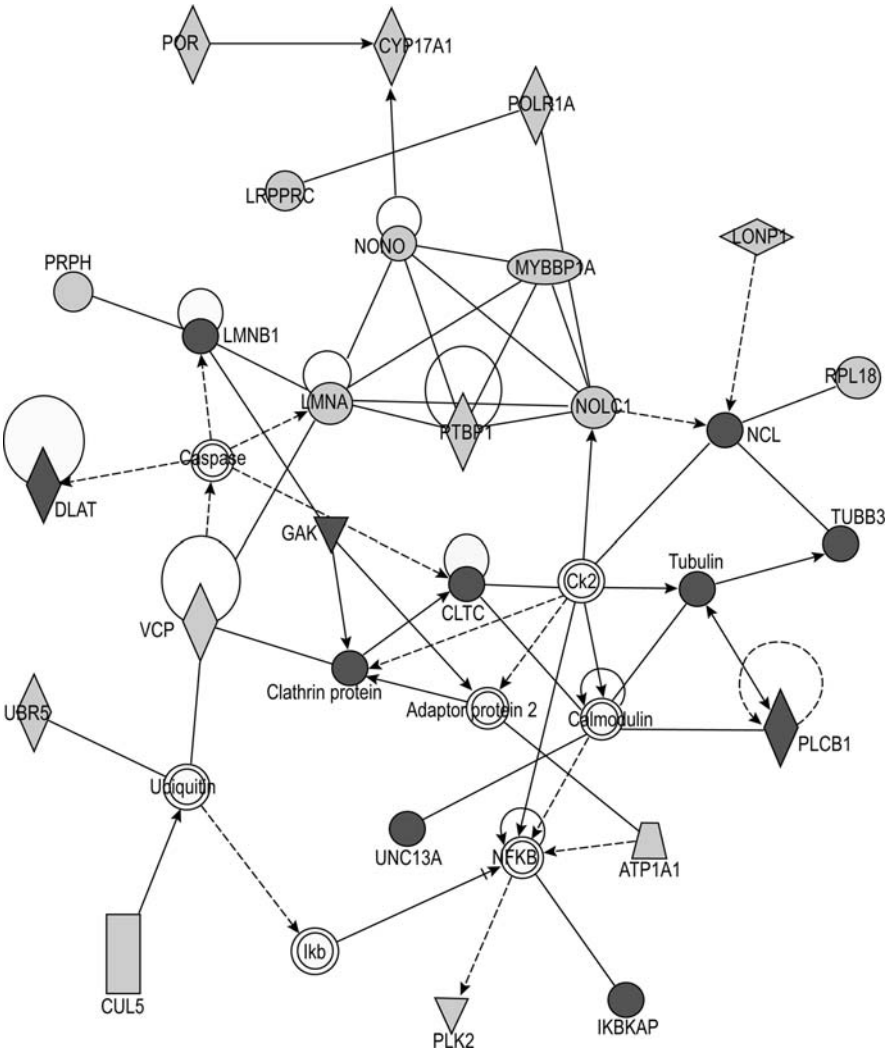
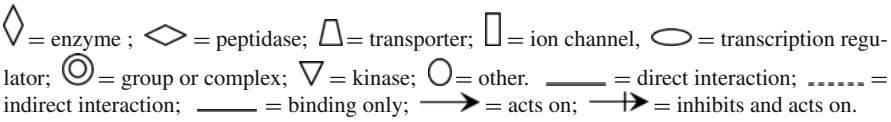


Fig. 16.3 Ingenuity pathway network obtained on a set of differentially regulated proteins detected in INS-1E cells exposed to 25 mM glucose compared to the cells cultured in the presence of 5.5 mM glucose. Proteins with a gray background were down-regulated by high glucose while other interacting proteins with a dark background were up-regulated. ATP1A1, ATPase, Na^+–K^+ transporting, α1 polypeptide; CK2, casein kinase 2; CLTC, clathrin, heavy chain; CUL5, cullin 5; CYP17A1, cytochrome P450, family 17, subfamily A, polypeptide 1; DLAT, dihydrolipoamide *S*-acetyltransferase; GAK, cyclin G-associated kinase; Ikb, inhibitor of nuclear factor of κ light polypeptide gene enhancer in B-cells, beta; IKBKAP, inhibitor of kappa light polypeptide enhancer in B-cells, kinase complex-associated protein; LMNA, lamin A/C; LMNB1, lamin B1; LONP1, lon peptidase 1, mitochondrial; LRPPRC, leucine-rich PPR-motif containing; MYBBP1A, MYB-binding protein (P160) 1a (p53-activated protein-2); NCL, nucleolin; NFKB, nuclear factor of κ light polypeptide gene enhancer in B-cells; NOLC1, nucleolar and

metabolism, protein synthesis, DNA replication, recombination and repair, cell signaling, and metabolic disease. Using IPA we mapped biological networks affected by the differentially expressed proteins between 5.5 mM and 25 mM glucose-exposed INS-1E cells. Figure 16.3 shows the network involved in endocrine system development and function, lipid metabolism, and small molecule biochemistry. In INS-1E cells exposed to 25 mM glucose, N-methylpurine DNA glycosylase (MPG) showed significant (> 2-fold) up-regulation while carboxypeptidase E (CPE) was 4-fold down-regulated. Other substantially down-regulated proteins in response to high glucose exposure included chromogranin A (CGA), membrane-associated guanylate kinase (MAGI1), ubiquitin protein ligase E3 component n-recognin 5 (UBR5), and mitofusin (MFN). Although fold change is a commonly used criterion in quantitative proteomics, it does not provide an estimation of false-positive and false-negative rates that are often likely in a large-scale quantitative proteomic analysis such as in label-free LC-MS/MS quantitation. It is therefore essential for the islet researchers to adopt effective significance analysis of proteomic data which is particularly useful in the estimation of false discovery rates [140]. The proteomic data from 2DG and LC-MS/MS analysis of the glucotoxic studies provide a comprehensive overview of the orchestrated changes in expression of multiple proteins involved in nutrient metabolism, energy production, nucleic acid metabolism, cellular defense, glycoprotein folding, molecular transport, protein trafficking, RNA damage and repair, DNA replication, apoptosis signaling, and mDNA stability. Farnandez et al. [141] have correlated proteomic data with metabolomic findings in glucotoxic conditions in INS-1 β-cell line. While 75 proteins showed differential expression in the presence of high glucose, only 5 of those proteins were found to be involved in the observed metabolomic alterations, suggesting allosteric regulation and/or posttranslational modifications are more important determinants of metabolite levels than enzyme expression at the protein level [141]. Combined SELDI-TOF and 2DGE approach identified 11 different proteins coupled to altered insulin release in response to high glucose (20 mM) (Sol EM, personal communication).

Fig. 16.3 (continued) coiled-body phosphoprotein 1; NONO, non-POU domain containing, octamer-binding protein; PLCB1, phospholipase C, β 1; PLK2, polo-like kinase 2; POLR1A, polymerase (RNA) I polypeptide A; POR, P450 (cytochrome) oxidoreductase; PRPH, peripherin; PTBP1, polypyrimidine tract-binding protein 1 (heterogeneous nuclear ribonucleoprotein I); RPL18, ribosomal protein L18; TUBB3, tubulin β-3; UBR5, ubiquitin protein ligase E3 component n-recognin 5; UNC13A, unc-13 homolog A; VCP, valosin-containing protein.



16.3.4 Type 1 Diabetes and Islet Proteomics

Type 1 diabetes (T1D) is an autoimmune disorder characterized by selective destruction of insulin-producing β-cells in the pancreas resulting from the action of environmental factors on genetically predisposed individuals [142]. The prevailing view for the pathogenesis of type 1 diabetes is that an autoimmune reaction, where cytokines play an important role, causes destruction of the β-cells [143]. Numerous reports have demonstrated both in rodent and in human islets that interleukin-1β (IL-1β) alone or in combination with interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α) affects the transcription and translation of genes, which have been implicated in β-cell destruction [144]. To search for novel proteins involved in cytokine-induced destruction of β-cells 2DGE has been used [145]. This approach has detected up-regulation of 29 proteins on 2DG image of rat islets exposed to IL-1β compared to control islets, and addition of nicotinamide reduced the up-regulation of 16 IL-1β-induced proteins [145]. In a subsequent study [146], on 2D gels of ^{35}S-methionine-labeled rat islets 52 spots were up-regulated, 47 down-regulated, and 6 synthesized de novo by IL-1β. Among these 105 differentially expressed proteins, 23 protein spots were found to be significantly affected when nitric oxide (NO) production was prevented, suggesting a major role of NO-independent IL-1β-mediated regulation of gene expression [147]. Mass spectrometric analysis allowed identification of 15 proteins, which were most profoundly altered by cytokine treatment [147]. Also, on the transcription level similar approaches have been employed to search for genes involved in the cytokine-induced alterations [148]. Both these powerful approaches have yielded important information about putative genes/proteins involved in the development of the disease. Larsen et al. [149] identified 57 different proteins from IL-1β-exposed rat islets and categorized them into several functional groups including (1) energy transduction; (2) glycolytic pathway; (3) protein synthesis, chaperones, and protein folding; and (4) signal transduction. Results of this differential expression analysis suggest that islet exposure to cytokines induces a complex pattern in β-cells comprising protective (e.g., up-regulation of stress proteins) as well as deleterious (e.g., iNOS induction and NO production) events [150]. The overall picture of the proteomic studies of type 1 diabetes is complex and do not allow us to predict which protein changes may be considered ‘primary’ or ‘secondary’ in importance, time, and sequence [149]. An integrative analysis method was developed combining genetic interactions using type 1 diabetes genome scan data and a high-confidence human protein interaction network [151]. Using this network analysis of the differentially expressed proteins in INS-1E cells exposed to cytokines, 42 of the differentially expressed proteins constituted a significant interaction network suggesting extensive cross talk between the different proteins and the pathways in which they are involved with some proteins such as the chaperones GRP78, HSPA8, and GRP75 and the RNA synthesis/turnover proteins placed at the center of different networks. In fact all these islet proteomic studies strongly suggest a protective role of the chaperones in regulating β-cell dysfunction.

16.3.5 Pharmacoproteomics and Pancreatic Islets

A potential application of proteomics in islet research is the detection of molecular alterations in diabetes and further characterization of existing or new drug [152]. One of the prime targets for the treatment of diabetes is to enhance the insulin sensitivity so that the tissues can precisely utilize glucose and keep its plasma level within physiological limit. Rosiglitazone, a member of the thiazolidinedione (TZD) class of antidiabetic agents, improves insulin sensitivity both in liver and in peripheral tissues. TZDs bind to and activate the peroxisome proliferator-activated receptor (PPARγ) and regulate the coordinated expression of multiple genes that integrate the control of energy, glucose, and lipid homeostasis, therefore contribute to increased insulin sensitivity. Rosiglitazone has been shown to prevent islet cell hyperplasia and protects islets from toxic agents [153–155]. In an elegant study using 2DGE, Sanchez et al. [156] compared protein expression profiles of pancreatic islets from obese diabetic C57BL/6J *lep*/*lep* mice and their lean littermates treated with rosiglitazone. They identified 9 differentially expressed proteins between lean and obese, diabetic, untreated mice. The expression levels of four of those nine proteins (tropomyosin 1, profilin, profilin fragment, and fatty acid-binding protein) were significantly modulated by rosiglitazone treatment of the obese mice. In a second set of experiments designed to identify proteins potentially associated with a low islet cell mass, they compared the islet protein expression between C57BL/6J and C57BL/Ks mice. The C57BL/Ks mice have a 2-fold less islet cell mass as compared with the C57BL/6J [157] and, as a consequence, were more susceptible to diabetes [158, 159]. Thirty-one proteins were found to be differentially expressed between the two mouse models and two of them, tropomyosin 1 and profilin, showed the same differential pattern between C57BL/Ks and obese diabetic C57BL/6J *lep*/*lep* mice. Taken together, these results suggest that actin-binding proteins could play an important role in defective islet function. We have a long way to go for the development of novel actin-modulating drugs for treatment of diabetes similar to microtubule-interacting or microtubule-stabilizing drugs developed for cancer treatment [160, 161]. In a recent study, the effects of imidazolines have been tested on rat islet proteome [162] with the optimism that if it were possible to develop one of the them into a drug. This compound may be effective without risk of insulin shock from hypersecretion in subjects with low or normal blood glucose as imidazolines increase insulin release selectively at high glucose concentrations [163]. The 2DG analysis revealed 53 differentially expressed proteins between imidazoline-treated and imidazoline-nontreated islets. Of special interest among the differentially expressed proteins are those involved in protecting cells from misfolded proteins (HSP60, PDI, and calreticulin), Ca^{2+} binding (calgizzarin, calcyclin, and annexin A1), and metabolism or signaling (pyruvate kinase, α enolase, and protein kinase C inhibitor 1). However, elucidation of exact mechanism of action of imidazolines and validation of targets require further studies.

Natural medicinal plant extracts and active components have antidiabetic activity [164], and the extracellular polysaccharides (EPS) obtained from mycelia culture

of *Phellinus baumii* has strong hypoglycemic activity. Proteomic study provided insights into the mechanism of antidiabetic activity of the EPS in type 1 diabetes [165]. The 2DGE image analysis and mass spectrometry identified 10 down-regulated and 16 up-regulated proteins in streptozotocin-treated diabetic mice islets. The altered level of all these differentially expressed proteins was partially or fully restored to normal level by EPS treatment. The interesting down-regulated proteins in diabetic model include cholesterol esterase, PDI and islet regenerating protein, whereas the up-regulated proteins were Cu/Zn superoxide dismutase, carbonyl reductase, GRP58, hydroxymethylglutaryl-CoA synthase, similar to α glucosidase II, α subunit, and putative human mitogen-activated protein kinase activator with WD repeats-binding protein. One advantage of this study is that the proteomic data was indeed supported by transcriptomics. It would be interesting to know how alteration of certain specific protein targets modulates the development and progress of type 1 diabetes. In a recent study, using proteomic approaches it has been demonstrated that Rho-GDI-α/JNK pathway might be the focus of therapeutic target for the prevention of mycophenolic acid-induced islet apoptosis [166].

16.4 Conclusion

During the last decade state-of-the-art proteomic technologies including the 2DGE and label-free LC-MS/MS quantitation have been applied to dissect the pathophysiology of islet function in an increasingly manner. A vast array of proteomics data has emerged from these studies providing molecular and comprehensive snapshot of complex disease process involving the pancreatic islet cells – but just like a trace of light through an age-old dark cave, coming from the gleaming endless ocean. Careful analysis and powerful bioinformatic tools are still required for functional summary of the data sets and generation of novel hypothesis. These proteomic studies are indeed very early steps toward better understanding of the mechanism of pathophysiology of diabetes and providing new approaches for the prevention and treatment of the disease. Almost no functional proteomics has been performed in islet research. However, improvement and easy availability of high-throughput proteomic techniques will hopefully draw the attention of more islet biologist and generate significant functional data. An important feature of diabetes is that it is a progressive condition. Pancreatic β-cell function, in particular, shows a progressive decline in the pre-diabetic phase and in established diabetes. To clearly define islet function, therefore, we need to measure it over a period of time amalgamating multiple platforms and involving cell biologists, physiologists, geneticists, and biochemists working together with proteomics specialists. A large-scale study will allow this, together with the detection of changes in islet protein patterns and other metabolic traits will lead to a better understanding of how susceptible gene variants and their protein products predispose to diabetes. This will also help to explore novel biomarkers to predict future diabetes, for better understanding of the pathophysiology of diabetes, to reveal drug targets, as well as to optimize the selection of molecules that interact with these targets.

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Chapter 17
Wnt Signaling in Pancreatic Islets

Zhengyu Liu and Joel F. Habener

Abstract The Wnt signaling pathway is critically important not only for stem cell amplification, differentiation, and migration, but also is important for organogenesis and the development of the body plan. Beta-catenin/TCF7L2-dependent Wnt signaling (the canonical pathway) is involved in pancreas development, islet function, and insulin production and secretion. The glucoincretin hormone glucagon-like peptide-1 and the chemokine stromal cell-derived factor-1 modulate canonical Wnt signaling in β-cells which is obligatory for their mitogenic and cytoprotective actions. Genome-wide association studies have uncovered 19 gene loci that confer susceptibility for the development of type 2 diabetes. At least 14 of these diabetes risk alleles encode proteins that are implicated in islet growth and functioning. Seven of them are either components of, or known target genes for, Wnt signaling. The transcription factor TCF7L2 is particularly strongly associated with risk for diabetes and appears to be fundamentally important in both canonical Wnt signaling and β-cell functioning. Experimental loss of TCF7L2 function in islets and polymorphisms in TCF7L2 alleles in humans impair glucose-stimulated insulin secretion, suggesting that perturbations in the Wnt signaling pathway may contribute substantially to the susceptibility for, and pathogenesis of, type 2 diabetes. This review focuses on considerations of the hormonal regulation of Wnt signaling in islets and implications for mutations in components of the Wnt signaling pathway as a source for risk-associated alleles for type 2 diabetes.

17.1 The Diabetes Problem

The prevalence of diabetes mellitus and its accompanying complications is increasing in populations throughout the world [1]. Diabetes results from a deficiency of the β-cells of the islets of Langerhans to produce insulin in amounts sufficient to meet

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the body's needs, either absolute deficiency (type 1 diabetes) or relative deficiency (type 2 diabetes). In type 2 diabetes the remaining β-cells are placed under stress by (1) being forced to overproduce insulin to compensate for the lost β-cells, (2) insulin resistance, and (3) by the glucotoxic effects of prolonged, sustained hyperglycemia. In the USA, 20 million individuals are currently afflicted with some form of diabetes, while an estimated 12 million additional people in the USA have diabetes but do not know it yet [2]. Worldwide, an estimated 190 million people have the disease and this global figure is expected to skyrocket to 366 million by 2030 [3]. Type 2 diabetes is the most prevalent form of diabetes comprising >90% of all diabetes. Most individuals who develop type 2 diabetes do so in association with obesity [4]. Because a common feature of both type 1 and type 2 diabetes is a reduction in β-cell mass, understanding the factors and the cellular mechanisms that govern β-cell growth and survival may lead to new effective treatments for diabetes.

In adult rats and mice the entire mass of the β-cells in the pancreas turns over approximately every 50 days (2–3% per day) by processes of apoptosis counterbalanced by replication from existing β-cells and neogenesis from progenitor cells believed to be located in the pancreatic ducts and possibly within the islets [5–7]. The adult pancreas of rodents, including the endocrine islets, has a substantial capacity for regeneration [8]. Rodent models of pancreatic injuries are followed by partial to nearly complete regeneration of the exocrine and endocrine pancreas. Such models of pancreas regeneration include partial pancreatectomy [9], streptozotocin-mediated ablation of the β-cells [10, 11], duct ligation, and caerulein treatments [12]. However, it remains controversial whether progenitors exist in the adult pancreas. A slow cycling, multi-potent stem cell in the pancreas has not yet been identified convincingly. Compelling evidence found that the majority of new β-cells derive from preexisting insulin-expressing cells after partial pancreatectomy [13], but recent evidence suggested that another form of surgical injury duct ligation activates Ngn3-positive β-cell precursors in the ductal epithelium [14]. Therefore, the activation of adult pancreatic progenitors might depend on the specific experimental model.

Genome-wide scans of several large populations of diabetic cohorts have begun to uncover some of the genes associated with type 2 diabetes [15–20]. Of note, the majority of the candidate genes identified thus far appear to be involved in islet functions, and most notably, the insulin-producing β-cells in the islets [19, 20]. Furthermore, as discussed later in this chapter, several of these genes appear to be involved in the Wnt signaling pathway; either components of the Wnt signaling system itself or target genes for downstream Wnt signaling by beta-catenin and TCF7L2. The Wnt signaling pathway may be involved in the dysfunction of β-cells in type 2 diabetes [21]. Attention is directed to recent reviews on the role of Wnt signaling in pancreas development and function [18–20] and the importance of the transcription factor TCF7L2 in pancreatic islet function and diabetes [20, 25–36]. In this review evidence is considered for the regulation of islet β-cell functions by beta-catenin/TCF7L2 induced by glucagons-like peptide-1 and stromal cell-derived factor-1. Speculations are presented on the potential involvement of the Wnt signaling pathway in the genetic predisposition to type 2 diabetes.

17.2 Wnt Signaling Pathways

The Wnt signaling cascade controls several cellular functions, including differentiation, proliferation, and migration [37–43]. Useful brief summaries of the Wnt signaling pathways are provided in [44] and [45]. The Wnt proteins form a large family of cell-secreted factors that control diverse aspects of development and organogenesis. Wnt proteins exert their effect by binding to cell surface G protein-coupled Frizzled (Fz) receptors and the lipoprotein receptor-like proteins, LRP5/6 co-receptors, and modulate the expression of various target genes through a series of intracellular processes ultimately leading to the regulation of transcription. There are currently several recognized Wnt signaling pathways: the beta-catenin-dependent, so-called canonical Wnt pathway that is dependent on the activation of the transcriptional complex of proteins consisting of beta-catenin and TCF/LEF (Fig. 17.1) and several (at least nine) distinct and complex beta-catenin, TCF/LEF-independent, noncanonical pathways (Fig. 17.2, Ref. [41]).

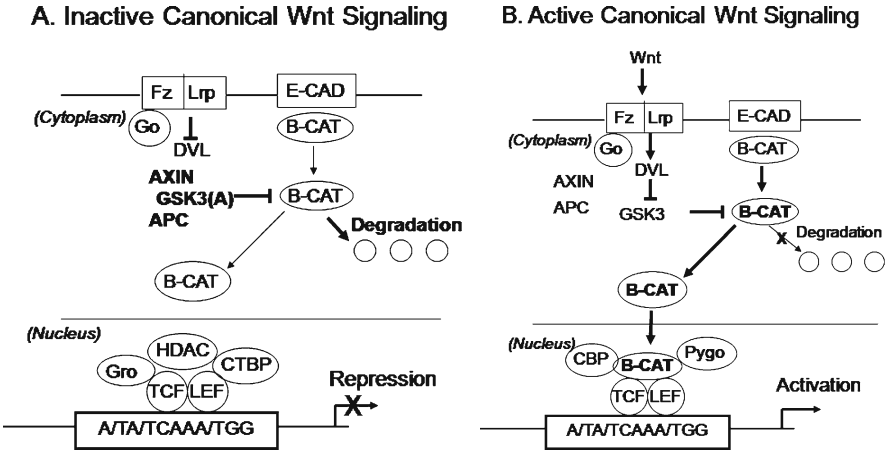

Fig. 17.1 Models depicting the canonical, beta-catenin/TCF/LEF-dependent Wnt signaling pathway in inactive and active states. **A**. Inactive Wnt signaling. In the absence of Wnt ligand-mediated activation of its receptor frizzled (Fz), beta-catenin in the cytoplasm is phosphorylated by the protein kinases glycogen synthase kinase-3beta (GSK3beta) and casein kinase Ialpha (CKIa) leading to its degradation by proteasome complexes. GSK3beta and CKIalpha are constitutively activated by the cofactors adenomatous polyposis coli (APC) and Axin that along with GSK3beta and CKIalpha are known as the destruction complex. In the absence of sufficient levels of cytosolic beta-catenin, nuclear levels are depleted and the DNA-binding transcription factors TCF and LEF act as repressors of gene transcription by the recruitment of corepressors such as Groucho and CtBP. **B**. Active Wnt signaling. In the presence of Wnt ligands Fz is activated via G protein G alpha i/o and small GTPases leading to the activation of disheveled (DVL) that disrupts the destruction complex composed of GSK3, CKI, APC, and Axin, thereby inhibiting the activities of GSK3 and CKI. In the absence of phosphorylation, unphosphorylated beta-catenin is stabilized, translocated to the nucleus where it non-covalently associates with TCF/LEF DNA-binding proteins, recruits coactivators such as CBP and Pygo resulting in the activation of gene transcription

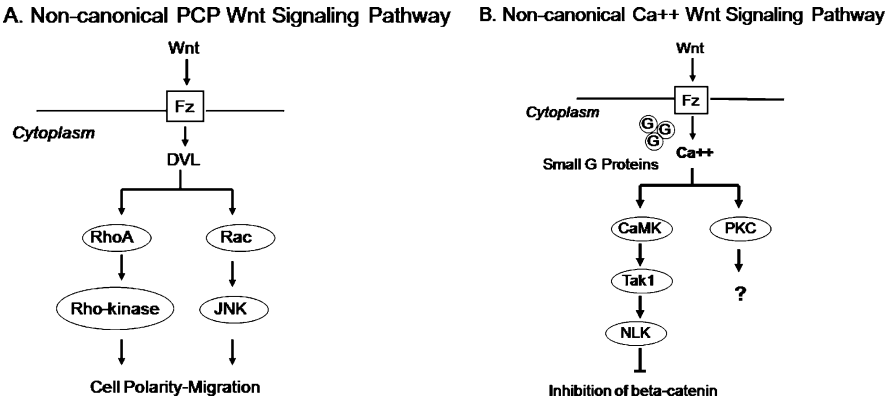

Fig. 17.2 Models depicting noncanonical beta-catenin-independent Wnt signaling pathways. **A**. The planar cell polarity (PCP) pathway. The activation of Fz by Wnts leads to the activation of DVL and small G proteins such as rhoA and Rac and the kinases Rho-kinase and Jun kinase (JNK). Through as yet undefined pathways Rho-kinase and JNK modulate changes in the cytoskeleton involved in cell migration and polarity. **B**. The Ca^{2+} pathway. Wnt ligands such as Wnt 5a activate Ca^{2+}-activated calmodulin kinases. CaMK and downstream kinases TAK1 and NLK. This pathway inhibits the canonical beta-catenin-dependent Wnt signaling pathway and is active during gastrulation. The Ca^{2+} pathway also activates protein kinase C (PKC)

17.2.1 The Canonical Wnt Signaling Pathway

The downstream canonical Wnt signaling pathway is defined as the pathway that ends in the formation of active, productive transcriptional transactivation complexes composed of beta-catenin and the DNA-binding proteins TCF (T-cell factor) and LEF (lymphocyte enhancer factor) (Fig. 17.1). It involves beta-catenin that when stabilized translocates to the nucleus where it associates with the TCF/LEF family of transcription factors to regulate the expression of canonical Wnt target genes. In the absence of a Wnt signal, beta-catenin is efficiently captured by the scaffold protein Axin, which is present within a protein complex (referred to as the destruction complex) that also harbors adenomatous polyposis coli (APC), glycogen synthase kinase (GSK)-3, and casein kinase 1 (CSNK1) (Fig. 17.1a). The resident CSNK1 and GSK3 protein kinases sequentially phosphorylate conserved serine and threonine residues in the N-terminus of beta-catenin subsequently targeting it for ubiquitination and degradation. The efficient suppression of beta-catenin levels ensures that Groucho proteins are free to bind members of the lymphocyte enhancer factor (LEF)/T cell factor (TCF) family of transcription factors occupying the promoters and enhancers of Wnt target genes in the nucleus. These transcriptionally repressive complexes actively suppress the Wnt target genes such as c-Myc and cyclin D1, thereby silencing an array of biological responses, including cell proliferation. Rapid activation of the canonical pathway occurs when Wnt proteins interact with specific receptor complexes comprising members of the Frizzled family of proteins and the low-density lipid co-receptor LRP5 or LRP6 (Fig. 17.1b). The

ligand-receptor binding activates the intracellular protein, Disheveled (Dvl), which inhibits APC-GSK3beta-axin activity and subsequently blocks degradation of beta-catenin. This stabilization of beta-catenin allows it to accumulate and translocate to the nucleus where it forms a transcriptionally active complex with the DNA-binding TCF transcription factors to activate the expression of Wnt signaling target genes. In pancreatic β-cells TCF7L2 is a major form of TCF involved in downstream Wnt signaling responsible for the activation of growth-promoting genes in response to glucagon-like peptide-1 (GLP-1) agonists [46, 47]. Notably, TCF7L2 has recently been found to be a major susceptibility factor for the development of T2D manifested by diminished insulin production [24, 25, 30, 32, 33, 48].

17.2.2 Noncanonical Wnt Signaling

Wnt signaling via frizzled receptors can also lead to the activation of noncanonical pathways that are independent of beta-catenin and TCF/LEF complexes [45]. Two of the several recognized [45] beta-catenin-independent pathways are considered (Fig. 17.2). One such noncanonical pathway consists of the release of intracellular calcium. Other intracellular second messengers associated with this pathway include heterotrimeric G proteins, phospholipase C (PLC), and protein kinase C (PKC). The Wnt/Ca^{2+} pathway is important for cell adhesion and cell movements during gastrulation [49]. The Wnt/Ca^{2+} pathway is also known to control cell migration and is involved in regulating endothelial cell migration. Interestingly, the Wnt/Ca^{2+} pathway may antagonize the canonical Wnt/beta-catenin pathway. The canonical and noncanonical Wnt pathways are likely to have opposing effect on endothelial cells and probably antagonize each other in order to finely balance endothelial cell growth.

The WNT/planar cell polarity (PCP) signaling pathway is a second noncanonical Wnt signaling pathway [49, 50, 51]. PCP controls tissue polarity and cell movement through the activation of RHOA, c-Jun N-terminal kinase (JNK), and nemo-like kinase (NLK) signaling cascades. In the planar cell polarity pathway Wnt signaling through frizzled receptors mediates asymmetric cytoskeletal organization and the polarization of cells by inducing modifications to the actin cytoskeleton.

17.3 Wnt Signaling in Pancreas Development and Regeneration

Expression of components of the Wnt signaling pathway, including Wnt ligand family members and various frizzled receptors, is well documented in the developing mouse, rat, chick, fish, and human pancreas [52–56]. A description of the subsets of the dozen or so Wnt ligands, Frizzled receptors, and the Wnt/FZ regulators, secreted frizzle-related proteins, and dickkopfs is provided in Heller et al. [52]. Endogenous Wnt signaling also occurs in mouse and rat β-cell lines [46]. Detailed information on the cellular distributions of expression of the various Wnt ligands, receptors, and

regulators is not available. From the findings of Heller et al. [52] it is clear that Wnt signaling factors are expressed both in epithelium and in mesenchyme. Several studies confirm that functional Wnt signaling is active in islets throughout development. A Wnt reporter strain of mice, in which lacZ was inserted into the locus of the Wnt target gene conductin/axin2, expressed beta-galactosidase, the product of the LacZ gene, throughout the islets [57]. Expression of the conductin gene is transcriptionally activated by the canonical Wnt pathway via TCF binding sites in its promoter. Furthermore, the beta-galactosidase (LacZ) reporter activity is maintained in islets of mice up to 6 weeks after birth. A monoclonal antibody specific for the non-phosphorylated form of beta-catenin revealed a strong immunoreactivity in the pancreatic epithelium of the mouse at embryonic day 13 [58]. Taken together, human and rodent islets and rodent β-cell lines are known to express members of the Wnt ligand and frizzled receptors families, along with modulators of Wnt signaling, the LRP co-receptors, and secreted Dkk (dickkopf) proteins.

Another source of Wnt ligands is adipose tissue [59]. Adipocytes secrete a wide range of signaling molecules including Wnt proteins. Fat cell-conditioned media from human adipocytes increases the proliferation of INS-1 β-cell and induces Wnt signaling, which could contribute to the β-cell hyperplasia that occurs in humans and rodents in response to obesity. Interestingly, inhibitory noncanonical Wnt ligand Wnt5b gene is associated strongly with obesity and type 2 diabetes [59]. Expression of Wnt5b in preadipocytes increases adipogenesis and the expression of adipokine genes through the inhibition of canonical Wnt signaling [59]. Thus, alterations in Wnt5b levels in humans could alter adipogenesis and, consequently, affect the risk of diabetes onset.

17.3.1 Wnt Signaling Loss-of-Function Studies

Following early pancreas specification, Wnt signaling appears to be indispensable for pancreas development, although its precise role remains controversial. The majority of studies have shown that Wnt signaling is essential in the development of the exocrine pancreas. Disruption of the Wnt signaling pathway results in an almost complete lack of exocrine cells [57, 58, 60, 61]. However, its role in endocrine cell development is still uncertain. Several studies in which Wnt signaling is abolished by conditional beta-catenin knockout in the developing mouse pancreas have revealed that the endocrine component of the pancreas develops normally and is functionally intact in the studies of Murtaugh et al. [60] and Wells et al. [61] in which the beta-catenin gene in the epithelium of the pancreas and duodenum was specifically deleted, pancreatic islets are intact and contain all lineages of endocrine cells. In contrast, using a different beta-catenin knockout approach Dessimoz et al. [57] found a reduction in endocrine islet numbers. It is worth noting that knockout studies should be interpreted with some caution because of the potential occurrence of adaptive compensatory mechanisms that could alter the phenotype. Furthermore, the use of different strains of mice expressing PDX-Cre, which have different

recombination efficiencies, are expressed at different stages of development and are shown to have mosaic expression in the pancreata of transgenic mice [62]. It seems possible that beta-catenin and Wnt signaling have several different roles throughout the development of the pancreas. Since the timing of the activation or inactivation of Wnt signaling is crucial for its effects on pancreas development, the currently available Cre-based recombinant technology might not be adequate to fully explore the role of Wnt signaling. Collectively, the loss-of-function studies have not yet provided a definitive role for beta-catenin in the development and/or maintenance of function of adult islets. Nonetheless, these results underscore the possible dual nature of Wnt signaling in pancreas growth and development. Excessive Wnt signaling activation prevents proper differentiation and expansion of early pancreatic progenitor cells during early, first transition specification. During the second transition, beta-catenin acts as a pro-proliferative cue that induces gross enlargement of the exocrine and/or endocrine pancreas.

17.3.2 Wnt Signaling Gain-of-Function Studies

Gain-of-function experiments suggest an inhibitory role for Wnt pathway in pancreas specification, a stage when cells at the appropriate regions of the foregut begin to form a bud. Heller et al. [52] showed that forced misexpression of Wnt1 driven by PDX-1 promoter in mice induces a block in the expansion and differentiation of PDX-1-positive cells and causes ensuing reduction in endocrine cell number and a lack of organized islet formation. Excessive Wnt signaling in the epithelia limits the expansion of both the mesenchyme and the epithelium and inhibits growth of the pancreas and islets. Using a different approach, Heiser et al.'s [62] study reached a similar conclusion. The conditional knock-in of stable beta-catenin in early pancreatic development of mice using PDX-1-driven Cre recombinase efficiently targets all three pancreatic lineages – endocrine, exocrine, and duct – and results in up-regulation of Hedgehog and leads to a loss of PDX1 expression in early pancreatic progenitor cells [62]. This genetic model of forced over-expression of beta-catenin prevents normal formation of the exocrine and endocrine compartments of the pancreas. Using a Xenopus model, McLin et al. [63] found that forced Wnt/beta-catenin signaling in the anterior endoderm, between gastrula and early somite stages, inhibits foregut development. By contrast, blocking beta-catenin activity in the posterior endoderm is sufficient to initiate ectopic pancreas development [62]. These genetic manipulations of Wnt signaling in mice suggest a contribution of both inhibitory and facilitating roles of Wnt signaling during pancreas development. The gain-of function studies by Dessimoz et al. [57] show a distinctive role of Wnt signaling in endocrine development. Wnt3A induces the proliferation of islet and MIN-6 cells [64]. The addition of the soluble Wnt inhibitor, Fz 8-cysteine-rich domain (Fz8-CRD), eliminated this stimulatory effect of Wnt3a on cell proliferation [64]. The treatment of islets with Wnt3a significantly increased mRNA levels of cyclin D1, cyclin D2, and CDK4, all of which have Wnt-responsive elements in the promoter regions of their genes [56]. Conditional knock-in of active

beta-catenin in mice promotes the expansion of functional β-cells [62] whereas the conditional knock-in of the Wnt inhibitor Axin impaired proliferation of neonatal β-cells [64].

Surprisingly, recent studies found that Wnt signaling may play a role in regulating the secretory function of mature β-cells [65]. The Wnt co-receptor, LRP5, is required for glucose-induced insulin secretion from the pancreatic islets. The knockout of LRP5 in mice resulted in glucose intolerance [65]. Treatment of isolated mouse islets with purified Wnt3a and Wnt5a ligands causes potentiation of glucose-stimulated insulin secretion. Thus, LRP5 together with Wnt proteins appear to modulate glucose-induced insulin secretion. Furthermore, Schinner et al. [59] reported that activating Wnt signaling increases insulin secretion in primary mouse islets and activates transcription of the glucokinase gene in both islets and INS-1 cells. The consummate evidence came in isolated mouse and human islets, in which reducing levels of TCF7L2 by siRNA decreases glucose-stimulated insulin secretion, expression of insulin and PDX-1, and insulin content [47, 66, 67].

17.4 Role of Wnt Signaling in β-Cell Growth and Survival

In addition to its potential role in regulating glucose-stimulated insulin secretion, the Wnt pathway is involved in β-cell growth and survival. The activation of Wnt signaling in β-cell lines or primary mouse islets results in an expansion of the functional β-cell mass, findings consistent with the up-regulation of pro-proliferative genes including cyclin D1 and D2 [46]. Furthermore, the misexpression of a negative regulator of Wnt signaling, axin, impairs the proliferation of neonatal β-cells, demonstrating a requirement for Wnt signaling during β-cell expansion [64]. Axin expression impaired normal expression of islet cyclin D2 and pitx2, a transcriptional activator that directly associates with promoter regions of the cyclin D2 gene. Shu et al. [47] provide further evidence in support of a role for Wnt signaling in β-cell growth and survival in both mouse and human islets. Depletion of TCF7L2 in human islets causes a decrease in β-cell proliferation, an increase in levels of apoptosis, and a decline in levels of active Akt, an important β-cell survival factor [46]. Similarly, in INS-1 cells, expression of dominant-negative TCF7L2 decreases proliferation rates [46]. Furthermore, over-expression of TCF7L2 in both mouse and human islets protects β-cells against glucotoxicity or cytokine-induced apoptosis [47].

17.5 Roles of Non-Wnt Hormonal Ligands in the Activation of the Wnt Signaling Pathway in Islets

Several hormones and growth factors, such as insulin, insulin-like growth factor-1, platelet-derived growth factor, parathyroid hormone, and prostaglandins, are known to activate the canonical and noncanonical Wnt signaling pathways. However, these

observations have been made in non-islet tissues such as intestine, cancer cell lines, osteoblasts, and fibroblasts [68]. It has been proposed that a primary function of Wnt signaling is to maintain stem cells in a pluripotent state and that growth factors such as FGF and EGF augment their proliferation [69]. Very little is known, however, about the hormonal activation of Wnt signaling in pancreatic islets. Recent studies of glucagon-like peptide-1 (GLP-1) and stromal cell-derived factor-1 (SDF-1) actions on islet β-cell demonstrate that both hormones activate downstream Wnt signaling via beta-catenin/TCF7L2-regulated gene transcription and that downstream Wnt signaling is required for the pro-proliferative actions of GLP-1 [46] and the anti-apoptotic actions of SDF-1 [70].

17.5.1 Downstream Wnt Signaling Requirement for GLP-1-Induced Stimulation of β-Cell Proliferation

Glucagon-like peptide-1 (GLP-1) is a glucoincretin hormone released from the intestines in response to meals and stimulates glucose-dependent insulin secretion from pancreatic β-cells [71, 72]. GLP-1 also stimulates both the growth and the survival of β-cells. GLP-1 is produced in the enteroendocrine L-cells that reside within the crypts of the intestinal mucosa by selective posttranslational enzymatic cleavages of the prohormonal polypeptide, proglucagon, the protein product of the expression of the glucagon gene (Gcg). Notably, the same proglucagon expressed from Gcg in the α-cells of the pancreas is alternatively cleaved to yield the hormone glucagon, rather than GLP-1. Glucagon functions as an insulin counter-regulatory hormone to stimulate hepatic glucose production and thereby to maintain blood glucose levels in the postabsorptive, fasted state.

Genes expressed in Wnt signaling in β-cells were examined using a focused Wnt signaling gene microarray and the clonal β-cell line INS-1 [46]. Of the 118 probes represented on the Wnt signaling gene array, 37 were expressed above background in cultured INS-1 cells. Exposure of the cells to GLP-1 enhanced the expression of 14 of the genes, including cyclinD1 and c-myc, strongly suggesting that GLP-1 agonists activate components and target genes of the Wnt signaling pathway. GLP-1 agonists activate beta-catenin and TCF7L2-dependent Wnt signaling in isolated mouse islets and INS-1 β-cells and antagonism of beta-catenin by siRNAs and of TCF7L2 by a dominant negative form of TCF7L2-inhibited GLP-1-induced proliferation [46]. These findings suggest that Wnt signaling is required for GLP-1-stimulated proliferation of β-cells. Although INS-1 cells maintain high basal levels of Wnt signaling via Wnt ligands and Frizzled receptors, GLP-1 agonists specifically enhance Wnt signaling through their binding to the GLP-1 receptor (GLP-1R), a G protein-coupled receptor coupled to GalphaS and the activation of cAMP-dependent protein kinase A (PKA). Although PKA is not involved in maintaining basal levels of Wnt signaling, it is essential for the enhancement of Wnt signaling by GLP-1 [46]. In addition, the pro-survival protein kinase Akt, along with active MEK/ERK signaling, is required for maintaining both basal- and GLP-1-induced

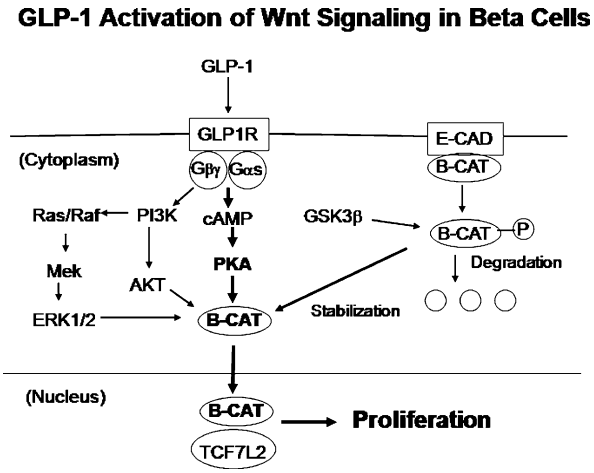

Fig. 17.3 Diagram summarizing the signaling pathway in pancreatic β-cells by which GLP-1 actions couple to the downstream Wnt signaling pathway [37]. The interaction of GLP-1 with the GLP-1 receptor (GLP-1R) activates G protein alpha S (GalphaS) resulting in cAMP formation and activation of the cAMP-dependent protein kinase A (PKA). Remarkably, by the GLP-1-activated pathway beta-catenin is stabilized by direct phosphorylation by PKA, rendering it resistant to degradation in response to phosphorylations by GSK3beta. This stabilization of beta-catenin by PKA-mediated phosphorylation is a distinct departure from the canonical Wnt pathway in which phosphorylation of beta-catenin by GSK3beta results in its degradation. Beta-catenin thus stabilized by PKA-mediated phosphorylation is resistant to degradation in response to phosphorylation by GSK3beta, accumulates in the cytoplasm, and is translocated to the nucleus where it associates with TCF7L2 to form a productive transcriptional activation complex. Beta-catenin/TCF7L2 complexes activate the expression of target genes involved in β-cell proliferation

Wnt signaling [46] (Fig. 17.3). In summary, both beta-catenin and TCF7L2 appear to be required for GLP-1-mediated transcriptional responses and cell proliferation.

17.5.2 Downstream Wnt Signaling Requirement for SDF-1-Induced Promotion of β-Cells Survival

SDF-1 is a chemokine originally identified as a bone marrow (BM) stromal cell-secreted factor and now recognized to be expressed in stromal tissues in multiple organs [73–76]. The most extensively studied function of the SDF-1/receptor CXCR4 axis is that of chemoattraction involved in leukocyte trafficking and stem cell homing in which local tissue gradients of SDF-1 attract circulating stem/progenitor cells. SDF-1/CXCR4 signaling in the pancreas remains relatively unexplored. Kayali and coworkers reported expression of SDF-1 and CXCR4 in the fetal mouse pancreas and CXCR4 in the proliferating duct epithelium of the regenerating pancreas of the nonobese diabetic mouse [77]. The cross talk between the

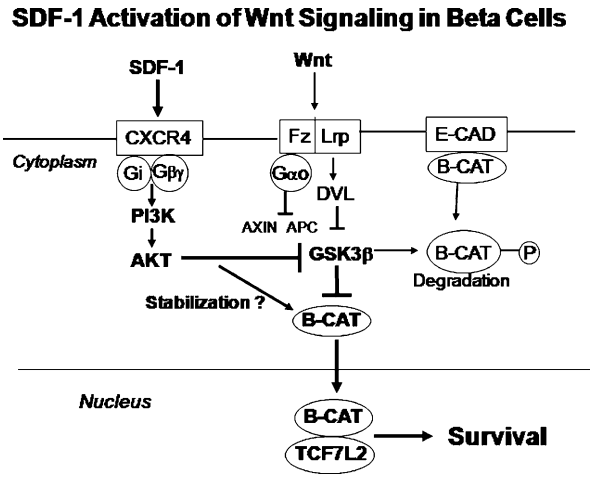

Fig. 17.4 Schematic model of signaling pathways utilized by SDF-1/CXCR4 in the activation of beta catenin/TCF7L2-mediated transcriptional expression of genes involved in β-cell survival. Interactions of SDF-1 with its G protein-coupled receptor CXCR4 activates G protein i/o that activates the phosphoinositol kinase 3 (PI3K) and the downstream pro-survival kinase Akt. Akt is a potent inhibitor of the Wnt signaling destruction complex composed of Axin, APC, and GSK3beta. Inhibition of GSK3beta by Akt results in the inhibition of phosphorylation of beta-catenin by GSK3, prevents the degradation of beta-catenin, and thereby results in the stabilization of beta-catenin which accumulates in the cytoplasm, enters the nucleus, where it associates with TCF7L2. The beta-catenin/TCF7L2 forms a transcriptional activation complex that activates the expression of genes that promote β-cell survival. A direct action of Akt on the stabilization of beta-catenin remains conjectural

SDF-1-CXCR4 axis and the Wnt signaling pathway was first demonstrated by Luo et al. [78] in studies of rat neural progenitor cells. Transgenic mice expressing SDF-1 in their β-cells (RIP-SDF-1 mice) are protected against streptozotocin-induced diabetes through activation of the pro-survival protein kinase Akt and resulting downstream pro-survival, anti-apoptotic signaling pathways [79]. An examination of SDF-1-activated Wnt signaling in both isolated islets and INS-1 cells using a beta-catenin/TCF-activated reporter gene assay revealed enhanced Wnt signaling through the Galphai/o-PI3K-Akt axis, suppression of GSK3beta, and stabilization of beta-catenin [70] (Fig. 17.4). Phosphorylation of GSK3 by Akt represses its phosphorylating activities on beta-catenin and thereby to reduce the degradation of beta-catenin. Moreover, SDF-1 signaling in INS-1 β-cells stimulates the accumulation of beta-catenin mRNA, likely due to an enhancement the transcription of the beta-catenin gene [70]. Recent evidence also suggests that active Wnt signaling mediates, and is required for, the cytoprotective, survival actions of SDF-1 on β-cells [70].

17.5.3 Potential Mechanisms by Which GLP-1 and SDF-1 May Act Cooperatively on Wnt Signaling to Enhance β-Cell Growth and Survival

There appear to be differences in the mechanisms of the interactions of SDF-1/CXCR4 signaling and GLP-1/GLP-1R signaling with the Wnt signaling pathway in β-cells. Although both SDF-1 and GLP-1 activate the downstream pathway of Wnt signaling, consisting of beta-catenin/TCF7L2-mediated gene expression, they do so by way of different pathways of interactions with the more upstream components of the Wnt signaling pathway. These proposed different upstream pathways of signaling utilized by GLP-1 and SDF-1 raises the possibility of additive or synergistic effects on downstream Wnt signaling in the promotion of β-cell growth and survival. SDF-1 inhibits the destruction complex of the canonical Wnt signaling pathway consisting of Axin, APC, and the protein kinases, glycogen synthase kinase-3 (GSK3) and casein kinase-1 (CSNK1). This inhibition of GSK3 and CSNK1 by SDF-1 is likely mediated by the well-known actions of Akt to inhibit these kinases, resulting in the stabilization and accumulation of beta-catenin. In marked contrast to the actions of SDF-1 on β-cells, GLP-1 activates beta-catenin/TCF7L2 complexes via the stabilization of beta-catenin by a different mechanism involving the phosphorylation and stabilization of beta-catenin by the cAMP-dependent protein kinase A (PKA). PKA activated by GLP-1/GLP-1R phosphorylates beta-catenin on Serine-675, resulting in its stabilization and accumulation. Thus, unlike SDF-1, GLP-1-induced activation of gene expression by beta-catenin/TCF7L2 in β-cells occurs independently of the destruction box and the activities of GSK3. It also remains possible that beta-catenin may be stabilized by its direct phosphorylation by Akt.

Beta-catenin is the activation domain and TCF7L2 is the DNA-binding domain of the transactivator. It is tempting to speculate that different phosphorylations of beta-catenin provided by SDF-1 signaling versus GLP-1 signaling result in different conformations of beta-catenin. When different conformers of beta-catenin interact with TCF7L2 they confer different conformations to the DNA-binding domains of TCF7L2, resulting in differing affinities of TCF7L2 for its cognate enhancer binding sites on the promoters of various Wnt signaling target genes. Such a combinatorial mechanism could account for the difference in genes regulated by beta-catenin/TCF7L2 in β-cells in response to SDF-1 compared to GLP-1. Wnt signaling may be a final downstream pathway for both SDF-1 and GLP-1 signaling in β-cells. However, gene expression targets diverge so that SDF-1 predominately regulates genes involved in cell survival, whereas GLP-1 regulates genes involved in cell cycle control (proliferation). If this circumstance proves to be valid, our findings raise the possibility of a dual therapeutic approach for increasing β-cell mass. GLP-1 is predominantly pro-growth and SDF-1 is predominantly pro-survival. Thereby the two peptides may act synergistically to promote both the growth and the survival of β-cells and to conserve, or even enhance, β-cell mass in response to injury.

17.6 Type 2 Diabetes Genes

Genome-wide scans in several large populations have uncovered associations of specific genetic loci with the development of type 2 diabetes [15–20, 27, 80–91]. At least 19 genes have associations with diabetes that are consistent among various population studies (Table 17.1). Of note, the majority of these genes (14 of 19) are expressed in pancreatic β-cells. Furthermore, several of the genes (seven) appear to be involved in the Wnt signaling pathway. TCF7L2, the DNA-binding component of the downstream transcription factor complex, appears to have a particularly strong association with type 2 diabetes.

17.6.1 Genes Associated with Islet Development/Function and Wnt Signaling

17.6.1.1 TCF7L2 (Transcription Factor 7-Like 2)

Grant and coworkers provided the index report on an association of polymorphisms in TCF7L2 with type 2 diabetes [92]. Epidemiology studies from Icelandic, Danish, and US cohorts reported that the inheritance of a specific single nucleotide polymorphism (SNPs), at the region DG10S478, within the intron 3 region of TCF7L2 gene is related to an increased risk of type 2 diabetes [25–36]. Then two other SNPs within introns 4 and 5 of TCF7L2, namely rs12255372 and rs7903146, were found in strong linkage disequilibrium with DG10S478 and showed similarly robust associations with type 2 diabetes patients with glucose intolerance. In Asian populations, the frequencies of SNPs rs7903146 and rs12255372 are quite low, but two novel SNPs-rs290487 and rs11196218 are associated with the risk of type 2 diabetes in a Chinese population. The most likely candidate is the rs7903146 single nucleotide polymorphism that has a strong association with type 2 diabetes [93]. This polymorphism resides in a noncoding region of the gene and no clear mechanism for its effects on TCF7L2 expression is apparent. It has been reported that nondiabetic carriers of the risk-associated TCF7L2 SNPs do not have defects in GLP-1 secretion. The risk alleles are associated with impaired insulin secretion, incretin effects, and an enhanced rate of hepatic glucose production. As mentioned previously, knockdown of TCF7L2 with small interfering RNAs reduces glucose-stimulated insulin secretion from β-cells [66, 67]. However, a study from Lyssenko et al. [25] demonstrates that TCF7L2 mRNA transcripts are more abundant in the islets of diabetic patients and the level of TCF7L2 expression in islets negatively correlates with insulin secretion. This finding indicates that increased levels of TCF7L2 in islets would increase the risk of diabetes onset by the inhibition of insulin secretion. However, it has not yet been determined whether the increase in TCF7L2 mRNA levels in human islets translates to an increase in protein levels of TCF7L2.

The glucoincretin hormone GLP-1 appears to be involved in the pathogenesis of diabetes in individuals who carry TCF7L2 risk alleles. These carriers of TCF7L2 risk alleles have impaired insulin secretion as a major contributor to impaired

Table 17.1 Type 2 diabetes genes identified by Genome-Wide Association Studies

Gene symbol	Functions	β-cell functions	Wnt signaling
Genes associated with islet development function and Wnt signaling			
TCF7L2	HMG transcription factor-7L2	β-cell proliferation and survival Insulin secretion	Canonical Wnt signaling regulates target genes in association with beta-catenin
FTO	Fatso. Fused toes locus includes FTS, FTM	Pancreas development obesity	FTS, a target gene for Wnt signaling
NOTCH 2	Delta/Notch signaling	Pancreas development	Wnt signaling interaction via phosphorylation by GSK3
IGF2BP2	Insulin growth factor 2 mRNA-binding protein 2	Islet growth	Expression induced by beta-catenin and TCF7L2
HHEX	Homeodomain transcription factor	Early pancreas development	Repressed by beta-catenin and TCF7L2
TCF2	Hepatocyte nuclear factor 1 beta (MODY 5)	Early islet progenitor cell specification. T2D pancreas development	Regulates beta-catenin
CDKN2A/N2B	Cyclin-dependent kinase Inhibitor, P16, INK4A	Islet regeneration regulates CDK4 in β-cells	Cross talk with Wnt signaling induced by beta-catenin
Genes associated with islet development/function, Wnt signaling unknown			
PPARgamma	Peroxisome proliferator activating receptor gamma	Insulin resistance insulin secretion	PPARdelta, Wnt target gene
KCJN11	Inward rectifying K+ channel	Regulates insulin secretion along with Sur1 (ABCC8)	Not known
WFS1	Wolfram syndrome 1 transmembrane protein	Insulin secretion, endoplasmic reticulum protein trafficking	Not known
CDKAL1	Cyclin-dependent kinase 5 homolog inhibitor	Islet glucotoxicity inhibitor, impaired insulin secretion	Not known
SLC30a8	Solute carrier 38a8 zinc transporter	Insulin granules, secretion	Not known
KCNQ1	Potassium channel	Insulin secretion	Not known
MTNR1B	Melatonin receptor 1b	Insulin secretion	Not known

Table 17.1 (continued)

Gene symbol	Functions	β-cell functions	Wnt signaling
Genes not known to be involved in either islet development/function or Wnt signaling			
TSPAN8/LGR5/ GPR 49	Tetraspanin 8. Leucine-rich G protein-coupled receptor 5. G protein-coupled receptor 49	Unknown	Wnt signaling target gene in intestinal crypt stem cells
JAZF1	Nuclear zinc finger transcriptional repressor	β-cell apoptosis?	Not known
CDC123/CAMK1D	Calcium-dependent calmodulin kinase	β-cell apoptosis?	Not known, planar cell polarity, noncanonical Wnt signaling?
THADA	Thyroid adenoma associated	Unknown	Not known
ADAMTS9	Metallopeptidase with thrombospondin 9	Unknown	Not known

glucose tolerance or diabetes [25–36]. Glucose clamp studies on a large cohort of carriers of TCF7L2 polymorphisms revealed both reduced insulin secretion in response to oral glucose tolerance tests and impaired GLP-1-induced insulin secretion [48]. However, in these studies plasma GLP-1 levels were not influenced by the TCF7L2 variants [48]. These findings are of interest because two pathogenetic mechanisms involving GLP-1 have been proposed: impaired GLP-1 production in the intestine [29, 68] and impaired GLP-1 actions on pancreatic β-cells [46]. The studies of Schafer et al. [48] suggest that the defect in the enteroinsular axis in individuals with defective TCF7L2 functions lies at the level of impaired actions of GLP-1 on insulin secretion from pancreatic β-cells, rather than the level of impaired production of GLP-1 by intestinal L-cells. Evidence is reported from studies in vitro that support an important role for beta-catenin/TCF7L2-mediated Wnt signaling in both the expression of the proglucagon gene in intestinal cells [94] and in the regulation of insulin secretion [47, 66, 67] and β-cell proliferation [46]. Interestingly, there is some reported evidence that TCF7L2 may be expressed at low levels [94, 95], or not at all [96] in β-cells. These reports conflict with those of the Rutter [67] and Maeder [47] laboratories, and our own observations [46]. Based on the findings currently available, the contributions of TCF7L2 functions to the enteroinsular axis may occur at the levels of both the production of GLP-1 by intestinal L-cells and the actions of GLP-1 on pancreatic β-cells. The two levels of involvement of TCF7L2 actions are not necessarily mutually exclusive.

17.6.1.2 FTO (Fat Mass and Obesity-Associated Protein)

FTO encodes a protein that is homologous to the DNA repair AlkB family of proteins that are involved in the repair of alkylated nucleobases in DNA and RNA [97]. The FTO gene is up-regulated in orexigenic neurons in the feeding center of the hypothalamus [98]. Genetic variants in FTO result in excessive adiposity and insulin resistance, as well as a markedly increased predisposition to the development of diabetes [99]. A 1.6 Mb deletion mutation in the mouse results in the deletion of a locus containing FTO, FTS (fused toes), FTM, and three members of the Iroquois gene family, Irx3, Irx5, and Irx6 [100], resulting in multiple defects in the patterning of the body plan during development [100, 101]. The Irx (Iroquois) proteins are homeodomain transcription factors. The FTO, FTS, and IRX locus is implicated in Wnt signaling. FTS is a small ubiquitin-like protein with conjugating protein ligase activity that is known to interact with the protein kinase Akt, a potent inhibitor of GSK3beta activity in the Wnt signaling pathway. Moreover, Wnt signaling is reported to induce the expression of Irx3 [102]. Irx1 and Irx2 are expressed in the endocrine pancreas of the mouse under the control of Neurogenin-3 (Ngn3) expression [103].

17.6.1.3 NOTCH2

The delta/notch signaling pathway is an important cell–cell interactive signaling pathway (lateral inhibition) involved in embryonic stem cell amplification, differentiation, and in determination of organogenesis. Notch2 is expressed in pancreatic

ductal progenitor cells and may be involved in early branching morphogenesis of the pancreas [104]. The conditional ablation of Notch2 signaling in mice moderately disturbed the proliferation of epithelial cells during early pancreas development [105]. Evidence is presented linking Notch2 to Wnt signaling [106]. GSK3beta phosphorylates Notch2, thereby inhibiting the activation of Notch target genes.

17.6.1.4 IGF2BP2 (Insulin-Like Growth Factor 2 Binding Protein 2)

IGF2BP2 is a paralog of IGF2BP1, which binds to the 5' UTR of the insulin-like growth factor 2 (IGF2) mRNA and regulates IGF2 translation [107]. IGF2 is a member of the insulin family of polypeptide growth factors involved in the development, growth, and stimulation of insulin action.

Wnt1 is reported to induce the expression of IGF2 in preadipocytes [108].

17.6.1.5 HHEX (Hematopoietically Expressed Homeobox)

HHEX is a homeodomain protein that regulates cell proliferation and tissue specification underlying vascular, pancreatic, and hepatic differentiation [109–111]. Variants in the Hhex gene manifest in impaired β-cell function [112]. Hhex is associated with Wnt signaling during pancreas development, as it acts with beta-catenin to serve as a corepressor of Wnt signaling [113, 114].

17.6.1.6 TCF2 (Hepatocyte Nuclear Factor 1 Beta, HNF1beta, MODY 5 Gene)

Tcf2 is a critical regulator of a transcriptional network that controls the specification, growth, and differentiation of the embryonic pancreas [115]. Mutations in the TCF2 gene result in hypoplasia of the pancreas, resulting in exocrine pancreas dysfunction to varying degrees [115–117]. Some mutations manifest as a form of Maturity Onset Diabetes of the Young (MODY 5).

17.6.1.7 CDKN2A/B (Cyclin-Dependent Kinase Inhibitor 2A/B, ARF, p16INK4a)

The CDKN2A/B gene generates several transcript variants which differ in their first exons. CDKN2A is a known tumor suppressor and its product, p16 INK4a, inhibits CDK4 (cyclin-dependent kinase 4), a powerful regulator of pancreatic β-cell replication [118–120]. Over-expression of Cdkn2a leads to decreased islet proliferation in ageing mice [121]. Cdkn2b over-expression is also causally related to islet hypoplasia and diabetes in murine models [122]. P16(Ink4a) is linked to the Wnt signaling pathway as stabilized beta-catenin silences the p16(Ink4a) promoter in melanoma cells [123].

17.6.2 Genes Associated with Islet Development/Function, Wnt Signaling Unknown

17.6.2.1 PPARgamma (Peroxisome Proliferator-Activated Receptor Gamma)

PPARgamma is involved in insulin signaling in insulin-responsive target tissues [124] and is implicated in β-cell growth and survival. PPARgamma mediates growth arrest and survival of β-cells [125]. Islets of mice in which PPARgamma is specifically ablated display a marked reduction in the expression of the transcription factor PDX-1 and develop glucose intolerance, impaired glucose-stimulated insulin secretion, and a loss of actions of PPARgamma agonists to enhance PDX-1 expression [125]. PPARgamma is not yet linked to the Wnt signaling pathway, although PPARdelta is a known target gene for activation by Wnt signaling [126].

17.6.2.2 KCNJ11 (Inward Rectifying Potassium Channel)

KCNJ11 is an important component of the ATP-sensitive potassium channel on β-cells responsible for the regulation of insulin secretion [127]. KCNJ11 exists in a complex with the sulfonylurea-regulated receptor SUR1. In response to elevated glucose and other insulin secretagogues, the ATP-sensitive potassium channel closes and allows for a decrease in the resting potential (depolarization) of β-cells resulting in the opening of voltage-sensitive calcium channels. The inward flux of Ca^{2+} into β-cells is believed to be an important stimulus for the exocytosis of insulin. A deficiency of the numbers and/or functions of ATP-sensitive channels, either KCNJ11 or SUR-1, due to genetic mutations results in a chronic depolarized state of β-cells and unregulated excessive insulin secretion [128]. As of now no direct evidence implicates Wnt signaling with KCNJ11.

17.6.2.3 WFS1 (Wolfram Syndrome 1)

WFS1 encodes a transmembrane protein of 890 amino acids that is highly expressed in the endoplasmic reticulum of neurons and pancreatic β-cells [129]. Mutations in WFS1 result in Wolfram syndrome, an autosomal recessive neurodegenerative disorder. Disruption of the WFS1 gene in mice causes progressive β-cell loss and impaired stimulus-secretion coupling in insulin secretion [130]. The reduction in β-cell mass is likely a consequence of enhanced endoplasmic reticulum stress resulting in the apoptosis of β-cells [131–133]. Impaired proinsulin processing to insulin and insulin transport through the secretory pathway may also be involved in the impaired insulin secretion. To date no information is available on the mechanisms that regulate WFS1 expression or of an involvement of Wnt signaling in its expression.

17.6.2.4 CDKAL1 (CDK5 Regulatory Subunit-Associated Protein-1-Like 1)

CDKAL1 encodes a protein of unknown functions. However, the protein is similar to CDK5 regulatory subunit-associated protein 1 (encoded by CDK5RAP1), expressed in neuronal tissues. CDKAL1 inhibits cyclin-dependent kinase 5 (CDK5) activity by binding to the CDK5 regulatory subunit p35 [134]. Variants in the CDKAL1 gene in humans are associated with decreased pancreatic β-cell functioning. [112]. CDK5 has a role in the loss of β-cell function in response to glucotoxicity as the inhibition of the CDK5/p35 complex prevents a decrease of insulin gene expression that results from glucotoxicity [135]. Therefore, it seems possible that CDKAL1 may have a role in the inhibition of the CDK5/p35 complex in pancreatic β-cells similar to that of CDK5RAP1 in neuronal tissue. One may conjecture that a reduced expression and inhibitory function of CDKAL1 or reduced inhibitory function could exacerbate β-cell impairment in response to glucotoxicity.

17.6.2.5 SLC30a8 (Solute Carrier 30a8)

SLC30A8 transports zinc from the cytoplasm into insulin secretary vesicles [136, 137] where insulin is stored as a hexamer bound with two Zn^{2+} ions prior to secretion [138]. Variation in SLC30A8 may affect zinc accumulation in insulin granules, affecting insulin stability, storage, or secretion. In high-glucose conditions, over-expression of SLC30A8 in INS-1E cells enhanced glucose-induced insulin secretion. SLC30A8 is specific to the pancreas and is expressed in β-cells, where it facilitates accumulation of zinc from the cytoplasm into intracellular vesicles [139].

17.6.2.6 KCNQ1 (Potassium Channel Q1)

KCNQ1 encodes the pore-forming alpha subunit of the voltage-gated potassium channel KvLQT1 [140]. It is expressed in pancreatic islets and blockade of the channel stimulates insulin secretion [141].

17.6.2.7 MTNR1B (Melatonin Receptor 1B)

The melatonin receptor 1b is expressed throughout the nervous system and in the β-cells of the pancreatic islets [142]. Melatonin is secreted in a circadian pattern from the pineal gland with high nocturnal levels of secretion. Since melatonin suppresses insulin secretion from β-cells it is suggested that it may suppress insulin secretion during the night [143]. The risk allele for diabetes results in an increase of the receptor in β-cells perhaps leading to an inappropriate inhibition of insulin secretion [143]. It has been suggested that melatonin receptor antagonists may be an effective therapy for patients with diabetes linked to defects in MTNR1B [143].

17.6.3 Genes Not Known to be Involved in Either Islet Development/Function or Wnt Signaling

17.6.3.1 TSPAN8/LGR5/GPR49

The protein encoded by this gene is a member of the transmembrane 4 superfamily, also known as the tetraspanin family. Most of these members are cell surface proteins that have a role in the regulation of cell development, activation, growth, and motility. LGR5/GPR49 is a leucine-rich repeat-containing G protein-coupled receptor. A role for TSPAN8 in the pancreas is as yet unknown. However, Tspan8/Lrg5 is a recognized Wnt signaling target gene in small intestinal and colonic stem cells [144].

17.6.3.2 JAZF1 (Zinc Finger 1, TIP27)

JAZF1 is a zinc finger transcriptional repressor, corepressor [145]. The gene is susceptible to chromosomal recombination in endometrial stromal tumors with resultant transcription of chimeric mRNAs encoding fusion proteins of JAZF1 with JJAZF1 and SUZ12 (suppressor of zeste 12) [146]. Remarkably, the RNA transcripts from the JAZF1 and SUZ12 genes in noncancerous tissues undergo trans-splicing resulting in the translation of an identical protein [147]. This protein exerts strong both pro-proliferative and anti-apoptotic actions in cells. It remains unknown whether JAZF1 proteins are expressed in the pancreatic islets, but if they are, it seems likely that they may contribute to their growth and survival.

17.6.3.3 CDC123/CAMK1D

The CAMK1D gene encodes a member of the Ca^{2+}/calmodulin-dependent protein kinase 1 subfamily of serine/threonine kinases [148]. The encoded protein may be involved in the regulation of granulocyte function through the chemokine signal transduction pathway. Alternatively spliced transcript variants encoding different isoforms of this gene have been described [149]. Camk1d is implicated in the apoptosis of cells [150]. No information is available about a possible role of CAMK1D in the pancreas or any connections with Wnt signaling. It is tempting to speculate, however, that it may be a competent of the noncanonical Ca^{2+} Wnt signaling pathway.

17.6.3.4 THADA (Thyroid Adenoma Associated)

THADA is identified as the target gene of 2p21 aberrations in thyroid adenomas. The gene spans roughly 365 kb, and based on preliminary results, it encodes a death receptor-interacting protein [151]. Chromosomal rearrangements lead to alterations in the gene and encoded protein, one of which consists of a fusion of an intronic sequence of PPARgamma to exon 28 of THADA [152]. Associations of THADA with islets and/or Wnt signaling are unknown.

17.6.3.5 ADAMTS9

The ADAMTS9 gene encodes a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) protein family [153]. Members of the ADAMTS family have been implicated in the cleavage of proteoglycans, the control of organ shape during development, and the inhibition of angiogenesis. ADAMTS8 is widely expressed during mouse embryo development [154]. Functions for ADAMTS9 in pancreas or in Wnt signaling are heretofore unrecognized.

17.7 Future Directions

Continued studies of the involvement of the Wnt signaling pathway in islet development and function may reveal novel factors important in β-cell growth and survival. A prerequisite for understanding the potential importance of Wnt signaling in islets is the identification of the specific Wnt signaling factors that are expressed in islets. Identification of these factors may provide opportunities for development of small molecules that target specific components of the pathways to promote growth and survival. Ongoing high-throughput screening studies of hundreds of thousands of compounds using islet tissues containing fluorescence reporter genes and growth or apoptosis-responsive promoters may uncover such small molecules.

Anti-diabetogenic therapies consisting of combinations of GLP-1 and SDF-1 agonists may provide additive benefits in promoting both the growth and the survival of β-cell, thereby preserving or enhancing β-cell mass. Recent findings suggest that both the pro-proliferative actions of GLP-1 and the anti-apoptosis actions of SDF-1 are mediated by the activation of beta-catenin and TCF7L2 in β-cells. Although both the GLP-1/GLP-1R and the SDF-1/CXCR4 axes converge on downstream Wnt signaling at the level of the formation of transcriptionally productive complexes of beta-catenin/TCF7L2, the target genes activated by GLP-1 and by SDF-1 differ. GLP-1-mediated activation of beta-catenin/TCF7L2 results in the expression of genes involved in the cell division cycle, whereas SDF-1 actions result in the activation of the expression of genes engaged in cell survival. Furthermore, downstream beta-catenin/TCF7L2 activation is a requisite for the pro-proliferative actions of GLP-1 and the anti-apoptotic actions of SDF-1. The two hormones, GLP-1 and SDF-1, acting together may provide additive benefits in promoting the regeneration and maintenance of β-cell mass in diabetes.

Genome-wide association studies in search of risk alleles for type 2 diabetes are just beginning. It is estimated that 80–90% of the human genome remains yet to be explored for the existence of diabetes-associated genes in the population. Predictably, further genome-wide scans in the future will uncover even more than the current 19 genes, many will likely be involved in islet and β-cell development and functions. It is tempting to speculate that the additional risk genes for type 2 diabetes that remain to be discovered in the future will include genes encoding components of the Wnt signaling pathway.

Intriguing current evidence warrants further investigations of Wnt ligands and Wnt signaling in the cross talk between adipose tissue and islets. Possibilities arise suggesting that Wnt ligands produced and secreted by adipocytes act on β-cells to stimulate Wnt signaling.

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Chapter 18
Molecular Pathways Underlying the Pathogenesis of Pancreatic α-Cell Dysfunction

Dan Kawamori, Hannah J. Welters, and Rohit N. Kulkarni

Abstract Glucagon plays a critical role in glucose homeostasis by counteracting insulin action, especially during hypoglycemia. Glucagon secretion from pancreatic α-cells is regulated by various mechanisms including glycemia, neural input, and secretion from neighboring β-cells. However, glucagon secretion is dysregulated in diabetic states, causing exacerbation of glycemic disorders. Recently, new therapeutic approaches targeting excess glucagon secretion are being explored for use in diabetes treatment. Therefore, understanding the molecular mechanism of how glucagon secretion is regulated is critical for treating the α-cell dysfunction observed in diabetes.

Keywords Diabetes · Hypoglycemia · Pancreatic islets · α-cells · Glucagon · Secretion · Insulin · GABA · Zinc · Somatostatin · GLP-1 · Counter regulation · Neurotransmitters and nervous systems · Development · Hypertrophy

18.1 The Pancreatic α-Cell and Glucagon

18.1.1 The Pancreatic α-Cell

Pancreatic islets, scattered within the exocrine pancreas, collectively form the endocrine pancreas. The islets are composed of five endocrine cell types each of which secretes hormones that contribute to the overall regulation of glucose metabolism. The glucagon-secreting α-cells account for approximately 20% of islet cells. In adults, β-cells, which secrete insulin, are restricted to the islet core while α-cells, somatostatin-secreting δ-cells, pancreatic polypeptide-secreting PP-cells, and

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ghrelin-secreting ε-cells are scattered along the periphery of the islet. This architecture is typical of rodent islets, while in humans, non-β-cells are often observed both at the periphery and also seemingly at the center of islets [1]. On closer inspection however the arrangement of the different cell types in human islets has been noted to be similar to that in rodents. Interestingly human islets appear to consist of several "rosettes," with each rosette resembling the basic islet architecture seen in rodent islets [2]. It is likely that the distribution and arrangement of different islet cell types are important for normal islet microcirculation. Thus in rodent islets, and likely within each human islet "rosette," the blood flows from the center of the β-cell core toward the non-β-cells in the periphery [3, 4], suggesting that insulin impacts the release of hormones from other cell types in the islets.

18.1.2 Functions of Glucagon

Glucagon is a 29 amino acid peptide hormone, secreted from α-cells, which exerts biological effects on a wide range of organs (Fig. 18.1). The amino acid sequence is preserved almost identically among mammalian species indicating that glucagon is a fundamentally required hormone. Interestingly, guinea pigs have a mutant form of glucagon with reduced activity (1/1000), but can survive without defects in glucose homeostasis since they also express a mutant form of insulin which has reduced receptor binding efficiency and counterbalances the effects of glucagon [5]. Glucagon has important functions in vivo for sustaining appropriate blood glucose and these functions are also preserved among species. Therefore, it is conceivable that the structure and function of glucagon have been strictly preserved in the process of evolution. In physiological states, glucagon is released into the bloodstream in response to hypoglycemia to oppose the action of insulin in peripheral tissues, predominantly in the liver, and works as a counter-regulatory hormone to restore normoglycemia. Glucagon promotes hepatic gluconeogenesis, glycogenolysis, and simultaneously inhibits glycolysis and glycogenesis [6, 7], ultimately leading to an increase in blood glucose levels, to counter hypoglycemia. In contrast, in the fed state, insulin action dominates leading to suppression of hepatic glucose output while enhancing hepatic glucose intake to maintain normoglycemia. Thus, the insulin to glucagon ratio is a critical determinant of hepatic glucose metabolism in the overall maintenance of glycemia. Glucagon can also stimulate insulin secretion from pancreatic β-cells [8] and indirectly impact hepatic glucose output. Furthermore, glucagon has been suggested to play a role in the development of islets, although the molecular mechanisms underlying these effects during embryogenesis and in the adult organism are not fully understood [9, 10]. Taken together, these actions indicate an important role for glucagon in maintaining glucose homeostasis.

The glucagon receptor is a G-protein (Gs/Gq)-coupled receptor [11] and is widely expressed in insulin target organs, such as liver, adipose, β-cells, and brain, with the exception of skeletal muscle [12]. Following binding and conformational

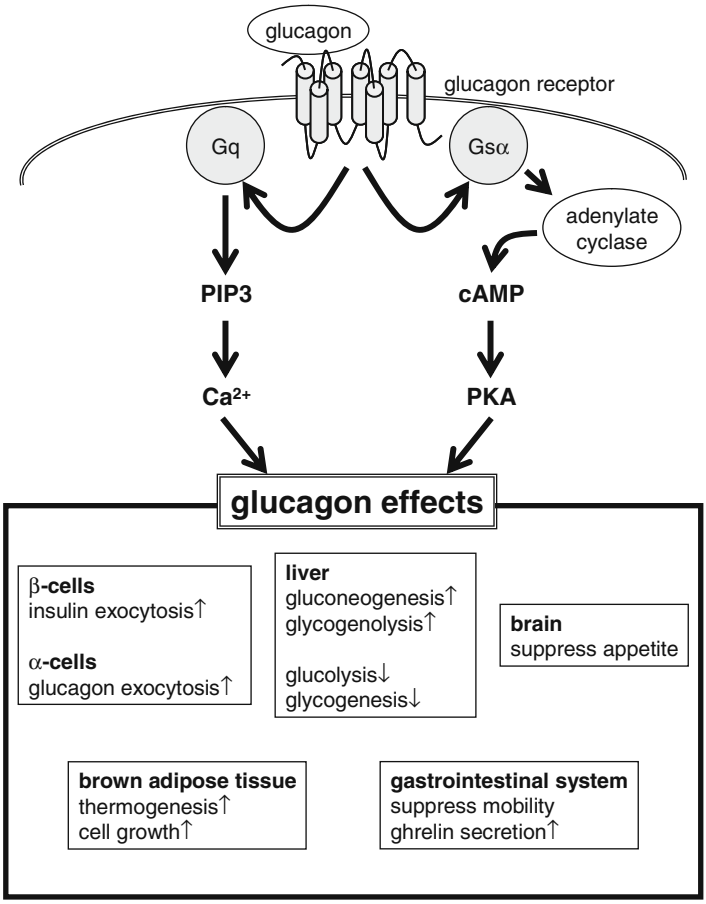

Fig. 18.1 Glucagon signaling and effects on various target tissues. Binding of glucagon to the glucagon receptor induces activation of G proteins. Activation of Gq leads to induction of the phospholipase C-inositol 1,4,5-triphosphate (PIP3)-cytosolic calcium cascade, whereas activation of Gsα causes an increase in the levels of cyclic adenosine monophosphate (cAMP) which activates protein kinase A (PKA). The effects of glucagon are induced through one or more of these pathways

changes of the receptor the activation of Gs leads to recruitment of adenylate cyclase to the cell membrane, causing an increase in intracellular cyclic adenosine monophosphate (cAMP) levels and subsequent activation of protein kinase A (PKA) [13]. On the other hand, activation of Gq induces activation of phospholipase C, upregulation of inositol 1,4,5-triphosphate, and the subsequent release of intracellular calcium (Ca^{2+}) [14] (Fig. 18.1). The action of glucagon is relatively complex and involves the coordinate regulation of transcription factors and signal transduction networks which converge to regulate amino acid, lipid, and carbohydrate metabolism. For example, in the liver, elevated PKA activity

triggers various downstream targets leading to the suppression of glycolysis and glycogenesis, and the enhancement of gluconeogenesis and glycogenolysis [15]. In islet cells, the elevation of cAMP by glucagon has been reported to stimulate insulin and glucagon secretion from β- and α-cells, respectively [16, 17], by PKA-dependent and PKA-independent mechanisms. Upregulation of cAMP activates cAMP-regulated guanine nucleotide exchange factors (cAMPGEFs/Epac), which modulate intracellular Ca^{2+}-ion mobilization, enhancing exocytosis [17, 18].

18.2 Dysregulation of α-Cell Function in Diabetes

18.2.1 Excess Glucagon Secretion

Glucagon plays a critical role in glucose homeostasis largely by regulating hepatic glucose metabolism. Circulating glucagon levels are often elevated in both type 1 and type 2 diabetes and suggested to contribute to the development of glucose toxicity and exacerbation of diabetes [19–22]. In addition, the absence of postprandial glucagon suppression in diabetes patients also contributes to postprandial hyperglycemia [23–25]. One potential contributor to the excess glucagon levels is a relative increase in α-cells compared to β-cells in pancreatic islets in both type 1 [19] and type 2 diabetes [26, 27]. Moreover, in type 1 diabetic islets, an increase in α-cell area and number and cell-type distribution in islets are dysregulated due to specific β-cell destruction. Although the precise mechanism(s) of relative hyperglucagonemia in the diabetic state is still obscure, β-cell dysfunction is a possible candidate since β-cell secretory products, including insulin, are known to suppress glucagon secretion (see Section 18.3.2.4). Thus altered β-cell function in diabetes can potentially induce inappropriately elevated glucagon in hyperglycemic states by impairing the intraislet influence of β-cells on glucagon regulation [28].

18.2.2 Defective Glucagon Response to Hypoglycemia

Diabetes patients (both type 1 and type 2) frequently develop defective counterregulatory responses to hypoglycemia that is associated with reduced or absent glucagon secretory responses. A defective glucagon secretory response to hypoglycemia in hyperinsulinemic states frequently exacerbates a hypoglycemic attack and limits intensive glucose control by insulin therapy [29, 30]. Moreover, hypoglycemia-associated autonomic failure is induced especially in patients with frequent exposure to hypoglycemia leading to a worsening phenotype [31]. This defective response to hypoglycemia includes sympathoadrenal and neurohormonal responses against hypoglycemia such as epinephrine, cortisol, and growth hormone that act to decrease blood glucose further, finally leading to sudden states of hypoglycemia and hypoglycemia unawareness [32–34]. How diabetes induces

these defective responses to hypoglycemia is still under investigation and suggested theories include alteration in brain glucose transport and metabolism by frequent exposure to hypoglycemia [35] and/or defective intraislet β-cell effects on α-cell function, such as the "switch-off" of insulin [36, 37] or zinc iron [38] (see Section 18.3.2.4).

18.2.3 Defective α-Cell Function in Islet Transplantation Grafts

Islet transplantation into the liver has been attempted as a potential approach to cure type 1 diabetes [39, 40]. While the transplanted islets are able to secrete insulin in response to alterations in blood glucose levels, they have been reported to lack an appropriate glucagon response [41, 42]. The disruption of the normal physiological islet environment including blood flow and nervous regulation might explain, in part, the dysfunction of the α-cells in transplanted islet grafts. Indeed some studies report that the intra-hepatic site is not appropriate for optimal α-cell function to counteract hypoglycemia [43], due to an altered glycogenolysis-derived glucose flux in the liver [44].

18.2.4 Glucagonoma Syndrome

The glucagonoma syndrome is a rare disorder caused by a functional pancreatic endocrine tumor [45]. In a manner similar to diabetes patients, patients with glucagonoma exhibit a glycemic disorder due to abnormally elevated glucagon [46]. Typically, the patients manifest dermatitis (necrolytic migratory erythema) [47], and altered glucose levels (diabetes) and weight loss due to the catabolic effects of excess glucagon. In addition to those symptoms, deep vein thrombosis and depression [48] are also observed, leading to the classic description as "4D's." Several patients also display hypoaminoacidemia, cheilosis, normocytic anemia, and neuropsychiatric symptoms. Generally, the correction of hyperglucagonemia, by removal of the tumor, eliminates these symptoms indicating that they are directly induced by glucagon. However, the mechanisms by which elevated glucagon induces these symptoms are not fully understood.

18.3 Mechanisms Regulating Glucagon Expression and Secretion

18.3.1 Regulation of Glucagon Processing and Gene Expression

18.3.1.1 Processing

Glucagon is processed from its larger biosynthetic precursor, proglucagon. The proglucagon gene is expressed in pancreatic α-cells, intestinal L-cells, and some neurons in the brain including those in the hypothalamus. The proglucagon gene

encodes a 180 amino acid preproglucagon molecule, differential processing of which leads to the production of several derivative hormones including, glucagon, glicentin, oxyntomodulin, GLP-1, and GLP-2 (reviewed [49]). The expression of each of these hormones is cell type dependent, due to the differential expression of prohormone convertase (PC) enzymes, which cleave the preproglucagon molecule at different sites [50]. The exclusive expression of PC2 in α-cells leads to the predominant production of glucagon (with minor amounts of GLP-1) [51, 52]. Conversely, in intestinal L-cells and in the brain the presence of PC1/3 allows the production of the incretin hormone, GLP-1, the intestinotrophic hormone, GLP-2, glicentin, and oxyntomodulin [49, 53].

18.3.1.2 Gene Expression

The transcription factors Pax6, Cdx2/3, large Mafs, Brain4 (Brn4), and Foxa2 are all implicated in regulating glucagon gene expression in α-cell lines, but their relative contributions to regulating gene expression in vivo is as yet unclear (reviewed [49, 54, 55]). Pax6 can promote proglucagon gene expression [56], but as it is expressed in all islet cells it is unlikely to be solely responsible for the specificity of glucagon gene expression to α-cells. In contrast, Pax4 can impair glucagon gene transcription by inhibiting Pax6-mediated transcription [57, 58]. Thus, α-cell-specific expression of the glucagon gene likely requires the presence of specific transcription factors (such as Pax6) and the absence of others (such as Pax4).

In addition to its important role in the regulation of proglucagon gene expression, Pax6 was recently discovered to play a role in glucagon processing [59]. Interestingly, Pax6 was found to regulate the expression of PC2 and its molecular chaperone, 7B2. Pax6 has been reported not only to activate 7B2 transcription directly but also to indirectly regulate PC2 and 7B2 levels through the activation of *cMaf* and *BETA2/NeuroD1* genes [59]. It is worth noting that the PPARγ agonists, such as the thiazolidinedione (TZD) class of drugs, which are insulin-sensitizing drugs used in the treatment of type 2 diabetes, have been shown to inhibit glucagon gene transcription [60]. This is by a ligand-specific, but DNA binding, independent mechanism that involves direct protein–protein interaction of PPARγ–RXR with the Pax6 transactivation domain, resulting in Pax6 inhibition [61].

Glucagon gene expression is negatively regulated by insulin [62] through the activation of PI3K and PKB which causes inhibition of Pax6 [63, 64]. Indeed, in type 2 diabetes, the elevated glucagon levels may be due to insulin resistance in α-cells, preventing the insulin inhibition of glucagon gene expression that occurs normally. The peripheral location of α-cells in islets and their potential exposure to high levels of insulin, particularly in diabetic patients with hyperinsulinemia, provide teleological support for this observation. Chronic exposure of α-cells to insulin has also been reported to prevent insulin-stimulated inhibition of glucagon gene transcription due to decreased insulin receptor expression via enhanced degradation and a subsequent reduction in IRS-1 phosphorylation [65]. A role for insulin in the regulation of α-cell gene expression is supported by recent studies. High glucose stimulated glucagon gene expression in islets isolated from α-cell-specific insulin

receptor knockout (αIRKO) mice, whereas there was a minimal response to glucose in control islets, presumably due to the inhibition of glucagon gene transcription by glucose-induced insulin secretion. Conversely, in low-glucose conditions glucagon gene expression was reduced in αIRKO islets, suggesting that insulin signaling also controls basal glucagon gene expression [66].

18.3.2 Regulation of Glucagon Secretion

18.3.2.1 Ion Channels and Electrical Activity

Similar to insulin secretion from β-cells, glucagon secretion from α-cells is regulated by electrical communication between various ion channels. The ATP-sensitive K^+ (K^+_{ATP}) channel is considered to be the primary channel and the main regulatory component of glucagon secretion [67]. The K^+_{ATP} channel triggers depolarization of cellular membrane potential leading to activation of low-voltage T-type Ca^{2+} channels [68], the opening of tetrodotoxin (TTX)-sensitive Na^+ channels and further depolarization which in turn activates high-voltage L- or N-type Ca^{2+} channels, and ultimately induction of exocytosis of glucagon-containing secretory granules [68, 69]. Recent studies revealed that the K^+_{ATP} channel activity is regulated within a narrow range of membrane potential for its optimal function [67]. Interestingly, some studies also reported that, in a manner similar to glucose-induced insulin secretion in β-cells, high glucose increases ATP in isolated α-cells causing closure of K^+_{ATP} channels and a subsequent increase in glucagon exocytosis [70, 71]. These studies suggest that the regulation of glucagon secretion by glucose in α-cells is complex and that other regulatory mechanisms, in addition to glucose itself, play significant roles.

Recently, a study utilized purified α-cells, sorted by yellow fluorescent protein expression, to investigate the role of the K^+_{ATP} channel on glucagon secretion. The authors report that K^+_{ATP} channels are already closed at low-glucose concentrations, and high glucose which induces modest decrease in Ca^{2+} influx does not affect glucose metabolism and K^+_{ATP} channel activity [72]. These interesting findings suggest that the mechanism of Ca^{2+} influx regulation by glucose is independent of K^+_{ATP} channels which regulate Ca^{2+} entry. Thus, direct high-glucose-induced reduction of Ca^{2+} influx [73] might be insufficient and relatively less important for the suppression of glucagon secretion during hyperglycemia. This also suggests that factors other than glucose play a role in high-glucose-induced suppression of glucagon secretion [72].

18.3.2.2 Glucose and Other Nutrients

The secretion of glucagon from α-cells is elevated in response to hypoglycemia and suppressed by hyperglycemia in vivo. However, the regulation of glucagon secretion by glucose concentration is complex and the contribution of neural, hormonal, and intraislet interactions is also important (Figs. 18.2 and 18.3). While some studies

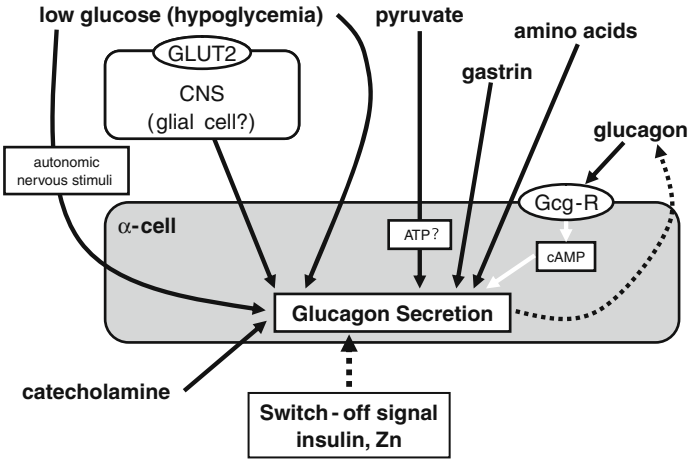

Fig. 18.2 Proposed mechanisms for stimulation of glucagon secretion. Low glucose/ hypoglycemia stimulates glucagon secretion directly and indirectly through central and autonomic nervous system. In addition to various direct stimulators, the "switch-off" signal of insulin/zinc ion stimulates glucagon secretion

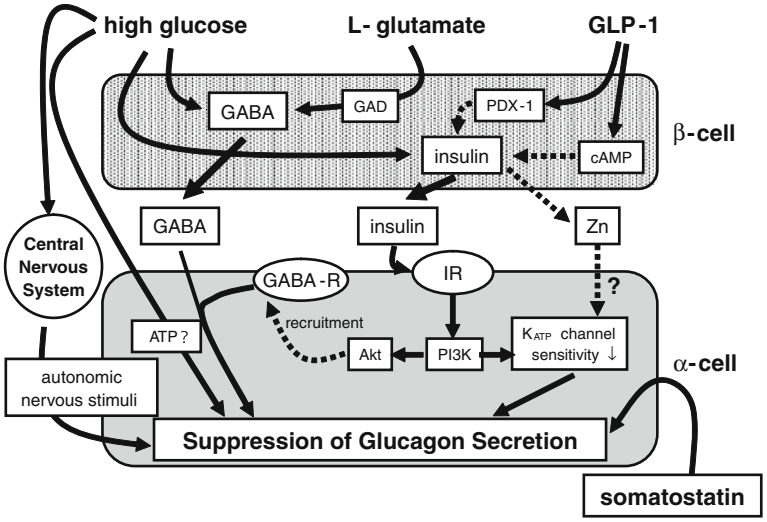

Fig. 18.3 Proposed mechanisms for inhibition of glucagon secretion. β-Cells play a critical role in suppression of glucagon secretion from α-cells via a paracrine mechanism. The β-cell secretes insulin, γ-amino-butyric acid (GABA), and zinc irons (Zn) which suppress glucagon secretion. High glucose/hyperglycemia suppresses glucagon secretion through the nervous system and by stimulation of β-cell secretion. Somatostatin also suppresses glucagon secretion

suggest a direct suppressive effect of glucose on α-cell secretory function [73, 74] the paradoxical stimulation of glucagon secretion by high glucose in isolated islets and α-cell lines [70, 71, 75] suggests that additional mechanisms contribute to the secretion of glucagon in response to glucose.

Amino acids such as L-arginine are potent stimulators of glucagon secretion [76] (Fig. 18.2). This may be physiologically relevant to prevent hypoglycemia after protein intake since amino acids also stimulate insulin secretion. L-glutamate is produced, secreted by various cell types including neural cells, and acts as a neurotransmitter. In islet α-cells, glutamate is contained in glucagon secretory vesicles [77]. Interestingly, a recent study shows that glutamate secreted by α-cells functions as an autocrine positive feedback signal for glucagon secretion [78], as α-cells express glutamate transporters and receptors [79]. Low glucose stimulates glutamate release from α-cells, which in turn acts on α-cells in an autocrine manner leading to membrane depolarization and glucagon secretion [78].

18.3.2.3 Nervous System and Neurotransmitters

While glycemia can modulate glucagon secretion directly, several reports indicate the involvement of the central and/or autonomic nervous systems in the regulation of glucagon secretion (Figs. 18.2 and 18.3) [80–83]. Hypoglycemia is a critical condition for body especially since glucose is an essential fuel for the brain. Thus in response to hypoglycemia, the nervous response immediately triggers various counter-regulatory mechanisms to protect the brain from energy deprivation, including the stimulation of glucagon secretion.

The dense innervations of the islets suggest that both α- and β-cells are regulated by the nervous system [81]. The autonomic nervous system (ANS) transmits stimuli to promote glucagon secretion especially under hypoglycemia when blood glucose must be increased to supply fuel for the body. The ANS can modulate all islet cells and regulate glucagon secretion directly via the parasympathetic pathway or indirectly by pathways that can modulate islet paracrine factors (see Section 18.3.2.4) [81]. In addition, circulating autonomic neurotransmitters epinephrine and norepinephrine have been reported to stimulate glucagon secretion from α-cells through adrenergic receptors [84, 85]. Glucagon secretion is also modulated by other neurotransmitters including GABA (see Section GABA) and glutamate (see Section 18.3.2.2).

The precise mechanism by which the central nervous system (CNS) senses blood glucose and affects glucagon secretion is not fully understood, although several possibilities have been suggested. Glucose sensing in the CNS is suggested to be an interaction between neurons and glial cells. For example, neurons in the ventromedial hypothalamus (VMH) have been reported to play a role in sensing hypoglycemia in the brain and triggering the responses of counter-regulatory hormones to impact hypoglycemia [86], through AMPK [87], K^+_{ATP} channels [82], and corticoptrophin-releasing factor receptors [88] in rat models. Moreover, it has also been reported that GLUT2 in cerebral astrocytes acts as a central glucose sensor in the modulation of glucagon secretion in mice [83].

18.3.2.4 Intraislet Regulation and Other Hormones

Given that the intraislet microcirculation is designed to flow from the core to the periphery [3, 89], intraislet autocrine effects between islet cells have been widely investigated (Fig. 18.3).

Insulin

Insulin, the major secretory product of β-cells, has been proposed as one of the intraislet paracrine factors that can modulate the secretion of glucagon from neighboring α-cells [90–92]. α-Cells are located downstream in terms of intraislet blood flow and are potential direct targets of secreted insulin from β-cells. Furthermore, proteins in the insulin signaling pathway are expressed in α-cells supporting an important role for insulin signaling in α-cells [71, 93, 94].

Several in vivo and ex vivo studies have suggested that insulin suppresses glucagon secretion [4, 90–92, 95, 96], and recent in vitro studies, using gene knockdown techniques, indicate a role for the insulin receptor and its signaling pathway in the regulation of glucagon secretion. In insulinopenic animal models, exogenous insulin suppressed glucagon secretion [4, 90, 95]. Conversely, suppression of insulin action by infusion of an anti-insulin antibody increased glucagon release [92]. In humans, insulin has been suggested to suppress glucagon secretion [28, 91, 96–98]. In α-cell lines with a knockdown of the insulin receptor, suppression of glucagon secretion by high glucose and stimulation of glucagon secretion by low glucose are abolished [74, 99]. These reports suggest a direct effect of insulin in suppressing glucagon secretion, and this possibility has been tested in α-cell-specific insulin receptor knockout (αIRKO) mice. Adult αIRKO mice exhibit mild glucose intolerance, hyperglycemia, and hyperglucagonemia in the fed state, and enhanced glucagon secretion in response to L-arginine stimulation [66]. These data provide the first direct genetic evidence of a significant role for insulin signaling in the regulation of α-cell function in vivo [66]. Insulin has been reported to act either by reducing the sensitivity of K^+_{ATP} channels [71] through phosphatidyl inositol 3-kinase (PI3K) [100], or by modulating Akt, a critical downstream effector of PI3K, leading to recruitment of the GABA-A receptor to the cellular membrane to allow its ligand, GABA, to inhibit glucagon secretion [101] (Fig. 18.3).

Thus, it is conceivable that chronic and postprandial hyperglucagonemia seen in diabetes patients is due to a lack of the direct suppression of insulin on glucagon secretion induced either by an absolute lack of insulin and/or α-cell insulin resistance [28, 97].

In addition, insulin is reported to stimulate glucagon secretion through a "switch-off" mechanism [36, 37] (Fig. 18.2). During hypoglycemia, a decrease in intraislet insulin may act as a trigger for glucagon secretion as α-cells can sense the decrease in ambient insulin. This concept is proposed by studies wherein cessation of insulin administration in in vivo pancreas perfusion experiments in insulinopenic diabetic rats induces glucagon secretion in response to hypoglycemia [36, 37]. This observation has relevance to hypoglycemic attacks in diabetes patients that occur in

response to iatrogenic hyperinsulinemia. It is also possible that the defective secretory response of glucagon to hypoglycemia in diabetes patients occurs secondary to a defect in insulin sensing in α-cells.

GABA

γ-Amino-butyric acid (GABA) is produced from the excitatory amino acid glutamate by glutamic acid decarboxylase (GAD) and works as an important inhibitory neurotransmitter in neural synapses, mainly in the central nervous system [103]. In neurons, GABA is released by the presynaptic terminal into synaptic junctions and binds to GABA receptors on the postsynaptic membrane, inhibiting cellular electrical firing through modulation of ion channels and consequent membrane hyperpolarization [103]. Islets are also innerved by GABAergic neurons [104], suggesting that GABA is a potential inhibitor of α-cell function.

In addition, GABA has also been reported to be secreted from β-cells and suppress glucagon secretion from α-cells in an intraislet paracrine manner [101, 105, 106]. High glucose or glutamate levels stimulate secretion of GABA from β-cells and the secreted GABA then binds to its receptor expressed on α-cells (Fig. 18.3), inhibiting glucagon secretion through cellular membrane hyperpoloarization. Importantly, the GABA-A receptor is recruited to the cellular membrane by insulin-Akt signaling [101], and its activation suppresses glucagon secretion through desensitization of K^+_{ATP} channels. These observations suggest a cooperative role between insulin and GABA in the inhibition of glucagon secretion (Fig. 18.3).

Zinc

Zinc ions (Zn^{2+}), co-released with insulin by β-cells, in response to high glucose levels, have been reported to activate K^+_{ATP} channels on α-cells, desensitize the channels, and suppress glucagon secretion [71, 107] (Fig. 18.3). Zn^{2+} is also reported to stimulate glucagon secretion from α-cells when its concentration falls as part of a "switch-off" mechanism [38] (Fig. 18.2). While one report suggests an involvement of K^+_{ATP} channels in Zn^{2+}-induced suppression of glucagon secretion [108], another study reports a lack of inhibitory effect of exogenous Zn^{2+} on glucagon secretion [74], indicating that the effects of Zn^{2+} on glucagon secretion are complex and require further investigation.

Somatostatin

Somatostatin, an inhibitory hormone, secreted by neuronal and pancreatic δ-cells in islets inhibits both insulin and glucagon in a paracrine manner in the islet [76, 109, 110] (Fig. 18.3). Somatostatin is considered to exert its suppressive effect on glucagon secretion largely through interstitial communication between α- and δ-cells [4]. Following binding to its receptors on α-cells somatostatin inhibits

glucagon secretion by inducing plasma membrane hyperpolarization [111], suppression of cAMP elevation [112], and direct inhibition of the exocytotic machinery via a G-protein-dependent mechanism [113].

Somatostatin secretion from islet δ-cells is stimulated by glucose [114, 115], consistent with the report that the suppressive effect of high glucose on glucagon secretion may be mediated by glucose-induced secretion of somatostatin [116]. Interestingly, global somatostatin knockout mice exhibit enhanced insulin and glucagon secretion in vivo and ex vivo. In addition the ability of exogenous glucose to suppress glucagon secretion is lost in islets isolated from somatostatin knockout mice [116] and highlights the intraislet interactions between somatostatin, glucagon, and insulin. These observations from a global knockout of somatostatin should be interpreted with caution since extra-pancreatic neuronal effects cannot be ruled out. It should also be noted that somatostatin involvement in glucagon suppression during hyperglycemia might be less important than the effects of β-cell secretion in vivo according to the direction of intraislet microcirculation, β–α–δ [4, 117]. Further investigation is thus necessary to clarify the intraislet relationship of islet hormones.

Glucagon-Like Peptide-1 (GLP-1)

The incretin hormone, glucagon-like peptide-1 (GLP-1), is secreted by intestinal L-cells in response to food intake and is a potent stimulator of insulin secretion from β-cells [118]. GLP-1 is reported to suppress glucagon secretion indirectly either by stimulating insulin secretion or by modulating other hormones which are potential glucagon secretion suppressors [120] (Fig. 18.3). A recent study reported that GLP-1 inhibits glucagon secretion even in the absence of secretory products from β-cells and suggested the involvement of somatostatin. This was based on the observation that a highly specific somatostatin receptor subtype 2 (SSTR2) antagonist completely abolished the GLP-1 effect on glucagon secretion in isolated perfused rat pancreas [121]. A direct action of GLP-1 on α-cells is also suggested to suppress glucagon secretion [122, 123]. However, there are conflicting reports concerning the expression of GLP-1 receptors in α-cells [49, 124]. A recent study using in situ hybridization and immunofluorescence microscopy in mouse, rat, and human pancreas identified which islet cell types express GLP-1 receptors [125] and concluded that GLP-1 receptors are not expressed in α-cells. However, reports of GLP-1-induced suppression of glucagon secretion, in addition to its beneficial role on β-cells including augmentation of glucose-stimulated insulin secretion, promotion of β-cell proliferation, and protection of β-cells from various cytotoxicities, emphasize the potential of GLP-1 therapy for the treatment of diabetes.

Paradoxically, another incretin hormone, glucose-dependent insulinotropic polypeptide (GIP), can stimulate glucagon secretion despite stimulating insulin secretion from β-cells in a manner similar to GLP-1 [121, 126, 127]. On the other hand, GLP-2, although derived from the same proglucagon gene as GLP-1, in intestinal L-cells, has not been reported to affect the secretory properties of β-cells but stimulates glucagon secretion in human subjects [102], by activation of GLP-2 receptors on α-cells [128].

Glucagon

Glucagon itself stimulates glucagon secretion from α-cells in an autocrine manner [17] (Fig. 18.2). Upregulation of cAMP by glucagon signaling is suggested to stimulate glucagon exocytosis via a mechanism that is similar to the stimulatory effects of glucagon on insulin and somatostatin secretion [16, 129].

18.4 Growth of α-Cells

18.4.1 Development

In mice, glucagon-containing cells are among the first endocrine cells to be formed in the developing pancreas (for detailed reviews on pancreas development see references [54, 130–132]). The formation of mature functional α-cells requires the activation of a complex network of transcription factors that are expressed sequentially. One of the initial factors is the pancreatic duodenal homeobox-1 (PDX-1), a homeobox gene, which commits cells to the pancreatic lineage. In a similar manner, the LIM homeodomain gene, Isl-1, is also essential for pancreas development [133, 134]. The endocrine differentiation program is then initiated by Ngn3 (neurogenin3) a basic helix-loop-helix transcription factor that is transiently expressed in cells at early stages of development and controls the expression of a complex network of transcription factors required for the differential expression of the endocrine subtypes including: Arx, MafA, MafB, BETA2/NeuroD, Nkx6.1, Nkx6.2, Pax4, and Pax6 [135].

Pax6, a paired-homeodomain transcription factor, is expressed during the early stages of endocrine cell development and is essential for α-cell development [136]. In contrast Brain4 (Brn4) is expressed abundantly and exclusively in α-cells [137]; however, homozygous Brn4 knockout mice exhibit normal pancreatic bud formation and glucagon cell numbers suggesting that Brn4 is not essential for α-cell development [137]. Many initial islet cell fate decisions are regulated by the NK homeodomain protein, Nkx2.2, which is present in the earliest pancreatic progenitor cells [138]. Nkx2.2 knockout mice exhibit reduced numbers of α-cells, suggesting that the transcription factor is responsible for the differentiation of the majority of α-cells. In addition, Arx (aristaless-related homeobox) is necessary and sufficient to instruct α-cell fate [139]. Loss of Arx function causes hypoglycemia due to early onset loss of mature α-cells [119, 139] whereas the missexpression of Arx during development leads to loss of β- and δ-cells and a corresponding increase in α- and PP-cells [140]. The differentiation of α-cells is at least in part regulated by MafB (bZip protein) [141–143] and the forkhead/winged helix family member, Foxa2 (HNF3β). Foxa2 lacking mice are severely hypoglycemic with a 90% reduction in glucagon expression and a reduction in mature α-cells. However, the α-cell progenitors are not affected suggesting that Foxa2 acts at a late stage of α-cell development [144].

Likewise in the human fetal endocrine pancreas, PDX-1 is not expressed in glucagon-expressing cells, suggesting that the absence of PDX-1 is essential for α-cell development [145]. Ngn3 is co-localized with newly differentiated endocrine cells, but not in mature islets suggesting it is critical for the establishment of islet cell types. In all endocrine cell types ISL1, NeuroD1, Nkx2.2, and Pax6 are upregulated during development whereas Nkx6.1 is only expressed in β-cells [145].

18.4.2 Alterations in α-Cell Distribution in Human Type 1 and Type 2 Diabetes

An increase in the pancreatic α-cell mass has been reported in patients with type 2 diabetes [26, 27, 146]. A similar increase in α-cell mass has also been reported to occur in type 1 diabetes by some studies [19] but not others [27]. The mechanism(s) modulating the increased α-cell mass is unclear, although several possibilities have been proposed using animal models (described in Section 18.4.3).

18.4.3 Animal Models Exhibiting α-Cell Hypertrophy

Although several animal models of α-cell hyperplasia have been described the mechanisms that regulate α-cell growth in adults are not fully understood. For example 129/J mice on a high-protein diet display hyperplasia and hypertrophy of α-cells [147], while mice on a high-fat diet develop α-cell hyperplasia [148]. IL-6 has been proposed as a factor that might be involved in the increase in α-cell mass in diabetes as levels of this cytokine are increased in type 2 diabetes and the IL-6 receptor is highly expressed in α-cells [148]. Mice lacking IL-6 are unable to increase their α-cell mass in response to high-fat feeding and have decreased fasting glucagon levels compared to control animals [148].

In a similar manner to insulin resistance-induced β-cell hyperplasia, it has been suggested that α-cells can sense glucagon resistance and increase secretion and proliferation as a compensatory mechanism. Indeed α-cell hyperplasia is observed in animal models in which glucagon signaling has been inhibited, either by reduction of glucagon receptor expression or by a decrease in expression of components of the downstream signaling pathway [10, 149, 150]. For example, global glucagon receptor knockout mice manifest postnatal α-cell hyperplasia with very high circulating levels of glucagon and exhibit lower blood glucose levels and improved glucose tolerance, despite normal circulating insulin, due to reduced hepatic glucose output [10].

Antisense oligonucleotides (ASO) targeted to the glucagon receptor have been successful at reducing glucagon receptor expression in mouse livers [149, 151]. In db/db mice ASO treatment improved glucose tolerance and increased levels of circulating glucagon which, in one study, correlated with an accompanying increase in

α-cell mass [149], whereas another study reported an increase in glucagon content of the α-cells, but no increase in α-cell number [151].

The glucagon receptor, upon binding of glucagon, increases cAMP levels by activating Gsα. Mice with a liver-specific ablation of Gsα have elevated serum glucagon and GLP-1 levels and marked α-cell hyperplasia, presumably as a result of hepatic glucagon resistance [150]. These data suggest that decreased glucagon signaling in target organs likely feeds back to increase glucagon secretion and to promote α-cell proliferation.

Mice with disruption of the gene coding for the enzyme responsible for proglucagon processing, PC2, are unable to process glucagon to its mature form and exhibit altered development of α-cells, increased proliferation of proglucagon cells in the perinatal period, and a dramatic postnatal α-cell hyperplasia, which causes mild hypoglycemia [152]. The α-cell hyperplasia was corrected by exogenous glucagon delivered by a micro-osmotic pump, leading to increased α-cell apoptosis and a downregulation of proglucagon mRNA [153], suggesting that appropriate glucagon action/production is pivotal for α-cell homeostasis.

Although insulin inhibits glucagon gene expression, it is possible that it promotes α-cell survival and proliferation. Indeed mice with an α-cell-specific knockout of the insulin receptor failed to increase their α-cell mass with age compared to controls [66]. In addition, pancreas-specific insulin receptor substrate 2 (IRS2; a protein in the insulin signaling pathway) knockout mice display reduced α-cell mass and glucagon secretion [154]. Taken together these studies suggest that hyperinsulinemia in type 2 diabetes could be one factor that directly contributes to α-cell hyperplasia.

Mice lacking the micro-RNA MiR-375 have a slight reduction in β-cell mass and a concomitant increase in α-cells in the presence of increased circulating glucagon levels [155]. Despite the decreased β-cell mass, insulin secretion levels remained normal, suggesting that the increase in α-cell mass is due to a direct effect of MiR-375 in regulating α-cell proliferation. However it is possible that the increase in α-cell mass is a compensatory response to decreased β-cell number and/or due to increased glucagon secretion [155].

18.5 Strategies for Restoring Glucagon Secretion

The hyperglucagonemia seen in patients with type 2 diabetes likely contributes in part to systemic hyperglycemia by elevating hepatic glucose output. Thus therapies which block glucagon action would be a useful strategy for the treatment of type 2 diabetes. Although specific modulators of glucagon action are not yet in use, several approaches are being explored. This includes peptide glucagon receptor antagonists, which successfully normalized glucose homeostasis in diabetic rats providing proof of concept that inhibiting the glucagon receptor is beneficial for improving glycemic control [156]. Non-peptide small molecule inhibitors of the glucagon receptor have also been developed with the aim of limiting hypoglycemia in type 2 diabetes

[157–159]. Work is continuing to optimize the specificity of glucagon receptor antagonists. For example, Kodra et al. have created a compound with a 1000-fold greater selectivity for the glucagon receptor over the GLP-1 receptor, which is effective at lowering blood glucose levels in ob/ob mice [160]. As an alternative to glucagon receptor antagonists, antisense oligonucleotides (ASOs) have been designed against the glucagon receptor to reduce its expression. In rodent models of type 2 diabetes, antisense oligonucleotides (ASO) targeted to the glucagon receptor, decreased blood glucose levels, and improved glucose tolerance. This was accompanied by decreased expression of cAMP target genes in the liver and inhibition of glucagon-induced hepatic glucose output [149, 151].

Another strategy for limiting the levels of circulating glucagon is immunoneutralization using monoclonal antibodies against glucagon. In animal models a high-affinity monoclonal antibody was reported to eliminate free circulating glucagon [161]. In several models of diabetes including alloxan-induced diabetic rabbits, moderately hyperglycemic STZ rats, and ob/ob mice, acute treatment with a glucagon monoclonal antibody reduced plasma glucose concentrations [161, 162] and chronic treatment of ob/ob mice with the monoclonal antibody reduced A1c levels [163].

Several drugs already in use for the treatment of type 2 diabetes have been reported to impact upon glucagon action. The TZD class of drugs which primarily act as insulin sensitizers can also inhibit glucagon gene expression [61]. Furthermore, rosiglitazone has been shown to decrease glucagon mRNA levels in STZ-treated rats [164] and studies in humans indicate that pioglitazone either decreases [165] or has no effect [166] on circulating glucagon. The incretin hormone, GLP-1, decreases fasting and post-meal glucagon levels in healthy volunteers and patients with type 2 diabetes [167]. This suggests that GLP-1 therapy, in addition to enhancing insulin secretion, is useful in inhibiting excess glucagon secretion in type 2 diabetes patients. Several drugs designed to increase GLP-1 levels are currently used to treat type 2 diabetes patients, including the GLP-1 mimetic, exenatide [168], and the GLP-1 analogue, liraglutide [169]. In addition, DPP-4 (dipeptidyl peptidase-4) inhibitors, such as vildagliptin, increase levels of GLP-1 by preventing its breakdown by DPP-4 [170]. DPP-4 inhibitors can also increase GIP [171], which has been shown to increase glucagon secretion; however in patients with type 2 diabetes, the DDP-4 inhibitor vildagliptin suppressed meal-stimulated glucagon [172].

18.5.1 Potential Limitations

The data from animal and human studies clearly point to the potential for modulation of glucagon action as an effective approach to reduce hyperglycemia in diabetic patients. Some of the studies however also highlight the problems of this strategy. For example, experiments in which glucagon signaling was abolished resulted in an increase in α-cell hyperplasia [10, 149, 150]; whether this occurs also in

humans is not known. Any treatment that causes an increase in cell proliferation obviously requires careful evaluation of its safety. In addition glucagon signaling may be required for functions in metabolic tissues besides regulating glucose output in the liver. Glucagon has been shown to inhibit triglyceride synthesis and secretion and stimulate fatty acid oxidation in hepatocytes, and interestingly this effect is abolished in glucagon receptor knockout mice [173], suggesting that blocking glucagon signaling is associated with an increased risk of dyslipidemia and fatty liver. Glucagon signaling may also be important in promoting hepatocyte survival as glucagon receptor knockout mice are more susceptible to experimental liver injury [174].

Thus complete blockade of glucagon signaling as a means of treating diabetes may not be entirely beneficial. Instead, it would be desirable to design therapies that can attenuate the elevated levels of glucagon present in type 2 diabetes.

18.6 Perspective

Although several new modulators of α-cell function are being unraveled it is clear that the regulation of glucagon secretion is complex and requires one or more of these factors to act in concert to counter the effects of insulin. While this complexity is an advantage for the organism so that more than one fail-safe mechanism is available to maintain physiological levels of glucagon during stress, the multiple factors and pathways that can affect α-cell function have eluded therapeutic attempts to successfully modulate glucagon secretion in vivo. Further studies are necessary to explore whether cells in the central and/or autonomic nervous systems can be targeted to modulate glucagon secretion for therapeutic purposes.

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Chapter 19
Mechanisms of Pancreatic β-Cell Apoptosis in Diabetes and Its Therapies

James D. Johnson and Dan S. Luciani

Abstract Diabetes occurs when β-cells no longer function properly or have been destroyed. Pancreatic β-cell death by apoptosis contributes significantly in both autoimmune type 1 diabetes and type 2 diabetes. Pancreatic β-cell death can be induced by multiple stresses in both major types of diabetes. There are also several rare forms of diabetes, including Wolcott-Rallison syndrome, Wolfram syndrome, as well as some forms of maturity onset diabetes of the young that are caused by mutations in genes that may play important roles in β-cell survival. The use of islet transplantation as a treatment for diabetes is also limited by excessive β-cell apoptosis. Mechanistic insights into the control of pancreatic β-cell apoptosis are therefore important for the prevention and treatment of diabetes. Indeed, a substantial quantity of research has been dedicated to this area over the past decade. In this chapter, we review the factors that influence the propensity of β-cells to undergo apoptosis and the mechanisms of this programmed cell death in the initiation and progression of diabetes.

Keywords Clinical islet transplantation · Autoimmune diabetes · Glucotoxicity and lipotoxicity · Endoplasmic reticulum stress · Gene–environment interactions · Mitochondrial death pathway

Abbreviations

MODY	maturity onset diabetes of the young
NOD	non obese diabetic
UPR	unfolded protein response
VNTR	variable number of tandem repeats
GLP-1	glucagon-like peptide 1

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19.1 Introduction to β-Cell Apoptosis

To a large extent glucose homeostasis and susceptibility to diabetes are determined by a person's functional β-cell mass. Functional β-cell mass is the product of β-cell number, β-cell size, and the ability of individual β-cells to secrete mature insulin in a correct manner (Fig. 19.1). It has become increasingly evident that β-cell apoptosis contributes to the development of both type 1 diabetes (autoimmune diabetes), type 2 diabetes (adult-onset diabetes), as well as to the more rare forms of the disease such as the various types of maturity onset diabetes of the young (MODY) [1–5]. Basal β-cell apoptosis also plays a role in the remodeling and development of the normal endocrine pancreas. For example, β-cells undergo a wave of apoptosis around the time of birth [2], which is followed by a proliferation-driven postnatal expansion of β-cell mass [6]. At all stages of life, β-cell replication and death are tightly controlled by intrinsic and extrinsic factors that control how β-cell mass adjusts to meet metabolic demand [7]. Only when a combination of genetic and environmental influences causes this balance to fail does diabetes develop. Despite major advances in recent years, the nature of the gene–environment interactions that promote β-cell apoptosis in diabetes remain unclear, as do many aspects of the apoptotic pathways involved. In this chapter, we review some of the central mechanisms that have been implicated in the control of β-cell apoptosis to date, as well as current therapeutic efforts that target these pathways.

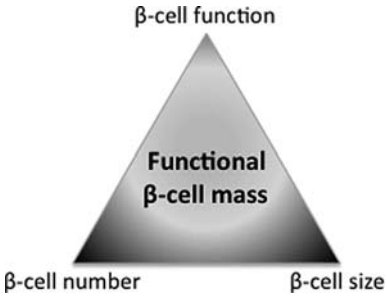

Fig. 19.1 Factors that dictate the functional β-cell mass

19.2 Increased β-Cell Apoptosis as a Trigger and Mediator of Type 1 Diabetes

Type 1 diabetes is an autoimmune disease in which the pancreatic β-cells are gradually destroyed, but the initial trigger for this destruction and the exact mechanisms of β-cell death remain enigmatic. Like necrosis, excessive apoptosis is capable of initiating an immune response in susceptible individuals. It has been suggested that a perinatal wave of β-cell apoptosis may promote the presentation of β-cell auto-antigens and thus provoke an autoimmune response against β-cells [2, 8, 9].

Clues to the cause and pathobiology of type 1 diabetes also come from the analysis of its genetics. In most cases, genes linked to type 1 diabetes are known to play specific roles in the immune system. IDDM1, is the human leukocyte antigen system

superlocus containing the major histocompatibility complex genes. This region of the human and the non-obese diabetic (NOD) mouse genome confers the majority of the risk for type 1 diabetes. Interestingly, the insulin gene itself (IDDM2) is the second most significant type 1 diabetes gene in humans. The genetic alterations are not in the coding sequence of insulin, but in an upstream regulatory region called the 'variable number of tandem repeats' or VNTR [10–12]. At-risk alleles appear to reduce the expression of the insulin gene in the thymus where it is thought to play a role in tolerance [10]. At the same time, VNTR sequences that confer diabetes risk increase insulin mRNA in the islets. High doses of insulin can have deleterious effects on the survival of β-cells under some culture conditions [13, 14]. If this was also the case in vivo, one might expect that the VNTR could increase type 1 diabetes risk via direct effects on β-cell apoptosis. Recently, genome-wide association studies have identified several single nucleotide polymorphisms that contribute modest risk to type 1 diabetes. While most of these would be expected to target immune cells specifically, the PTPN2 phosphatase was recently shown to modulate pancreatic β-cell apoptosis via effects on the transcription factor STAT1 [15]. Thus, genes that confer risk to type 1 diabetes may also affect β-cell death directly.

The mechanisms by which β-cells are selectively killed by the immune system have been studied extensively, and appear to involve multiple pathways (Fig. 19.2). One key mechanism is the activation of 'death receptors,' Fas and tumor necrosis factor receptor, by their respective ligands. Interestingly, Fas expression is negligible

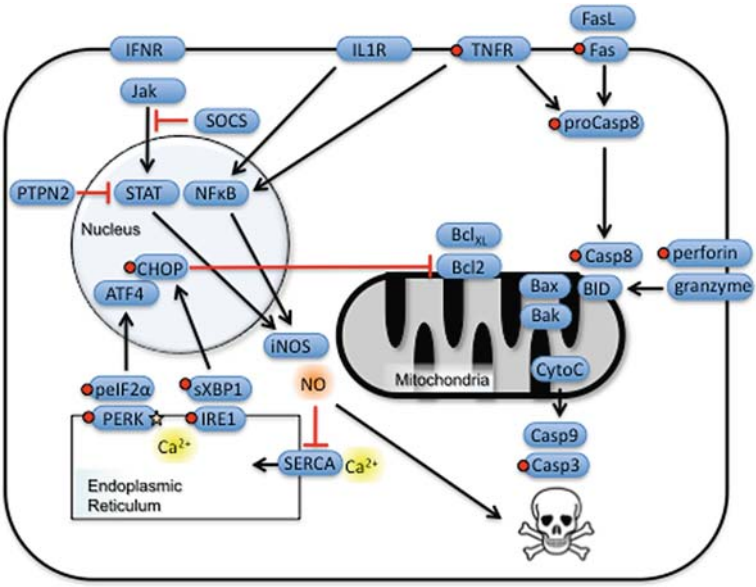

Fig. 19.2 Molecular mechanisms controlling β-cell apoptosis in type 1 diabetes. Shown is a partial description of signaling cascades that modulate β-cells survival in type 1 diabetes. Protein products of genes that are linked to human diabetes are denoted with a star. Genes that have been implicated in β-cell mass using in vivo or molecular loss-of-function experiments (i.e., knockout mice) are denoted with a dot

in normal β-cells and it may be up-regulated by cytokines such as IL-1 [16]. Activation of Fas by FasL converts pro-caspase-8 to active caspase-8 [2]. Caspase-8 then acts via the pro-apoptotic BH3-only Bcl family member Bid to promote mitochondrial outer membrane permeablization and cytochrome c release [17]. Bid may do so by interacting directly with the pro-apoptotic effector Bcl protein Bax and activate its channel-forming functions in the outer mitochondrial membrane [18].

Another pathway of β-cell apoptosis in type 1 diabetes involves perforin and granzyme B, cytotoxic components released by CD8+ T cells. Mouse models suggest CD8+ T cells to be major effectors of immune-mediated β-cell death and perforin knockout mice on an NOD background have reduced diabetes incidence compared with NOD controls [16]. Granzyme B cleaves multiple substrates in the target cell, including Bid and studies with islets from Bid knockout mice demonstrate that Bid is also key in this β-cell death cascade [19].

The involvement of other Bcl family members in type 1 diabetes and its animal models is less clear. Pancreatic islets isolated from Bax knockout mice are partially protected from death receptor-triggered β-cell apoptosis, in agreement with Bax as the downstream effector of mitochondrial outer membrane permeabilization following Bid activation [17]. Efforts to block diabetes using transgenic mice overexpressing Bcl-2 under the control of the rat insulin promoter have provided mixed results [16]. To date, no in vivo loss-of-function experiments have demonstrated an essential role for anti-apoptotic Bcl-2 or Bcl-x_L in β-cell survival. Interestingly, Bcl family proteins such as Bad may also play key roles in β-cell metabolic function [20], making studies into the joint role of these proteins especially important.

While the Bcl proteins collectively control mitochondrial outer membrane permeabilization and cytochrome c release, the majority of the β-cell 'execution' steps are triggered by the activation of effector caspases such as caspase-3. These proteases also co-ordinate the semi-ordered disassembly of β-cells with members of the calpain family of calcium activated proteases. Pancreatic β-cell apoptosis is promoted by caspase-3 and caspase-9, essential mediators in the intrinsic pathway of apoptosis. In cell culture models, β-cell death can be abrogated with inhibitors of caspase-3 activity [21]. In vivo, mice lacking caspase-3 in their β-cells are protected from type 1 diabetes [8]. Interestingly, isolated islets from β-cell-specific caspase-8 knockout mice are protected from Fas-induced apoptosis, but have increased 'basal' apoptosis and glucose intolerance in the absence of frank diabetes [22]. These results suggest that the action of caspases can be context-dependent in the β-cell.

19.3 Pancreatic β-Cell Apoptosis as a Complication of Diabetes: Glucose Toxicity

Pancreatic β-cells are exquisitely sensitive to metabolic stress, since they must transduce changes in blood glucose levels into insulin release via glycolytic and mitochondrial ATP production [7]. Since both hyperglycemia and hyperlipidemia are hallmarks of the diabetic state, β-cell apoptosis is also likely to be an important complication of diabetes. This downward spiral likely plays a significant role in the

rapid reduction in functional β-cell mass that precipitates the onset of both type 1 and type 2 diabetes. Chronically elevated glucose induces β-cell apoptosis via multiple mechanisms, including modulating the gene expression of multiple Bcl family members [23]. Toxic reactive oxygen species are produced by hyperactive mitochondria and β-cells contain relatively low levels of some key antioxidant proteins [23–25]. Moreover, elevated Ca^{2+} levels of overworked β-cells are toxic to the cells [26, 27]. This excitotoxicity may be the cause of the eventual clinical failure of long-term sulphonylurea treatment, which depolarizes β-cells by directly closing K_{ATP} channels [28]. Prolonged hyperglycemia may also activate Fas-mediated β-cell apoptosis [29] and pathways controlled by the pro-apoptotic protein TXNIP [30]. Moreover, chronic hyperglycemia increases secretory demand, which has been speculated to cause ER-stress due to the increased requirement for protein synthesis and processing (see below).

19.4 Apoptosis as a Contributing Factor in Type 2 Diabetes

It is established that pancreatic β-cell death is a key event in type 1 diabetes, but evidence has only recently emerged supporting an important role for β-cell apoptosis in the pathobiology of type 2 diabetes [3, 5, 31–37] (Fig. 19.3). Type 2 diabetes is

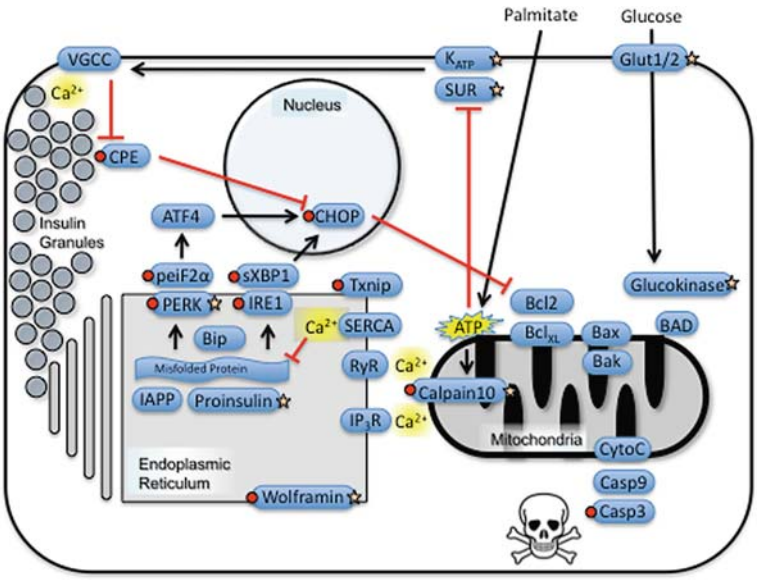

Fig. 19.3 Molecular mechanisms controlling lipid- and glucose-induced β-cell apoptosis in type 2 diabetes. Shown is a partial description of signaling cascades that modulate β-cell survival. Protein products of genes that are linked to human diabetes are denoted with a star. Genes that have been implicated in β-cell apoptosis or β-cell mass using in vivo loss of function experiments (i.e., knockout mice) are denoted with a dot

a disease of gene–environment interactions, with obesity and hyperlipidemia being the main manifestations of the 'environment'. Obesity is associated with inflammation and insulin resistance in a multitude of key metabolic tissues, including liver, fat, and muscle [38, 39]. In the majority of obese people, an expansion of β-cell mass and workload can effectively compensate for the increased insulin secretory demand [31, 36, 37]. However, if this compensatory increase in β-cell mass and function fails, the obese individual will progress to frank type 2 diabetes. Compared to weight matched controls, patients with type 2 diabetes exhibit a 60% reduction in β-cell mass associated with significantly increased β-cell apoptosis and ER-stress [36, 37]. A disruption of islet architecture and an accumulation of amyloid deposits are also associated with type 2 diabetes [40].

19.5 Mechanisms of β-Cell Apoptosis in Type 2 Diabetes: ER-Stress

Pancreatic β-cells are the body's only source of blood-borne insulin and therefore must produce and secrete large amounts of this hormone as well as other hormones such as amylin. This high secretory demand makes them susceptible to secretory pathway stress, especially when demand is increased by insulin resistance. Elevated protein flux through the ER and Golgi can result in misfolded proteins and activation of the unfolded protein response (UPR) [1, 41, 42]. Three main ER-resident signaling molecules, PERK, ATF6, and IRE1 act as sensors to trigger cellular adaptation responses, or ultimately β-cell apoptosis if the stress is not alleviated. Important components of the initial 'rescue response' are the PERK-triggered and eIF2α-mediated regulation of protein translation as well as an increased ER-associated degradation of misfolded proteins. When these rescue efforts fail, apoptosis is triggered. The relative sensitivity of β-cells to ER-stress-induced cell death is illustrated by humans and mice with mutations in PERK, since other cells in the body can be largely unaffected [42]. The transcription factor CHOP is a major mediator of ER-stress-induced apoptosis downstream of PERK and ATF6. Mice lacking the CHOP gene are resistant to β-cell apoptosis following ER-stress and are protected from developing diabetes under these conditions [41, 43]. Importantly, there is now increasing evidence of ER-stress in islets of human type 2 diabetes patients [41, 44], suggesting that ER-stress does in fact contribute to β-cell apoptosis during the progression of type 2 diabetes. It is less clear whether β-cell ER-stress can initiate β-cell death prior to the onset of type 2 diabetes.

19.6 Mechanisms of β-Cell Apoptosis in Type 2 Diabetes: Lipotoxicity

Obesity is thought to trigger type 2 diabetes by causing hyperlipidemia and insulin resistance. These events impose increased secretory demand on individual β-cells, which can activate the UPR, as outlined above. Moreover, elevated fatty acids such

as palmitate, have direct toxic effects on the β-cell via activation of a number of relatively separate apoptosis-inducing events, including the generation of ceramide and reactive oxygen species. Palmitate activates the caspase-3-dependent mitochondrial apoptosis pathway [33]. Some investigators have shown that the activation of caspase-3 by palmitate is synergistic with the detrimental effects of high glucose [45, 46], but it also triggers β-cell apoptosis in the absence of elevated glucose levels [33]. Palmitate also decreases the expression of the anti-apoptotic Bcl-2 protein [47]. The type 2 diabetes susceptibility gene, calpain-10 is also implicated in palmitate-induced β-cell death, since islets lacking calpain-10 have ~30% reduced apoptosis and mice with transgenic over-expression of calpain-10 are more susceptible to palmitate toxicity [35]. Moreover, palmitate has been demonstrated to directly act on the distal components of the insulin processing machinery of the β-cell. Specifically, palmitate induces a rapid, Ca^{2+}-dependent degradation of carboxypeptidase E, the final enzyme required for the conversion of proinsulin into mature insulin [33]. Carboxypeptidase E is also reduced in high fat fed mice and the transgenic MKR mouse model of insulin resistance [48]. A decrease in carboxypeptidase E is sufficient to induce CHOP-dependent ER-stress and β-cell apoptosis in vivo and in vitro. It is unclear how reduced carboxypeptidase E modulates β-cell apoptosis, but two possibilities can be considered. In one scenario, a backlog of unprocessed insulin induces the UPR from inside the cell. It is also possible that a reduction in local release of mature insulin could impair β-cell survival. Substantial evidence suggests local insulin levels at the right concentration may help protect β-cells against ER-stress and apoptosis [13, 49–51], and islets from patients with type 2 diabetes exhibit reductions in several critical insulin signaling components [52]. Fatty acids, including palmitate, also modulate secretory pathway stress by partially depleting ER Ca^{2+} stores. Although an incomplete ER Ca^{2+} reduction alone is not sufficient to induce ER-stress, this event activates PERK and it is likely that this could potentiate ER-stress induced by other factors [53].

19.7 Mechanisms of β-Cell Apoptosis in Type 2 Diabetes: Pro-inflammatory Cytokines

There is emerging evidence that pro-inflammatory cytokines and immune cell infiltration of the islet are common factors in type 1 diabetes and type 2 diabetes. The type 2 diabetic milieu of increased hyperglycemia and hyperlipidemia appears to stimulate the production of IL-1β from islets themselves. This has been suggested to have local inflammatory effects and advance subsequent islet infiltration by macrophages to promote apoptosis in type 2 diabetes [54]. There is evidence that pro-apoptotic cytokines (IL-1, TNFα, IFNγ) can act through nitric oxide to decrease the expression of the SERCA pumps that load Ca^{2+} into the ER, which in turn impairs Ca^{2+}-dependent protein processing and promotes ER-stress-induced β-cell apoptosis [41, 55]. This is in addition to changes in ER Ca^{2+}-release channels seen in the diabetic state [56]. Cytokines might thus promote similar types of

β-cell apoptosis in type 1 and type 2 diabetes, but the extent to which overlapping pathways are involved has been questioned [34].

19.8 Genetic Factors Affecting β-Cell Apoptosis in Type 2 Diabetes

Type 2 diabetes is a polygenic disease, with dozens of genes being implicated via both candidate studies and unbiased genome-wide approaches. Some of the first gene candidates studied for their role in type 2 diabetes were ones that play important roles in β-cell function. These included the components of the ATP-sensitive potassium channels (KCNJ11, ABCC8). PPARγ was also linked to type 2 diabetes risk, and recent experiments point to a role for PPARγ in β-cell apoptosis [57]. The first type 2 diabetes susceptibility gene discovery by unbiased linkage mapping was calpain-10 [58], although this association is not seen in all populations. In the β-cell, calpain-10 likely plays a pro-apoptotic role in addition to a role promoting insulin secretion [59]. Additional in vivo studies are required to determine the detailed roles of the calpain-10 gene, which encodes for 8 splice variants, in the maintenance of glucose homeostasis.

Newer genome-wide association studies have found about 20 single nucleotide polymorphisms that show significant and reproducible associations with type 2 diabetes [60]. The susceptibilities conferred by these loci are greater than those of the candidate genes or calpain-10. Most of these genes are expressed in the endocrine pancreas, suggesting β-cells should be considered the main target of the genetic component in type 2 diabetes. In European populations the strongest effect is associated with TCF7L2, a transcription factor involved in the development and survival of islet cells and enteroendocrine cells of the gut. In vitro studies implicate TCF7L2 in β-cell apoptosis associated with increased caspase-3 cleavage and decreased Akt activity [61]. Pancreatic β-cell function is also reduced in patients with TCF7L2 polymorphisms [62]. It is important to realize that each of the top 20 diabetes-linked genes has minimal effect on its own and that its combined effects are not synergistic. Also, its net contribution cannot explain the apparent heredity of type 2 diabetes, suggesting either that heredity has been overestimated or that epigenetic factors are dominant in the development of type 2 diabetes. The epigenetics of β-cell death in type 2 diabetes will be an important area for investigation in the future, given the persistent effects of fetal and early nutrition on β-cell function and survival.

19.9 The Role of β-Cell Apoptosis in Rare Forms of Diabetes

Although the common forms of type 1 and type 2 diabetes are polygenic, several rare forms of diabetes are caused by mutations in single genes. In most cases, these genes are important for β-cell survival or function. Monogenic causes of diabetes include

mutations in proinsulin that prevent its proper folding, cause ER-stress, β-cell death, and result in early onset diabetes [63]. Wolcott-Rallison syndrome is caused by mutations in the ER-stress-sensing protein PERK [41]. ER-stress-induced β-cell apoptosis may also be the cause of diabetes in Wolfram syndrome [64]. Several of the six MODY genes, may also influence β-cell survival. The prime example here appears to be Pdx-1 (MODY4). Mice lacking one allele of Pdx-1 have increased β-cell apoptosis, caspase-3 activation, a reduction in the Bcl-x_L to Bax ratio, and a 50% decrease in β-cell mass evident at one year of age [65]. This increase in apoptosis might reflect the fact that full expression of Pdx-1 is required for the pro-survival effects of insulin and incretin hormones in the β-cell [13, 66]. Other MODY genes, including HNF1α, have also been linked to β-cell apoptosis, [4, 67]. Pancreatic β-cells expressing a dominant-negative HNF1α exhibit caspase-3- and $Bclx_L$-dependent apoptosis [68]. Collectively, the genes implicated in monogenic diabetes illustrate the critical importance of β-cell function and survival in human glucose homeostasis.

19.10 Islet Engraftment and β-Cell Death in Islet Transplantation

Islet transplantation is severely limited by β-cell death at several stages of this clinical treatment. Since islets are isolated from cadaveric donors, a number of factors reduce the viability of islets even before they are isolated, including the age and health status of the donor as well as organ ischemia and the time from donor death to islet harvest. The process of islet isolation itself also causes significant β-cell death, by both necrosis and apoptosis. Islets are then cultured, typically at high density, and this is associated with a 2–20% apoptosis rate, which is markedly higher than what is observed in vivo [69, 70]. The implantation of islets into the liver is associated with rapid β-cell death, with only a fraction of islets engrafting with sufficient microvasculature. During and after the process of engraftment, β-cells also experience toxicity from the immunosuppressant drugs that are currently required to prevent allo- and auto-rejection of the transplant. A side-by-side comparison of three clinically significant immunosuppressant drugs revealed distinct differences in the mechanisms by which they impair β-cell function and survival [71]. Clinically relevant doses of rapamycin and mycophenolate mofetil increased caspase-3-dependent apoptosis and CHOP-dependent ER-stress in human islets, but did not have direct effects on glucose-stimulated insulin secretion. On the other hand, FK506, which had direct deleterious effects on insulin secretion, but caused relatively modest induction of caspase-3 activation and ER-stress, resulted in the worst graft function in vivo when transplanted into STZ-diabetic NOD.Scid mice. Treating islet cultures with the glucagon-like peptide 1 (GLP-1) agonist Exenatide ameliorated the effects of these drugs on human β-cell function and survival [71].

Thus, islet transplantation is associated with a cluster of related stresses including hypoxia and nutrient deprivation. The specific mechanisms that mediate β-cell death

from hypoxia remain to be fully elucidated, but likely involve hypoxia-inducible factors (HIF) [72]. Interestingly, von Hippel-Lindau factor and HIF1β have also been implicated in β-cell function [52, 73]. Pancreatic β-cells can undergo programmed cell death under hypoglycemic conditions, and this environment appears to regulate the expression of HIF1β [74]. The RyR2 Ca^{2+} channel and calpain-10 appear to be involved in β-cell death in hypoglycemia as well [35]. In adult islets, these genes form a network that also includes Presenilin, Notch, Neurogenin-3, and Pdx-1. This gene network appears to influence the basal rate of apoptosis, specifically under low glucose conditions [69, 70]. Whether hypoglycemia, nutrient deprivation, or hypoxia is involved in the progression of diabetes is not well understood. Such a scenario might occur under conditions where genetic or acquired defects in the extensive intra-islet vascular network restrict the delivery of oxygen and nutrients to the β-cells [75].

19.11 Survival Factors that Prevent β-Cell Apoptosis

A large number of endogenous and exogenous growth factors have been shown to promote β-cell survival, in vitro or in vivo. Some of the key factors are discussed here (Fig. 19.4). Examples of such anti-apoptotic signaling cascades are those activated by the gut hormones GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), which were first investigated for their positive effects on glucose-stimulated insulin secretion. The new diabetes drug Byetta acts by mimicking GLP-1 and has been shown to protect rodent β-cells from apoptosis when administered at high doses [66]. It is likely that other hormones that increase cAMP and activate RyR Ca^{2+} channels would also have anti-apoptotic effects on β-cells. It has also been suggested that inhibiting dipeptidyl peptidase-4, an enzyme that degrades GLP-1, might increase β-cell mass by preventing apoptosis and increasing proliferation. Nevertheless, caution is critical since this ubiquitous enzyme has many targets.

Many other β-cell growth factors, including hepatocyte growth factor, fibroblast growth factors, parathyroid hormone-related protein, gastrin, and delta/notch also promote β-cell survival [70, 76]. Furthermore, one of the most important endogenous β-cell growth factors appears to be insulin itself [13, 49, 50, 77–84]. Based on knockout mouse studies, the insulin receptor even appears more important than the IGF-1 receptor [83]. Insulin acts via a complex series of signaling events, including both the PI3-kinase/Akt pathway and the Raf-1/Erk pathway [13, 49, 77, 83]. Akt acts on multiple downstream targets, including Bad. In addition to stimulating Erk, Raf-1 can also phosphorylate and inactivate Bad at the mitochondria. Interestingly, signaling through IRS-2 rather than IRS-1 appears to play a role in β-cell survival [85]. While constitutive insulin signaling seems to be essential for β-cell survival under stressful conditions, excessive concentrations of insulin may be deleterious [49]. Further work is needed to understand the ideal way to harness this and other endogenous anti-apoptotic signaling pathways.

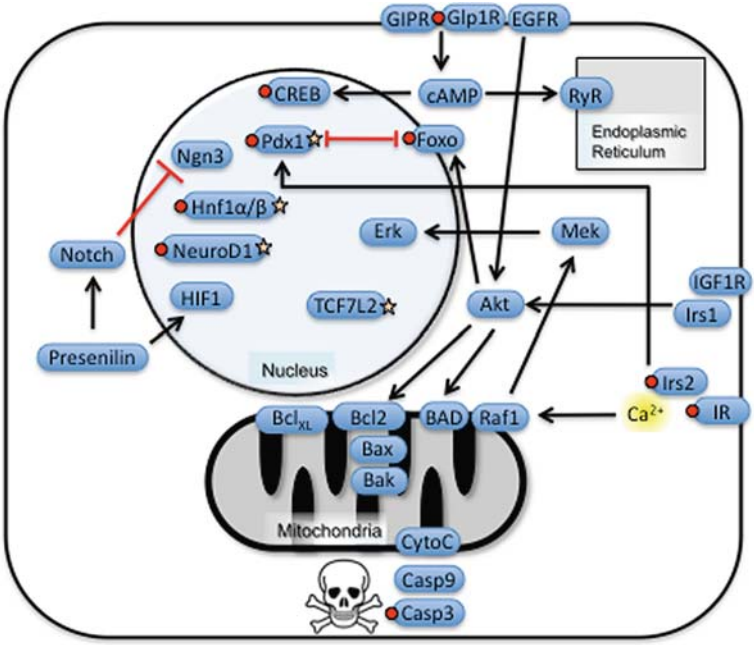

Fig. 19.4 Molecular mechanisms controlling basal β-cell apoptosis and survival factor signaling cascades. Shown is a partial description of signaling cascades that modulate β-cell survival. Protein products of genes that are linked to human diabetes are denoted with a star. Genes that have been implicated in β-cell apoptosis or β-cell mass using in vivo loss of function experiments (i.e., knockout mice) are denoted with a dot

19.12 β-Cell Apoptosis as a Therapeutic Target in Diabetes: Future Directions

The protection of existing β-cells and the regeneration of new ones is a major goal in diabetes research. Therapeutic strategies to protect β-cells could have an immediate impact on clinical islet transplantation, where close to half of the islets transplanted into the liver die before becoming engrafted. In future years we also expect drugs may be developed that improve endogenous β-cell survival in vivo. These treatments would theoretically slow the progression of, or perhaps reverse, type 1 diabetes or type 2 diabetes. Once the exact molecular defects are better known, specific components of the β-cell apoptosis system could be targeted more selectively. For diabetes caused by β-cell ER-stress, so-called molecular chaperones might be useful to decrease unfolded proteins in the ER. In cases where diabetes is associated with apoptosis controlled by cellular metabolism, we expect direct interventions at the level of β-cell mitochondria might be of benefit. Since islet amyloid formation can be found in type 2 diabetes and in transplantation, chemical inhibitors of this process might have therapeutic potential [86]. A thorough understanding

of survival signaling pathways induced by endogenous β-cell growth factors will hopefully provide new targets for intervention, based on the β-cells' own defenses. Moreover, unbiased and high-throughput methods promise to accelerate the pace at which we discover the mechanisms of β-cell apoptosis and treatments that target β-cell apoptosis in diabetes.

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Chapter 20
β-Cell Function in Obese-Hyperglycemic Mice [*ob/ob* Mice]

Per Lindström

Abstract This review summarizes key aspects of what has been learned about the physiology of pancreatic islets and leptin deficiency from studies in obese *ob/ob* mice. *ob/ob* Mice lack functional leptin. They are grossly overweight and hyperphagic particularly at young ages and develop severe insulin resistance with hyperglycemia and hyperinsulinemia. *ob/ob* Mice have large pancreatic islets. The β-cells respond adequately to most stimuli, and *ob/ob* mice have been used as a rich source of pancreatic islets with high insulin release capacity. *ob/ob* Mice can perhaps be described as a model for the prediabetic state. The large capacity for islet growth and insulin release makes *ob/ob* mice a good model for studies on how β-cells can cope with prolonged functional stress.

Keywords *ob/ob* · Mice · Leptin · Growth

20.1 The *ob/ob* Mouse

The discovery that the *ob/ob* mouse syndrome is caused by a defective adipocytokine leptin opened a whole new era of metabolic studies and understanding of the endocrine functions of adipose tissue. The *ob/ob* syndrome was found in 1949 in an outbred mouse colony at Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine [1] and was transferred to the already well-characterized C57Bl mice colony that had been established during the 1930s. Obesity is the most obvious characteristic of *ob/ob* mice. They are also hyperphagic, hyperinsulinemic, and hyperglycemic and have reduced metabolic rate and lower capacity for thermogenesis [2, 3]. The pancreatic islets are large and contain a high proportion of insulin-producing β-cells. It was soon discovered that *ob/ob* mice have a number of other traits except obesity. They are, e.g., infertile and have impaired immune functions.

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The *ob/ob* syndrome varies considerably depending on the genetic background [4, 5]. In this presentation *ob/ob* mice refers to 6J or Umeå *ob/ob* mice unless otherwise stated. On a 6J background hyperglycemia is relatively mild particularly at old age, and glycosuria is usually not present in the fasting state. They represent a mouse model for obesity and "diabetes" with moderate hyperglycemia, high insulin release capacity, and marked adiposity [6]. On a KsJ or BTBR background the mice have a higher food intake than *ob/ob* mice on a 6J background [7] and become overtly diabetic with a reduced life expectancy [8, 9]. On a 6J background the mice have a large lipogenic capacity in the liver [10], which may render them less susceptible to lipotoxic effects. β-Cells from *ob/ob* mice accumulate fat but only a small lipid increase is observed in β-cells from *ob/ob* mice on a 6J background [11], which is in keeping with the better-preserved function. The importance of a high insulin release capacity was evident from studies where the *ob* trait was transferred to DBA mice [12]. Mice with large islets and a high insulin release capacity maintained adiposity, whereas mice with lower serum insulin levels had diminished adiposity and a more severe diabetes [12]. There are differences between individual mice from the same colony of 6J and Umeå *ob/ob* strains with regard to hyperglycemia and other aspects of a "diabetes-like" condition. This can be used to also select subgroups of animals within the same strain for metabolic studies.

ob/ob Mice are indistinguishable from their lean littermates at birth, but within 2 weeks they become heavier and develop hyperinsulinemia. The syndrome becomes much more pronounced after weaning, and overt hyperglycemia is observed during the fourth week. The blood glucose rises to reach a peak after 3–5 months when the mice also have a very high food intake and a rapid growth [13–15]. After that, blood glucose values decrease and eventually become nearly normal at old age. Serum insulin levels are also very high and peak at a higher age than blood glucose values [13]. The animals remain insulin resistant but impaired glucose tolerance and glycosuria after a glucose load is observed mostly in the postweaning period of rapid growth [13, 16–18].

20.2 Discovery of Leptin

Elegant parabiosis experiments showed that *ob/ob* mice lack but are very sensitive to a circulating factor produced by their normal siblings [8, 19]. By extensive positional cloning experiments, this factor could be identified in 1994 by Friedman and co-workers as leptin produced in adipose tissue [20, 21]. The *ob/ob* syndrome can be reversed almost completely even in adult animals by exogenous leptin or transfection with the leptin gene [22–24]. There are cases with leptin deficiency also in obese humans but this is uncommon so *ob/ob* mice do not present a good model for the etiology of human obesity [25]. It has not been clarified if hyperglycemia and insulin resistance depend on the adiposity or are a consequence of leptin deficiency. However, the discovery of leptin has widened our understanding of the regulation of food intake, metabolic turnover, and obesity. We also have learned a lot more about

the interrelationship between metabolism and other functions such as reproduction and the immune system. Much of what we know about the physiology of leptin has been achieved through studies in *ob/ob* mice but also from observations in animal models with leptin receptor defects such as *db/db* mice and *fa/fa*-rats [26, 27].

20.3 Insulin Resistance and Absence of Leptin

ob/ob Mice have severe insulin resistance. Peripheral insulin resistance induces hyperglycemia and worsens the functional load on the β-cells. *ob/ob* Mouse β-cells also become insulin resistant [28, 29]. Insulin inhibits insulin release and insulin resistance coupled to reduced PI3K-dependent signaling may result in disinhibition of glucose-induced insulin release [29]. Insulin resistance can therefore be beneficiary for β-cell function.

β-cells have full-length leptin receptors and leptin inhibits insulin release and insulin biosynthesis in most studies [30–32]. Lack of leptin effects may enhance β-cell function and explain some of the functional differences between *ob/ob* mice and normal mice. The main signaling pathways for leptin are the JAK/STAT transduction cascade, the mitogen-activated protein kinase (MAPK) cascade, the phosphoinositide 3-kinase (PI3K), IRS, and the 5′-AMP-activated protein kinase (AMPK) pathways [33–35]. The role of these signal mediators in β-cell function has not been entirely clarified but the majority of findings suggest that AMPK [36] and p38 MAPK [37, 38] inhibit glucose-induced insulin release. There are different isoforms of the leptin receptor. The full-length leptin receptor present in pancreatic β-cells is required for the JAK/STAT response and activation is accompanied also by a rise in suppressor of cytokine signaling (SOCS) [39]. A shorter receptor form, which activates PI3K, is predominant in skeletal muscle [40] but PI3K activation is found also in β-cells [39]. Leptin signaling pathways may interact with insulin signaling at several points including JAKs, PI3K, and MAPK [41]. This interaction between insulin and leptin is complex but studies in *ob/ob* mice clearly indicate that the net effect of leptin is to increase insulin sensitivity [41, 42] and that leptin resistance worsens insulin resistance. Absence of leptin can therefore be one of the causes of insulin resistance in *ob/ob* mice. Obese individuals are usually both insulin resistant and leptin resistant. However, the total absence of leptin signaling already from the onset of obesity in *ob/ob* mice is in sharp contrast to obesity in humans and the cross talk between the cellular effects of insulin and leptin is obviously absent.

Leptin can also inhibit islet function through activation of sympathetic neurons [43, 44]. β-cells from *ob/ob* mice are more sensitive than lean mouse β-cells to the stimulatory effect of acetylcholine and the inhibitory effect of noradrenalin on glucose-induced insulin release [45]. This could be because of sympathetic disinhibition due to the lack of leptin. However, there is an age dependence for these effects of neurotransmitters. Islets from young *ob/ob* mice have an increased β-cell responsiveness to cholinergic stimulation already from 10–12 days of age [46]. The sensitivity to acetylcholine is reduced at old age, whereas the sensitivity to vagal

neuropeptides may be increased [47, 48]. A reduced cholinergic activity at old age paralleling improved glycemic control is consistent with the finding that M_3 receptor knock-out in *ob/ob* mice reduces the severity of most of the phenotype [49].

ob/ob Mouse islets have a rich supply of small vessels but a lower blood flow than lean mouse islets when calculated on the basis of islet size [50]. *ob/ob* Mouse islet vessels are also more sensitive to sympathetic inhibition of islet circulation [51]. This suggests that they have a reduced capacity to increase blood flow to meet metabolic demands [50] and this can increase β-cell stress. Amyloid deposits surrounding islet cells are observed in most islets from type 2 diabetics [52] and may be part of the pathogenesis for β-cell damage. Mice do not normally form islet amyloid deposits but *ob/ob* mice have high serum levels of the islet amyloid polypeptide (IAPP) [53] and the islet content of IAPP increases during *ob/ob* syndrome development [54]. The interaction between leptin and IAPP has not been much studied in *ob/ob* mouse islets but leptin inhibits IAPP release in lean mice [55]. Leptin deficiency could therefore increase IAPP content in *ob/ob* mice. IAPP inhibits insulin and glucagon release [56], and it has been suggested that IAPP also induces insulin resistance [57].

20.4 Pancreatic Islets

The islet volume is up to ten times higher in *ob/ob* mice than in normal mice [58, 59], and insulin producing β-cells are by far the most numerous [13, 59–61]. The islet hyperplasia is probably not caused by a primary abnormality in the islets due to leptin deficiency, although this can contribute; it is rather the consequence of an increased demand for insulin. The growth may be triggered not only by hyperglycemia but also by other blood-borne factors and nerve stimulation and is evident from the fourth week [15]. Partial pancreatectomy in *ob/ob* mice in a phase of rapid growth and severe hyperglycemia results in a huge expansion of islet area and islet number [62]. The islet growth normally continues for more than 6 months and is paralleled by reduced insulin content per islet volume during conditions of free access to food [63]. The large islets with many insulin-producing β-cells are in contrast to the decreased β-cell mass found in diabetes [64].

The cellular mechanisms for glucose-induced insulin release are not the subject of this article but islets isolated from *ob/ob* mice respond adequately to stimulators and inhibitors of insulin release in most experimental conditions [65, 66], and they have been used in several hundred papers as a rich source of β-cells in studies of islet function. After an overnight fast, the blood glucose is nearly normalized and *ob/ob* mouse islets release larger quantities of insulin after fasting when compared with normal mouse islets [67]. However, transplantation of coisogenic (+/+) islets to *ob/ob* mice lowered blood glucose values to nearly normal for 1 month [68]. The persistent hyperglycemia can therefore be a sign of insufficient β-cell function despite the high capacity to secrete insulin, and the *ob/ob* mouse can perhaps be

described to be in a constant prediabetic state. The threshold for glucose-induced insulin release occurs at a lower glucose concentration than in lean mouse islets [67, 69]. The mechanisms for this may in part be similar to the glucose hypersensitivity observed after prolonged exposure to elevated glucose in islets from normoglycemic animals and involve both metabolic and ionic events [70, 71].

20.5 Oscillatory Insulin Release

Serum insulin shows diurnal oscillations, and it is thought that the effect of insulin is improved on target organs when insulin is delivered in a pulsatile manner. We know little about the periodicity of serum insulin in *ob/ob* mice but serum insulin levels vary considerably in the same mouse also when sampled under tightly controlled conditions (Lindström unpublished). The oscillations can be triggered by several mechanisms including variations in cytosolic calcium and metabolic oscillations [72]. Variation in cAMP levels is also a likely candidate as evidenced from studies in *ob/ob* mouse β-cells [73] and β-cell lines [74]. *ob/ob* Mouse islets have a reduced capacity to accumulate cAMP [75, 76], but they are more sensitive to a rise in cAMP for stimulation of insulin release [76]. The β-cells have an increased Na/K-ATPase activity [77] and may be more sensitive to voltage-dependent events [78] perhaps due to a reduced activation of K_{ATP} channels [39]. However, the function of voltage-dependent Ca^{2+} channels is impaired [79], and there is a disturbed pattern of cytoplasmic calcium changes after glucose stimulation [80]. *ob/ob* Mouse β-cells also do not show the same type of cell-specific Ca^{2+} responses that are found in lean mouse islets [81]. There is an excessive firing of cytoplasmic Ca^{2+} transients when *ob/ob* mouse β-cells are stimulated with glucagon [82]. This effect could be a direct consequence of leptin deficiency because it was reduced when leptin was also added. Ryanodine receptors in the endoplasmic reticulum may be involved in β-cell calcium regulation and stimulation of insulin release but the precise role is controversial [83, 84]. In one study it was reported that β-cells from *ob/ob* mice have less ryanodine receptor activation than β-cells from lean mice [85].

An increased sensitivity to cAMP could also have other effects. UCP-2 was demonstrated in β-cells a decade ago, and it has been suggested that UCP-2 is important as a negative regulator of glucose-induced insulin release and protection against oxidative stress. cAMP could reduce the inhibitory effect of a rise in uncoupling protein-2 (UCP-2) through PKA-mediated inhibition of the K_{ATP} channel [86]. *ob/ob* Mouse β-cells have increased activity of UCP-2 [87, 88] when compared with lean mice from the same background. Inhibition of UCP-2 improved glucose tolerance [89] but knockdown of UCP-2 expression had no effect on glucose-induced insulin release in *ob/ob* mouse islets [87]. ACTH receptor activation is coupled to a rise in cAMP [90]. Leptin stimulates both CRF and ACTH secretions [91], but *ob/ob* mice have increased serum ACTH levels and islets from *ob/ob* mice respond with a larger increase in insulin release after stimulation with ACTH 1–39 [92].

20.6 β-Cell Mass

One of the features of *ob/ob* mice is that they have large pancreatic islets consisting of mostly β-cells (Fig. 20.1), and *ob/ob* mice have been used in studies of β-cell proliferation. β-Cell growth is probably stimulated by hyperglycemia directly or indirectly. There is a good correlation between the level of hyperglycemia and islet cell replication in rat [93] and obese-hyperglycemic mice [94] and the morphology of *ob/ob* mice islets reaggregated in vitro depends on the glucose concentration [95]. It has been suggested that cells recruited from bone marrow increase the insulin release capacity in *ob/ob* mice [96]. Duct progenitor cells can also be involved in the expansion of the β-cell mass, but mitotic figures have been demonstrated in β-cells from *ob/ob* mice [15, 59], and cells within existing islets are probably the most important source for expansion of the total islet mass [97]. *ob/ob* Mice have a growth-promoting environment for β-cells depending on (extra) pancreatic factors [98, 99], perhaps including insulin [100], and oncogenes stimulate *ob/ob* mice β-cell replication as a sign that they can be manipulated extrinsically [101]. Blood-borne factors involved probably include NPY [102] and GLP-1 [15, 103, 104] which both stimulate *ob/ob* mouse β-cell replication. Interestingly, NPY also inhibits insulin release and *ob/ob* mouse islets have reduced expression of NPY receptors [105]. Obesity probably also induces an indirect neuronal signal emanating from the liver which is important for stimulation of islet growth in *ob/ob* mice [106]. Cytokines

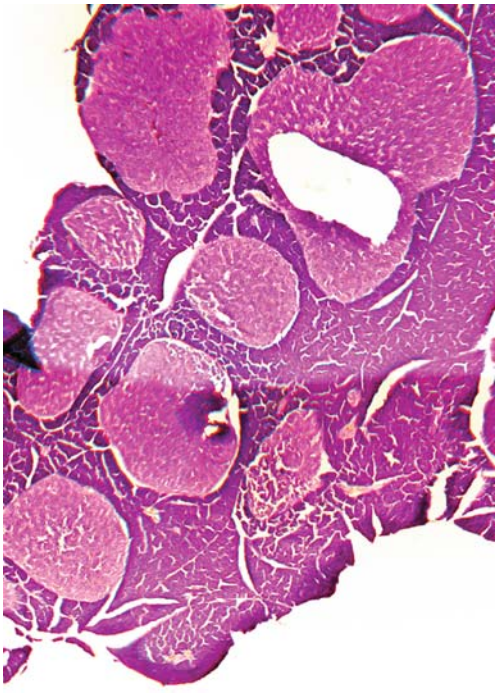

Fig. 20.1 This picture of a section from the pancreas of a 6-month-old *ob/ob* mouse shows the sometimes huge proportion of very large islets

and growth hormone may be important mitogens for β-cells [79, 107]. Intracellular signaling for GH receptors includes JAK/STAT activation and this is inhibited by SOCS that inhibit cytokine signaling [79, 108]. Inhibition of cytokine signaling by SOCS may prevent β-cell death induced by several cytokines such as IL-1β, TNFα, and IFNγ [108]. Leptin activates both JAK/STAT and SOCS and it is possible that the net effect of leptin deficiency is to stimulate β-cell growth through lowering of SOCS. Low-grade inflammation may be important for increased adiposity and for the pathogenesis of type 2 diabetes [109, 110]. Leptin stimulates the immune system and is involved in macrophage activation and release of cytokines [111]. This could be part of the explanation why leptin deficiency may prevent β-cell death. Few studies have specifically addressed the effect of cytokines in *ob/ob* mouse islets but they respond normally to cytokine activators and inhibitors [112–114].

20.7 Glucotoxicity and Lipotoxicity

Glucotoxicity caused by long-standing hyperglycemia may be one factor inducing β-cell death in the development of type 2 diabetes. The toxicity may be caused by induction of reactive oxygen species and by inducing endoplasmic reticulum (ER) stress [64]. ER stress is probably an important cause of β-cell dysfunction in diabetes [115]. We know little about ER stress in *ob/ob* mouse islets. ER stress can be an important cause of leptin resistance [116] but this may not be relevant to *ob/ob* mice since they lack leptin. However, *ob/ob* mice show clear signs of hepatocyte ER stress [117–119], and it is likely that also the β-cells have ER stress because of the increased demands for protein synthesis.

ob/ob Mice are living proof that prolonged hyperglycemia is not necessarily deleterious. It is possible that the insulin resistance and leptin absence protect the β-cells from the damage that constant glucose stimulation would otherwise cause. However, reported differences from lean mice with regard to β-cell metabolic signaling and enzyme activities are few. The mitochondrial enzyme FAD-linked glycerophosphate dehydrogenase (m-GDH) is thought to play a key role in the glucose-sensing mechanism of the insulin-producing B-cell. It catalyses a rate-limiting step of the glycerol phosphate shuttle but there was no difference between islets in enzyme activity between normal and *ob/ob* mice [120]. Perhaps *ob/ob* mouse β-cells are protected because they have an increased glucose cycling through glucose-6-phosphatase [121]. They also have lower levels of the glucose transporter (GLUT2) [122]. A reduced glucokinase activity could lessen β-cell stress but there are conflicting data as to whether glucokinase is lower [122] or higher [43] than in lean mouse islets. Elevated serum levels of free fatty acids in the presence of hyperglycemia and aberrant lipoprotein profiles could cause lipotoxic damage to β-cells. *ob/ob* Mouse islets show signs of a reduced fatty acid oxidation in the presence of high glucose [123] which could lead to toxic effects of lipids. However, *ob/ob* mice have low-serum VLDL levels and high HDL levels [124] and this can be protective. It is likely that the large capacity to accumulate fat in adipose tissue protects *ob/ob* mice against β-cell lipotoxicity [125].

20.8 Incretins

The incretins GLP-1 and GIP are released in response to food ingestion and play an important role in stimulating insulin release when blood glucose levels are elevated. The half-life in circulation is short because of enzymatic digestion through dipeptidyl peptidase-4 (DPP-4). GLP-1 and GLP-1 analogues stimulate β-cell proliferation [15, 103, 104] and glucose-induced insulin release in *ob/ob* mice [126–128] and inhibition of DPP-4 improves β-cell function [129]. On the other hand, chemical ablation of the GIP receptors causes normalization of hyperglycemia, serum insulin, insulin sensitivity, glucose tolerance, and islet hypertrophy in *ob/ob* mice [130, 131]. This indicates that different incretins can have both beneficiary and adverse effects in obesity-related hyperglycemia and insulin resistance. Glucagon levels are high in *ob/ob* mice [132]. It was early hypothesized that elevated glucagon secretion contributes to the altered metabolism of *ob/ob* mice [133] and immunoneutralization of endogenous glucagon improves metabolic control [134]. There is a correlation between serum glucagon levels and hepatic glucose output in type 2 diabetic patients [135] and reduction of serum glucagon may be a target for diabetes treatment.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. PPAR-γ and PPAR-α exert profound effects on lipid handling. PPAR-γ directs lipid toward adipose tissue and PPAR-α activation predominantly stimulates lipid oxidation. PPAR agonists have been used in the treatment of type 2 diabetes to reduce insulin resistance and improve β-cell function. Treatment with both PPAR-γ agonists [136] and PPAR-α agonists [137] improved glucose-stimulated insulin release in *ob/ob* mice. This is another indication that *ob/ob* mouse β-cells are normally under functional stress.

20.9 Conclusions

ob/ob Mouse islets are large and contain a high proportion of insulin-producing β-cells. They respond adequately to most stimulators and inhibitors of insulin release and have been used as a rich source of β-cells for in vitro studies of islet function. *ob/ob* Mouse β-cells show insulin resistance and other signs of leptin deficiency. The lack of leptin must always be taken into account when using *ob/ob* mice as a model. Nevertheless, *ob/ob* mice represent an excellent model for studies on how β-cells can adapt to increased demand and maintain a high insulin release capacity during prolonged functional stress.

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Chapter 21
Islet Structure and Function in the GK Rat

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Abstract Type 2 diabetes mellitus (T2D) arises when the endocrine pancreas fails to secrete sufficient insulin to cope with the metabolic demand because of β-cell secretory dysfunction and/or decreased β-cell mass. Defining the nature of the pancreatic islet defects present in T2D has been difficult, in part because human islets are inaccessible for direct study. This review is aimed to illustrate to what extent the Goto–Kakizaki rat, one of the best characterized animal models of spontaneous T2D, has proved to be a valuable tool offering sufficient commonalities to study this aspect. A comprehensive compendium of the multiple functional GK islet abnormalities so far identified is proposed in this perspective. The pathogenesis of defective β-cell number and function in the GK model is also discussed. It is proposed that the development of T2D in the GK model results from the complex interaction of multiple events: (i) several susceptibility loci containing genes responsible for some diabetic traits (distinct loci encoding impairment of β-cell metabolism and insulin exocytosis, but no quantitative trait locus for decreased β-cell mass); (ii) gestational metabolic impairment inducing an epigenetic programming of the offspring pancreas (decreased β-cell neogenesis and proliferation) transmitted over generations; and (iii) loss of β-cell differentiation related to chronic exposure to hyperglycaemia/hyperlipidaemia, islet inflammation, islet oxidative stress, islet fibrosis and perturbed islet vasculature.

Keywords Type 2 diabetes · GK rat · Islet cells · β-cell development · Differentiation and survival · Insulin release

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21.1 The Goto–Kakizaki Wistar (GK) Rat as Model of Spontaneous T2D

Type 2 diabetes (T2D) arises when the endocrine pancreas fails to secrete sufficient insulin to cope with the metabolic demand [1, 2] because of β-cell secretory dysfunction and/or decreased β-cell mass. Hazard of invasive sampling and lack of suitable non-invasive methods to evaluate β-cell mass and β-cell functions are strong limitations for studies of the living pancreas in human. In such a perspective, appropriate rodent models are essential tools for identification of the mechanisms that increase the risk of abnormal β-cell mass/function and of T2D. Some answers to these major questions are available from studies using the endocrine pancreas of the Goto–Kakizaki (GK) rat model of T2D. It is the aim of the present chapter to review the common features that make studies of the GK β-cell so compelling.

The GK line was established by repeated inbreeding from Wistar (W) rats selected at the upper limit of normal distribution for glucose tolerance [3–8].

Until the end of the 1980s, GK rats were bred only in Sendai [3]. Colonies were then initiated with breeding pairs from Japan, in Paris, France (GK/Par) [9], Dallas, TX, USA (GK/Dal) [10], Stockholm, Sweden (GK/Sto [6], Cardiff, UK (GK/Card) [11], Coimbra, Portugal (GK/Coi [12], Tampa, USA (GK/Tamp) [13]. Some other colonies existed for shorter periods during the 1990s in London, UK (GK/Lon) [14], Aarhus, Denmark, and Seattle, USA (GK/Sea) [15]. There are also GK rat colonies derived from Paris in Oxford, UK (GK/Ox) [16] and Brussels, Belgium (GK/Brus) [17]. Also, GK rats are available commercially from Japanese breeders Charles River Japan, Yokohama, Oriental Yeast, Tokyo, Clea Japan Inc, Osaka (GK/Clea), Japan SLC, Shizuoka (GK/SLC), Takeda Lab Ltd, Osaka (GK/Taked), and from Taconic, USA (GK/Mol/Tac).

In our colony (GK/Par subline) maintained since 1989, the adult GK/Par body weight is 10–30% lower than that of age- and sex-matched control animals. In male GK/Par rats, non-fasting plasma glucose levels are typically 10–14 mM (6–8 mM in age-matched Wistar (W) outbred controls). Despite the fact that GK rats in the various colonies bred in Japan and outside over 20 years have maintained rather stable degree of glucose intolerance, other characteristics such as β-cell number, insulin content and islet metabolism and secretion have been reported to differ between some of the different colonies, suggesting that different local breeding environment and/or newly introduced genetic changes account for contrasting phenotypic properties.

Presently it is not clear whether the reported differences are artefactual or true. Careful and extensive identification of GK phenotype within each local subline is therefore necessary when comparing data from different GK sources. For further details concerning the pathogenic sequence culminating in the chronic hyperglycaemia at adult age in the GK/Par rat, please refer to recent reviews [6–8].

21.2 A Perturbed Islet Architecture, with Signs of Progressive Fibrosis, Inflammatory Microenvironment, Microangiopathy and Increased Oxidative Stress

The adult GK/Par pancreas exhibits two different populations of islets in situ: large islets with pronounced fibrosis [5] and heterogeneity in the staining of their β-cells, and small islets with heavily stained β-cells and normal architecture. One striking morphologic feature of GK rat islets is the occurrence of these big islets characterized by connective tissue separating strands of endocrine cells [4, 18, 19]. Accordingly, the mantle of glucagon and somatostatin cells is disrupted and these cells are found intermingled between β-cells. These changes increase in prevalence with ageing [18].

No major alteration in pancreatic glucagon content, expressed per pancreatic weight, has been demonstrated in GK/Sto rats [20], although the total α-cell mass was decreased by about 35% in adult GK/Par rats [21]. The peripheral localization of glucagon-positive cells in W islets was replaced in GK/Sto rats with a more random distribution throughout the core of the islets [22]. Pancreatic somatostatin content was slightly but significantly increased in GK/Sto rats [20].

Chronic inflammation at the level of the GK/Par islet has recently received demonstration and it is now considered as a pathophysiological contributor in type 2 diabetes [23, 24]. Using an Affymetrix microarray approach to evaluate islet gene expression in freshly isolated adult GK/Par islets, we found that 34% of the 71 genes found to be overexpressed belong to inflammatory/immune response gene family and 24% belong to extracellular matrix (ECM)/cell adhesion gene family [25]. Numerous macrophages ($CD68^+$ and MHC class II^+) and granulocytes were found in/around adult GK/Par islets [25]. Upregulation of the MHC class II gene was also reported in a recent study of global expression profiling in GK/Takonic islets [26]. Immunolocalization with anti-fibronectin and anti-vWF antibodies indicated that ECM deposition progresses from intra- and peri-islet vessels, as it happens in microangiopathy [25]. These data demonstrate that a marked inflammatory reaction accompanies GK/Par islet fibrosis and suggest that islet alterations develop in a way reminiscent of microangiopathy [24]. The previous reports by our group and others that increased blood flow and altered vascularization are present in the GK/Par and GK/Sto models [27–29] are consistent with such a view. The increased islet blood flow in GK rats may be accounted for by an altered vagal nerve regulation mediated by nitric oxide, since vagotomy as well as inhibition of NO synthase normalized GK/Sto islet flow [28]. In addition, islet capillary pressure was increased in GK/Sto rats [30]; this defect was reversed after 2 weeks of normalization of glycaemia by phlorizin treatment. The precise relationship between islet microcirculation and β-cell secretory function remains to be established.

Immunohistochemistry on diabetic GK/Par pancreases (Fig. 21.1) showed, unlike Wistar islets, the presence of nitrotyrosine and HNE labellings, which identify ROS and lipid peroxidation, respectively. Marker-positive cells were

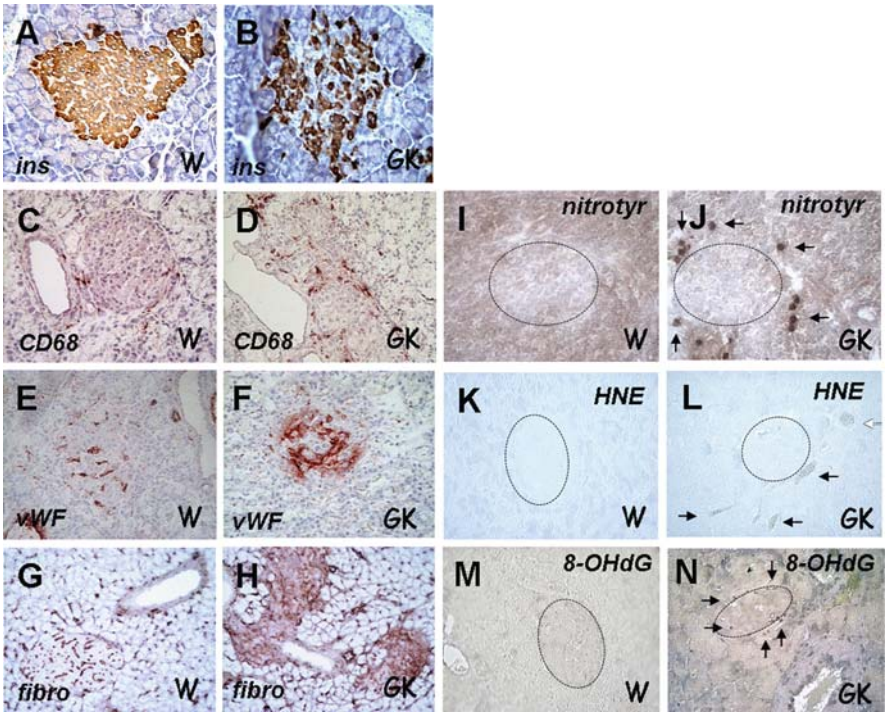

Fig. 21.1 Insulin labelling demonstrates the concomitant presence of large fibrotic islets (B) in adult GK/Par pancreas as compared with age-matched control Wistar (W) pancreas (A) (×500). Fibrosis is extensive in large GK/Par islets, as shown by fibronectin (H) labelling (×250). Small intact islets coexist with large fibrotic islets (not shown). Inflammatory cells infiltrate the islets of adult GK/Par rats. Compared with adult W rats, numerous macrophages are present in/around GK/Par islets, as shown by CD68 (D vs. C; ×500) and MHC class II (not shown) labellings. The concomitant presence of macrophages and granulocytes together with the quasi-absence of T and B cells and ED3 macrophages that are involved in autoimmune reaction (data not shown) suggests a pure inflammatory process. Islet vascularization is altered in adult GK/Par rats. Labelling for vWF, a factor known to be produced by endothelial cells, shows the normal organization of islet vascularization in adult W rats (E). Islet vascularization differs markedly in age-matched GK/Par rats and appears to be hypertrophied (F) (×500). Nitrotyrosine, 4-hydroxy-2-nonenal (HNE)-modified proteins and 8-hydroxy-2'-deoxyguanosine (8-OHdG) accumulate in the peri-islet vascular and inflammatory compartments of the adult GK/Par pancreas. Immunolabelling of nitrotyrosine, HNE-adducts or 8-OHdG (*arrows*) in pancreatic tissues of GK/Par (J, L, N) and W rats (I, K, M). An islet is encircled in each image (×250)

predominantly localized at the GK/Par islet periphery or along ducts and were accompanied by inflammatory infiltrates. Intriguingly, no marker-positive cell was detected within the islets in the same GK/Par pancreases [31]. Such was not apparently the case in GK/Taked pancreases, as 8-OHdG and HNE-modified proteins accumulation were described within the islets. In this last study, the animals were older as compared to our study and accumulation of markers was correlated to hyperglycaemia duration [32]. This suggest that the lack of OS-positive cells within

islets as found in the young adult diabetic GK/Par is only transient and represents an early stage for a time-dependent evolutive islet adaptation.

21.3 Less β-Cells Within the Pancreas with Less Replicative Activity but Intact Survival Capacity

In the adult hyperglycaemic GK/Par rats (males or females), total pancreatic β-cell mass is decreased (by 60%) [5, 21]. This alteration of the β-cell population cannot be ascribed to increased β-cell apoptosis but is related, at least partly, to significantly decreased β-cell replication as measured in vivo, in situ [5]. The islets isolated by standard collagenase procedure from adult GK/Par pancreases show limited decreased β-cell number (by 20% only) and low insulin content compared with control islets [33]. The islet DNA content was decreased to a similar extent, consistent with our morphometric data, which indicates that there is no major change in the relative contribution of β-cells to total endocrine cells in the GK islets. In addition, the insulin content, when expressed relative to DNA, remains lower in GK islets than in control (inbred W/Par) islets, which supports some degranulation in the β-cells of diabetic animals [33]. Electron microscopy observation of β-cell in GK/SLC pancreas revealed that the number of beta granules is decreased and that of immature granules increased. The Golgi apparatus was developed and the cisternae of the rough endoplasmic reticulum were dilated, indicating cell hyperfunction [34].

The distribution of various GK islet cell types appears to differ between some of the GK rat colonies. Thus, in the Stockholm colony, β-cell density and relative volume of insular cells were alike in adult GK/Sto rats and control W rats [6, 19, 20]. Similar results were reported in the Dallas colony (GK/Dal) [10]. Reduction of adult β-cell mass, to an extent similar to that we reported in GK/Par rats, was however mentioned in GK rats from Sendai original colony [4], in GK/Taked [35], in GK/Clea [36] and in GK/Coi [37]. Another element of heterogeneity between the different GK sources is related to the time of appearance of significant β-cell mass reduction when it is observed: It varies from foetal age in GK/Par to neonatal age in GK/Coi [12] or young adult age (8 weeks) in GK/Taked [35], GK/SLC [34]. The reason for such discrepancies in the onset and the severity of the β-cell mass reduction among colonies is not identified, but can be ascribed to differences in islet morphometric methodologies and/or characteristics acquired within each colony and arising from different nutritional and environmental conditions.

A meaningful set of data from our group [38–41] suggest that the permanently reduced β-cell mass in the GK/Par rat reflects a limitation of β-cell neogenesis during early foetal life and thereafter. Follow-up of the animals after delivery revealed that GK/Par pups become overtly hyperglycaemic for the first time after 3–4 weeks of age only (i.e. during the weaning period). Despite normoglycaemia, total β-cell mass was clearly decreased (by 60%) in the GK/Par pups when compared with age-related W pups [21]. Since this early β-cell growth retardation in the prediabetic

GK/Par rat pups can be ascribed neither to decreased β-cell replication nor to increased apoptosis [21], we postulated that the recruitment of new β-cells from the precursor pool (β-cell neogenesis) was defective in the young prediabetic GK/Par rat. A comparative study of the development of GK/Par and W pancreases indicates that the β-cell deficit (reduced by more than 50%) starts as early as foetal age 16 days (E16) [39]. During the time window E16–E20, we detected an unexpected anomaly of proliferation and apoptosis of undifferentiated ductal cells in the GK/Par pancreatic rudiments [39, 41]. Therefore, the decreased cell proliferation and survival in the ductal compartment of the pancreas, where the putative endocrine precursor cells localize, suggest that the impaired development of the β-cell in the GK/Par foetus could result from the failure of the proliferative and survival capacities of the endocrine precursor cells. Data from our group indicate that defective signaling through the IGF2/IGF1-R pathway is involved in this process at this stage. Importantly this represents a primary anomaly since Igf2 and IGF1-R protein expressions are already decreased within the GK/Par pancreatic rudiment at E13.5, at a time when β-cell mass (first wave of β-cell expansion) is in fact normal [41]. Low levels of pancreatic Igf2 associated with β-cell number deficiency are maintained thereafter in the GK/Par foetuses until delivery [42]. We have also published data illustrating a poor proliferation and/or survival of the endocrine precursors also during neonatal and adult life [38, 40]. Altogether these arguments support the notion that an impaired capacity of β-cell neogenesis (either primary in the foetus or compensatory in the newborn and the adult) results from the permanently decreased pool of endocrine precursors in the GK/Par pancreas [43].

21.4 Which Aetiology for the β-Cell Mass Abnormalities?

During the last few years, some important information concerning the determinants (morbid genes vs. environment impact) for the low β-cell mass in the GK/Par model has been supplied. Hyperglycaemia experienced during the foetal and/or early postnatal life may contribute to programming of the endocrine pancreas [44]. Such a scenario potentially applies to the GK/Par rat, as GK/Par mothers are slightly hyperglycaemic through their gestation and during the suckling period [45]. We have preliminary data using an embryo transfer strategy first described by Gill-Randall et al., [46], suggesting that GK/Par embryos transferred in the uterus of euglycaemic W mother still develop deficiency of β-cell mass when adults, to the same extent as the GK/Par rats from our stock colony [47]. While this preliminary conclusion rather favours a major role for inheritance of morbid genes, additional studies are needed to really eliminate the option that the gestational diabetic pattern of the GK/Par mothers does not contribute to establish and/or maintain the transmission of endocrine pancreas programming from one GK/Par generation to the next one. Moreover, studies on the offspring in crosses between GK/Par and W rats demonstrated that F1 hybrid foetuses, regardless of whether the mother was a GK or a

W rat, exhibit decreased beta mass and glucose-induced insulin secretion closely resembling those in GK/GK foetuses [45]. This finding indicates that conjunction of GK genes from both parents is not required for defective β-cell mass to be fully expressed. We have also shown that to have one GK parent is a risk factor for a low β-cell mass phenotype in young adults, even when the other parent is a normal W rat [48].

Search for identification of the morbid genes using a quantitative trait locus (QTL) approach has led to identification of six independently segregating loci containing genes regulating fasting plasma glucose and insulin levels, glucose tolerance, insulin secretion and adiposity in GK/Par rats [49]. The same conclusion was drawn by Galli et al., [50] using GK/Sto rats. This established the polygenic inheritance of diabetes-related parameters in the GK rats whatever their origin. Both studies found the strongest evidence of linkage between glucose tolerance and markers spanning a region on rat chromosome 1, called Niddm1 locus. Recent works using congenic technology have identified a short region on the Niddm1i locus of GK/Sto rats that may contribute to defective insulin secretion [51]. It has been recently reported that β-cell mass is intact in Niddm1i subcongenics [52]. These results are however inconsistent with the enhanced insulin release and increased islet size described in a GK/Ox congenic strain targeting a similar short region of the GK QTL Niddm1 [53]. Finally, no QTL association with β-cell mass or β-cell size could be found in the GK/Par rat [Ktorza and Gauguier, personal communication of unpublished data]. Therefore, the likelihood that a genotype alteration directly contributes to the low β-cell mass phenotype in the GK/Par rat is reduced. The raised question to be answered now is whether or not epigenetic perturbation of gene expression occurs in the developing GK/Par pancreas and programs a durable alteration of the β-cell mass as seen in the adult. *igf2* and *igf1r* genes are good candidates for such a perspective.

Finally, since the loss of GK/Taked β-cells was mitigated by in vivo treatment with the alpha-glucosidase inhibitors voglibose [54] or miglitol [36], or enhanced when the animals are fed sucrose [35, 55], pathological progression (β-cell number, fibrosis) of the GK β-cell mass is also dependent on the metabolic (glycaemic) control.

21.5 Multiple β-Cell Functional Defects Mostly Targeting Insulin Release

21.5.1 Insulin Biosynthesis Is Grossly Preserved

As for total pancreatic β-cell mass, there is some controversy regarding the content of pancreatic hormones in GK rats. In the adult hyperglycaemic GK/Par rats, total pancreatic insulin stores are decreased by 60–40% [5]. In other GK rat colonies (Takeda, Stockholm, Seattle), total insulin store values have been found similarly or more moderately decreased, compared with control rats [15, 20, 56–59]. The

islets isolated by standard collagenase procedure from adult GK/Par pancreases show lower insulin content compared with control islets [33]. In addition, when expressed relative to DNA, the GK/Par islet insulin content remains lower (by 30%) than in that control (inbred W/Par) islets, therefore supporting some degranulation in the diabetic β-cells [33].

Glucose-stimulated insulin biosynthesis in freshly isolated GK/Par, GK/Jap or GK/Sto islets has been reported grossly normal [22, 60, 61]. The rates of biosynthesis, processing and secretion of newly synthesized (pro)insulin were comparable [22]. This is remarkable in the face of markedly lower prohormone convertase PC2 immunoreactivity and expression in the GK/Sto islets, while the expression patterns of insulin, PC1 and carboxypeptidase E (CPE) remained normal [22]. Circulating insulin immunoreactivity in GK/Sto rats was predominantly insulin 1 and 2 in the expected normal ratios with no (pro)insulin evident. The finding that proinsulin biosynthesis and processing of proinsulin appeared normal in adult GK rats suggests that the defective insulin release by β-cells does not arise from a failure to recognize glucose as an activator of prohormone biosynthesis and granule biogenesis. Rather it points to an inability of the β-cell population as a whole to meet the demands on insulin secretion imposed by chronic hyperglycaemia in vivo. Although basal circulating GK insulin levels were similar or slightly elevated as compared to W rats, they were always inappropriate for the level of glycaemia, indicative of a secretory defect.

21.5.2 Glucose-Induced Activation of Insulin Release Is Lost

Impaired glucose-stimulated insulin secretion has been repeatedly demonstrated in GK rats (whatever the colony), in vivo [9, 49, 50, 59, 62], in the perfused isolated pancreas [6, 9 20, 57, 63, 64], and in freshly isolated islets [14, 57, 60, 65]. A number of alterations or defects have been shown in the stimulus secretion coupling for glucose in GK islets. GLUT2 is underexpressed, but not likely to the extent that it could explain the impairment of insulin release [10]. This assumption is supported by the fact that glucokinase/hexokinase activities are normal in GK rat islets [66–68]. In addition, glycolysis rates in GK rat islets are unchanged or increased compared with control islets [14, 57, 65, 60, 68, 69–71]. Furthermore, oxidation of glucose has been reported decreased [60], unchanged [14, 54, 57, 68, 71, 72] or even enhanced [69]. There exists however a common message between these data: the ratio of oxidized to glycolysed glucose was always reduced in GK islets compared to W islets. Also, lactate dehydrogenase gene expression [31] and lactate production [69] are increased and pyruvate dehydrogenase activity is decreased [73] in GK rat islets. In GK/Par islets, we showed that mitochondria exhibit a specific decrease in the activities of FAD-dependent glycerophosphate dehydrogenase [60, 68] and branched-chain ketoacid dehydrogenase [33]. Similar reduction of the FAD-linked glycerol phosphate dehydrogenase activity was reported in GK/Sto islets [66, 74]. These enzymatic abnormalities could work in concert to depress glucose oxidation. An inhibitory influence of islet fatty acid oxidation on glucose oxidation can be

eliminated since the islet triglyceride content was found normal and etomoxir, an inhibitor of fatty acid oxidation, failed to restore glucose-induced insulin release in GK/Sto islets [73].

We also found that the β-cells of adult GK/Par rats had a significantly smaller mitochondrial volume compared to control β-cells [75]. No major deletion or restriction fragment polymorphism could be detected in mtDNA from adult GK/Par islets [75]; however, they contained markedly less mtDNA than control islets. The lower islet mtDNA was paralleled by decreased content of some islet mt mRNAs such as cytochrome b [75]. In accordance with this, insufficient increase in ATP generation in response to high glucose was shown by our group [68]. This supports the hypothesis that the defective insulin response to glucose in GK islet is accounted for by an impaired ATP production, closure of the ATP-regulated K^+ channels [67] and impaired elevation of intracellular [Ca^{2+}] [72, 76, 77].

Such a view validated in the GK/Par β-cell is however contradictory to the reports in GK/Sto and GK/Sea islets that the rate of ATP production is unimpaired [15, 69]. Other energy metabolism defects identified in GK/Sto islets include increased glucose cycling due to increased glucose-6-phosphatase activity [57, 69] and decreased pyruvate carboxylase activity [74]. It is possible that these alterations may affect ATP concentrations locally. However, the enzyme dysfunctions were restored by normalization of glycaemia in GK/Sto rats [74; Ling et al., unpublished observations], but with only partial improvement of glucose-induced insulin release. Hence, it is likely that these altered enzyme activities result from a glucotoxic effect rather than being primary causes behind the impaired secretion. Also, lipotoxic effects leading to defective insulin release have been observed in GK rats on high-fat diet [78, 79], possibly mediated by a mechanism partly involving modulation of UCP-2 expression.

21.5.3 Insulin Secretion Amplifying Mechanisms Are Altered

Phosphoinositide [77] and cyclic AMP metabolism [77, 80] are also affected in GK/Par islets. While carbachol was able to promote normal inositol generation in GK/Par islets, high glucose failed to increase inositol phosphate accumulation. The inability of glucose to stimulate IP production is not related to defective phospholipase C activity per se (total activity in islet homogenates is normal) [77]. It is rather linked to abnormal targeting of the phosphorylation of phosphoinositides: The activity of phosphatidyl-inositol kinase, which is the first of the two phosphorylating activities responsible for the generation of phosphatidyl-inositol biphosphate, is clearly reduced (5, Giroix, unpublished data). Moreover, deficient calcium handling and ATP supply in response to glucose probably also contribute to abnormal activation of PI kinases and phospholipase C. A marked decrease in SERCA3 expression has also been described in the GK/Sto islets [81].

Concerning cAMP, it is remarkable that its intracellular content is very high in GK/Par β-cells already at low glucose [77]. This is related to increased expression (mRNA) of the adenylyl cyclase isoforms 2 and 3, and of the GαS and Gαolf, while

AC8 and phosphodiesterases PDE3B and PDE1C isoforms remain normal (Lacraz, unpublished data 2009). Furthermore, cAMP is not further enhanced at increasing glucose concentrations (at variance with the situation in normal β-cells) [77, 80]. This suggests that there exists a block in the steps linking glucose metabolism to activation of adenylate cyclase in the GK/Par β-cell. In the GK/Sto rat, it has been shown that increased AC3 is due to functional mutations in the promoter region of the Ac3 gene [82]. We do not retain this hypothesis in the GK/Par islet since we found that the expressions (mRNA) of AC 2 and AC 3, and of GαS and Gαolf, are not increased in the prediabetic GK/Par islets (Lacraz, unpublished data 2009).

The increased cAMP production has also offered the possibility to fully restore the β-cell secretory competence to glucose in GK/Par as well as GK/Sto islets [64, 80] with a clear biphasic response [80]. This also proves that the glucose incompetence of the GK/Par β-cell is not irreversible and emphasizes the usefulness of GLP-1 as a therapeutic agent in T2D. Also, cholinergic stimulation has been demonstrated to restore glucose-induced insulin secretion from GK/Sto as well as GK/Par islets [77, 83]. We have proposed that such a stimulation is not mediated through activation of the PKC pathway, but via a paradoxical activation of the cAMP/PKA pathway to enhance Ca^{2+}-stimulated insulin release in the GK/Par β-cell [77].

Other intriguing aspects of possible mechanisms behind defective glucose-induced insulin release in GK/Sto rat islets are the findings of dysfunction of islet lysosomal enzymes [59], as well as excessive NO generation [84, 85] or marked impairment of the glucose–haeme oxygenase–carbon monoxide signaling pathway [86]. Islet activities of classical lysosomal enzymes such as acid phosphatase, *N*-acetyl-beta-D-glucosaminidase, beta-glucuronidase and cathepsin D, were reduced by 20–35% in the GK rat. In contrast, the activities of the lysosomal alpha-glucosidehydrolases (acid glucan-1,4-alpha-glucosidase and acid alpha-glucosidase) were increased by 40–50%. Neutral alpha-glucosidase (endoplasmic reticulum) was unaffected. Comparative analysis of liver tissue did not display such a difference. Since no sign of an acarbose effect on GK alpha-glucosidehydrolase activity (contrarily to Wistar islet) was seen, it was proposed that dysfunction of the islet lysosomal/vacuolar system participates to impairment of glucose-induced insulin release in the GK/Sto rat [59]. An abnormally increased NO production in the GK/Sto islets might also be an important factor in the pathogenesis of β-cell dysfunction, since it was associated with abnormal iNOS expression in insulin and glucagon cells, increased ncNOS activity, impaired glucose-stimulated insulin release, glucagon hypersecretion and impaired glucose-induced glucagon suppression. Moreover, pharmacological blockade of islet NO production by the NOS inhibitor NG-nitro-L-arginine methyl ester greatly improved hormone secretion from GK/Sto islets, and GLP-1 suppressed iNOS and ncNOS expression and activity with almost full restoration of insulin release and partial restoration of glucagon release [84, 85].

Also carbon monoxide (CO) derived from β-cell haeme oxygenase (HO) might be involved in the secretory dysfunction. GK/Sto islets displayed a markedly decreased HO activity measured as CO production and immunoblotting revealed a 50% reduction of HO-2 protein expression [86]. Furthermore, a prominent

expression of inducible HO (HO-1) was found in GK/Sto [86] as well as GK/Par [31] islets. The glucose-stimulated CO production and the glucose-stimulated insulin response were considerably reduced in GK/Sto islets. Since addition of the HO activator hemin or gaseous CO to incubation media brought about a normal amplification of glucose-stimulated insulin release in GK/Sto islets, it was proposed that distal steps in the HO–CO signaling pathway are not affected [86].

A diminished pattern of expression and glucose-stimulated activation of several PKC isoenzymes (alpha, theta and zeta) has been reported in GK/Sto islets, while the novel isoenzyme PKC epsilon not only showed a high expression level but also lacked glucose activation [87, 88]. Since broad-range inhibition of the translocation of PKC isoenzymes by BIS increased the exocytotic efficacy of Ca^{2+} to trigger secretion in isolated GK/Sto β-cells [88], perturbed levels and/or activation of some PKC isoforms may be part of the defective signals downstream to glucose metabolism, involved in the GK insulin secretory lesion.

Peroxovanadium, an inhibitor of islet protein-tyrosine phosphatase (PTP) activities, was shown to enhance glucose-stimulated insulin secretion from GK/Sto islets [89, 90]. One possible target for this effect could be PTP sigma that is overexpressed in GK/Sto islets [91]. At present it is not known which exocytosis-regulating proteins are affected by the increased PTPase activity. In addition, defects in islet protein histidine phosphorylation have been proposed to contribute to impaired insulin release in GK/Sea islets [92].

Lastly, an increased storage and secretion of amylin relative to insulin was found in the GK/Sto rat [93] and GLP1 treatment in vivo was recently reported to exert a beneficial effect on the ratio of amylin to insulin mRNA in GK pancreas besides improvement of glucose-induced insulin release [94]. This is consistent with hypersecretion of amylin being one of the factor contributing to the impairment of glucose induced insulin release.

21.5.4 Insulin Exocytotic Machinery Is Abnormal

In addition to these upstream abnormalities, important defects reside late in signal transduction, i.e. in the exocytotic machinery. Indeed, glucose-stimulated insulin secretion was markedly impaired in GK/Taked, GK/Sto, GK/Sea and GK/Par islets also when the islets were depolarized by a high concentration of potassium chloride and the ATP-regulated K^+ channels kept open by diazoxide [15, 64, 95, Szkudelski and Giroix, unpublished data]. Similar results were obtained when insulin release was induced by exogenous calcium in electrically permeabilized GK/Jap islets [95]. In fact, markedly reduced expressions of the SNARE complex proteins (alpha-SNAP, SNAP-25, syntaxin-1, Munc13-1, Munc18-1, *N*-ethylmaleimide-sensitive fusion protein and synaptotagmin 3) have been demonstrated in GK/Sto and GK/Taked islets [61, 96, 97]. We also recently found similar results in the GK/Par islets [Tourrel-Cuzin, unpublished data 2009]. Thus, a reduced number of docking granules may account for impaired β-cell secretion [98] and this defect should partly

be related to glucotoxicity [96]. Actin cytoskeleton has also been implicated in regulated exocytosis. It has been proposed that in secretory cells, actin network under the plasma membrane acts as a physical barrier preventing the access of secretory granules to the membrane. However the role of the subcortical actin is certainly more complex as it is also required for final transport of vesicles to the sites of exocytosis. The level of total actin protein evaluated by western blotting has been found similar in GK/Par and W islets [99], at variance with reports in other GK rat lines [61, 96]. However, confocal analysis of the distribution of phalloidin-stained cortical actin filaments revealed a higher density of the cortical actin web nearby the plasma membrane in GK/Par islets as compared to W. Moreover preliminary functional results suggest that the higher density of actin cortical web in the GK/Par islets contribute to the defects in glucose-induced insulin secretion exhibited by GK islets [99].

21.5.5 Secretory Response to Non-glucose Stimuli Is Partly Preserved

Among the non-glucidic insulin stimulators, arginine has been shown to induce a normal or even augmented insulin response from perfused pancreases or isolated islets of GK/Clea, GK/Par, GK/Sto, and GK/Lon [9, 14, 63]. Since preperfusion for 50–90 min in the absence of glucose reduced the insulin response to arginine in the GK/Par but not in the control pancreas [9], it is likely that previous exposure to glucose in vivo or during the perfusion experiment potentiates arginine-induced insulin secretion. Insulin responses to another amino acid, leucine, and its metabolite, ketoisocaproate (KIC), were diminished in GK/Par and GK/Sto rats [6, 33, 60]. This was attributed to defective mitochondrial oxidative decarboxylation of KIC operated by the branched-chain 2-ketoacid dehydrogenase (BCKDH) complex [33]. However, in GK/Lon and GK/Taked islets, KIC induced normal insulin responses

Fig 21.2 Model for defective glucose-induced insulin release and the abnormal intracellular sites so far identified in the β-cell of the diabetic GK rats from the different sources (mostly the GK/Par and the GK/Sto sources). Where data are available, the impaired sites in the β-cell are indicated with the symbol: ✸Abbreviations: Glut2: glucose transporter isoform 2; Leu: leucine; KIC: ketoisocaproate; AC: adenylate cyclase isoforms; Gαs, Gαolf, Gαq: α subunits of heterotrimeric G proteins; Gβγ: β and γ subunits of heterotrimeric G proteins; PI, PIP, PIP2: phosphoinositides; PLC: phospholipase C; PKC: protein kinase C; DAG: diacylglycerol; IP3: inositol-3-phosphate; UCP2: uncoupling protein 2; ROS: reactive oxygen species; tSNARE, v-SNARE: SNARE proteins (syntaxin-1A, SNAP-25, VAMP-2, Munc-18); SERCA-3: endoplasmic reticulum Ca^{2+}-ATPase isoform 3; L-VOCC: L-type calcium channel modulated by the membrane polarization; CC/IP3R: calcium channel modulated by receptor to IP3; K^+/ATP-C: potassium channel modulated by the ATP/ADP ratio; Ach: acetylcholine; M3-R: muscarinic receptor isoform 3; GLP-1: glucagon-like peptide 1; GLP1-R: GLP1 receptor; PDE: cAMP-dependent phosphodiesterase isoforms

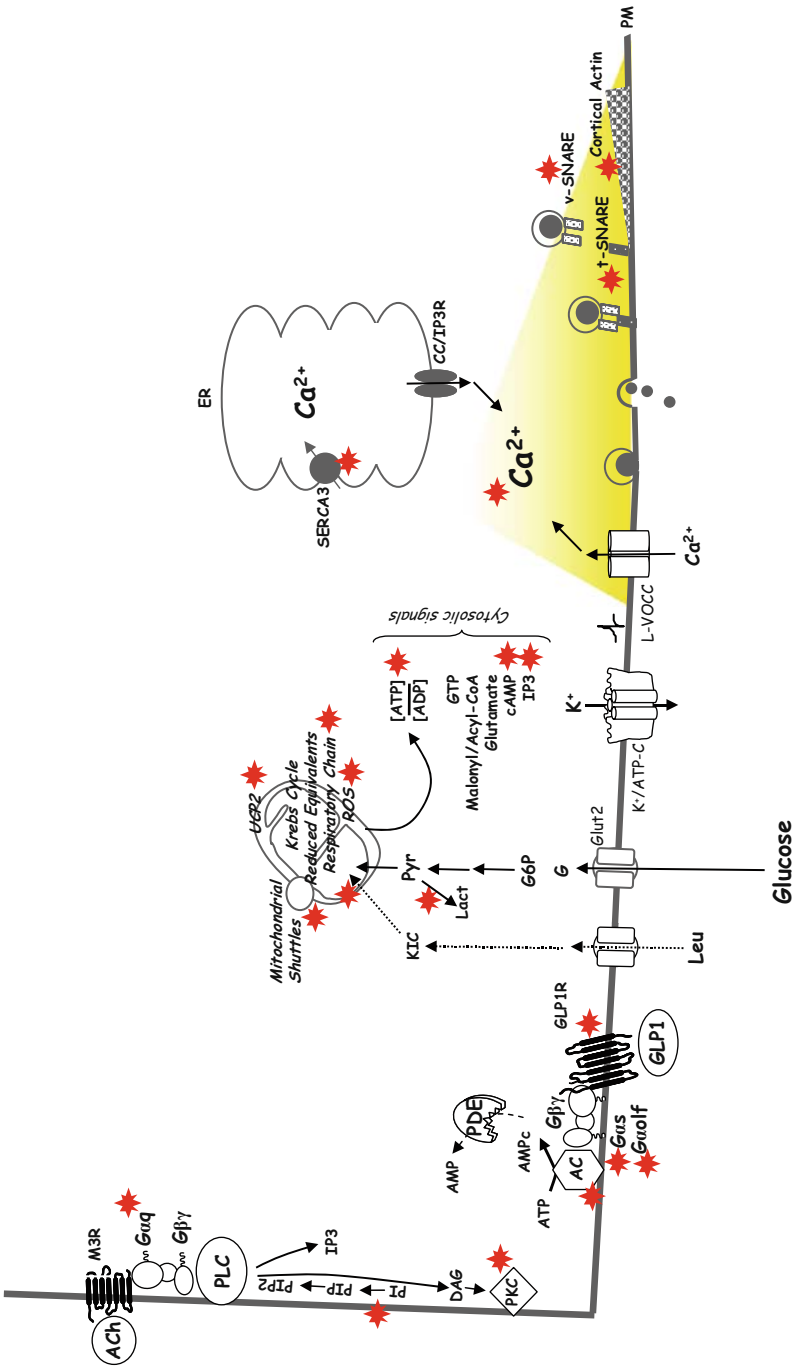

Fig 21.2 (continued)

[14, 67]. Finally it is of interest that GK islets are duly responsive to non-nutrient stimuli such as the sulfonylureas gliclazide (GK/Par) [60] and mitiglinide (GK/Sto) [100], the combination of Ba^{2+} and theophylline (GK/Par) [60], or high external K^+ concentrations (GK/Lon, GK/Sto, GK/Seat, GK/Par) [15, 64, 101, Dolz and Portha, unpublished data]. However this does not support the assumption from the molecular biology data that there exists a defect in the late steps of insulin secretion. As a tentative to elucidate this apparent contradiction, exocytosis assessment with high time-resolution membrane capacitance measurement in GK/Sto pancreatic slices showed a decreased efficacy of depolarization-evoked Ca^{2+} influx to trigger rapid vesicle release, contrasting with a facilitation of vesicle release in response to strong sustained Ca^{2+} stimulation [88].

21.5.6 Islet ROS Scavenging Capacity Is Increased

Considerable interest has recently been focused on the putative role of oxidative stress (OS) upon deterioration of β-cell function/survival in diabetes. Recent data from our group indicate that paradoxically GK/Par islets revealed protected against OS since they maintained basal ROS accumulation similar or even lower than non-diabetic islets. Remarkably, GK/Par insulin secretion also exhibited strong resistance to the toxic effect of exogenous H_2O_2 or endogenous ROS exposures. Such adaptation was associated to both high glutathione content and overexpression of a large set of genes encoding antioxidant proteins as well as UCP2 [8, 31].

Figure 21.2 illustrates a compendium of the abnormal intracellular sites so far identified in the diabetic GK islets from the different sources.

21.6 Which Aetiology for the Islet Functional Abnormalities?

There are several arguments indicating that the GK β-cell secretory failure is, at least partially, related to the abnormal metabolic environment (gluco-lipotoxicity). When studied under in vitro static incubation conditions, islets isolated from normoglycaemic (prediabetic) GK/Par pups amplified their secretory response to high glucose, leucine or leucine plus glutamine to the same extent as age-related W islets [5]. This suggests that there does not exist a major intrinsic secretory defect in the prediabetic GK/Par β-cells which can be considered as normally glucose competent at this stage, at least when tested in vitro. In the GK/Par rat, basal hyperglycaemia and normal to very mild hypertriglyceridaemia are observed only after weaning [5]. The onset of a profound alteration in glucose-stimulated insulin secretion by the GK/Par β-cell (after weaning) is time correlated with the exposure to the diabetic milieu. These changes in islet function could be ascribed, at least in part, to a loss of differentiation of β-cells chronically exposed to even mild chronic hyperglycaemia and elevated plasma non-esterified fatty acids. This view is supported by the reports that chronic treatments of adult GK rats with phlorizin [8, 61, 70, 96], T-1095

[102], glinides [100, 103], glibenclamide [103], gliclazide [104], JTT-608 [105, 106], voglibose [107], or insulin [103] partially improved glucose-induced insulin release, while hyperlipidaemia induced by high-fat feeding markedly impaired their insulin secretion [79].

The recent identification of TCF7L2 as a major predisposition gene for T2D and the predominant association of TCF7L2 variants with impaired insulin secretion have highlighted the importance of Wnt signaling in glucose homeostasis. In fact, two studies in human diabetic islets have reported that the expression of TCF7L2 is increased at mRNA [108, 109] and at protein levels [109] and it has been found that TCF7L2 overexpression in pancreatic β-cells is associated with reduced insulin secretion [108]. Islet TCF7L2 mRNA and protein levels revealed higher in GK/Par islets [Tourrel-Cuzin and Movassat, unpublished). Similar observation was reported in GK/Sto islets [52]. The functional link between the upregulation of TCF7L2 and the impairment of β-cell growth and function in the GK model remains to be uncovered.

Besides, there are indications in the GK/Sto rat that two distinct loci encode separately defects in β-cell glucose metabolism and insulin exocytosis [52]. Generation of congenic rat strains harbouring different parts of GK/Sto-derived Niddm1i has recently enabled fine mapping of this locus. Congenic strains carrying the GK genotype distally in Niddm1i displayed reduced insulin secretion in response to both glucose and high potassium, as well as decreased single-cell exocytosis. By contrast, the strain carrying the GK genotype proximally in Niddm1i exhibited both intact insulin release in response to high potassium and intact single-cell exocytosis, but insulin secretion was suppressed when stimulated by glucose. Islets from this strain also failed to respond to glucose by increasing the cellular ATP/ADP ratio. Since the congenics had not developed overt hyperglycaemia and their β-cell mass was found normal, it was concluded that their functional defects in glucose metabolism and insulin exocytosis were encoded by two distinct loci within Niddm1i [52]. These results in the GK/Sto are however inconsistent as previously mentioned (see Section 21.4), with the conclusion of a similarly designed congenic study indicating that the corresponding short region of the QTL Nidd/gk1 in GK/Ox congenics contributes to enhanced (and not decreased) insulin release [53]. Interestingly, the gene encoding for transcription factor TCF7L2 is also located in this locus and has recently been identified as a candidate gene for T2D in humans [110]. However, Tcf7l2 RNA levels were not different in the GK/Sto congenics displaying reduced insulin secretion compared with controls [52].

In conclusion, taking into account the diverse information so far available from the GK model through its different phenotype variants, it is proposed that the reduction of GK β-cell number and function reflects the complex interactions of different pathogenic items: multiple morbid genes causing impaired insulin secretion, early epigenetic programming of the pancreas by gestational diabetes (decreased β-cell neogenesis and/or proliferation) which is transmitted from one generation to the other and acquired loss of β-cell differentiation due to chronic exposure to hyperglycaemia/hyperlipidaemia, inflammatory mediators, oxidative stress and to perturbed islet microarchitecture. Last but not least, careful comparison of the alterations so

far detected in the diabetic GK β-cell population and those found in the T2D human β-cell population put into the front stage a number of striking commonalities [111]. Of course, the GK β-cell is not a blueprint for the diseased β-cell in human. There are however sufficient similarities with high value, to justify more efforts to understand the aetiopathogenesis of T2D in this rat model now widely used and, more specifically, the central role played by the GK islet cells.

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Chapter 22
The β-Cell in Human Type 2 Diabetes

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Abstract β-cell dysfunction is central to the onset and progression of type 2 diabetes. Reduced islet number and/or diminished β-cell mass/volume in the pancreas of type 2 diabetic subjects have been reported by many authors, mainly due to increased apoptosis not compensated for by adequate regeneration. In addition, ultrastructural analysis has shown reduced insulin granules and morphological changes in several β-cell organelles, including mitochondria and endoplasmic reticulum. Several quantitative and qualitative defects of β-cell function have been described in human type 2 diabetes using isolated islets, including alterations in early phase, glucose-stimulated insulin release. These survival and functional changes are accompanied by modifications of islet gene and protein expression. The impact of genotype in affecting β-cell function and survival has been addressed in a few studies, and a number of gene variants have been associated with β-cell dysfunction. Among acquired factors, the role of glucotoxicity and lipotoxicity could be of particular importance, due to the potential deleterious impact of elevated levels of glucose and/or free fatty acids in the natural history of β-cell damage. More recently, it has been proposed that inflammation might also play a role in the dysfunction of the β-cell in type 2 diabetes. Encouraging, although preliminary, data show that some of these defects might be directly counteracted, at least in part, by appropriate in vitro pharmacological intervention.

Keywords β-cell volume · β-cell mass · Insulin secretion · Apoptosis · Regeneration · Mitochondria · Endoplasmic reticulum · Gene polymorphisms · Gene expression · Protein expression · Glucotoxicity · Lipotoxicity · Inflammation

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22.1 Introduction

β-cell dysfunction is central to the development and progression of type 2 diabetes [1–3]. Reduced β-cell functional mass in diabetes and other categories of glucose intolerance has been described in patients, and decreased islet and/or β-cell volume in the pancreas of type 2 diabetic patients has been consistently observed [4–6]. These findings are in agreement with the results obtained with healthy humans who underwent hemipancreatectomy for the purpose of organ donation and 43% of cases developed impaired fasting glucose, impaired glucose tolerance, or diabetes on 3–10 years of follow-up [7]. In addition, studies in patients and the use of isolated islets have shown both quantitative and qualitative defects of glucose-stimulated insulin secretion in type 2 diabetes [8–10]. The importance of β-cell function (in the absence of obvious reduction of β-cell mass) is supported by the MODY2 type of diabetes, due to mutations of the enzyme glucokinase, leading to decreased glycolytic flux in the β-cell [11]. In this chapter, we describe the mass and functional defects of β-cells in type 2 diabetes and discuss the accompanying molecular alterations. Then, the role of a few genetic and acquired factors affecting the β-cell is briefly discussed, followed by the description of the beneficial effects that some compounds directly have on the diabetic β-cell.

22.2 β-Cell Mass Defects

Early work reported that total islet number was approximately 30% lower in pancreatic histology samples from type 2 diabetic subjects as compared to those from non-diabetic individuals [12]. The reduction in total islet volume in diabetic vs. non-diabetic pancreata (1.01 ± 0.12 vs. 1.60 ± 0.16 cm^3) was confirmed [13] and resulted even more marked when corrected for the presence of amyloid [13]. Successively, it was found that β-cell volume was 30–40% reduced in type 2 diabetic islets [14]. In the following years, although a few authors were not able to find differences in β-cell amount in diabetic vs. non-diabetic pancreas specimens [15, 16], several studies have consistently shown that β-cell mass is reduced in type 2 diabetes [17–21]. Clark and colleagues studied the pancreas of 15 type 2 diabetic and 10 control subjects, and observed 24% β-cell area reduction in the diabetic samples [17]. More recently, it has been reported that islet β-cell volume density and total β-cell mass were significantly lower (∼30%) in pancreatic specimens from Japanese type 2 diabetic patients in comparison with those obtained from non-diabetic individuals [18]. Accordingly, when pancreas samples following surgical removal were studied [19], it was found that in the non-diabetic cases β-cell volume was $1.94 \pm 0.7\%$, whereas specimens from type 2 diabetic patients contained a lower β-cell volume ($1.37 \pm 1.0\%$). In addition, in the diabetic samples, no correlation was found between β-cell volume and diabetes duration [19]. Pancreatic autoptic samples from type 2 diabetic patients, subjects with impaired fasting glycaemia (IFG), and non-diabetic individuals (the groups were subdivided

into lean or obese according to BMI) have been studied lately [20]. In normoglycaemic cases, obesity was associated with 50% higher β-cell volume, as compared to non-obese individuals. However, obese subjects with IFG or diabetes had 40–60% reduction in β-cell volume in comparison to BMI-matched, non-diabetic cases. This was due to β-cell number decrease, rather than changes in islet size. In the non-obese group, diabetes was associated with 41% reduction in the volume of the β-cells. A detailed study has been published very recently [21]. The authors analysed autoptic samples from 57 type 2 diabetic and 52 non-diabetic European subjects and confirmed that β-cell mass was lower (around 30%) in the former (Fig. 22.1). However, there was marked inter-subject variability and large overlap between the two groups (Fig. 22.1). No difference was found between diabetic patients treated with oral agents and insulin, whereas β-cell mass increased with BMI values and decreased with duration of diabetes [21]. Finally, a reduced number of β-cells in islets from type 2 diabetic subjects has been demonstrated by electron microscopy as well, which also showed that volume density of mature insulin granules was lower in type 2 diabetic than in non-diabetic β-cells [22].

It is generally assumed that β-cell loss in type 2 diabetes is mainly due to increased β-cell apoptosis [20, 23]. As a matter of fact, in autoptic samples, apoptosis was shown to be three- and tenfold higher in obese and lean type 2 diabetic samples, respectively, than in BMI-matched, normoglycaemic individuals [20], and increased β-cell apoptosis in diabetic islets has been reported following electron

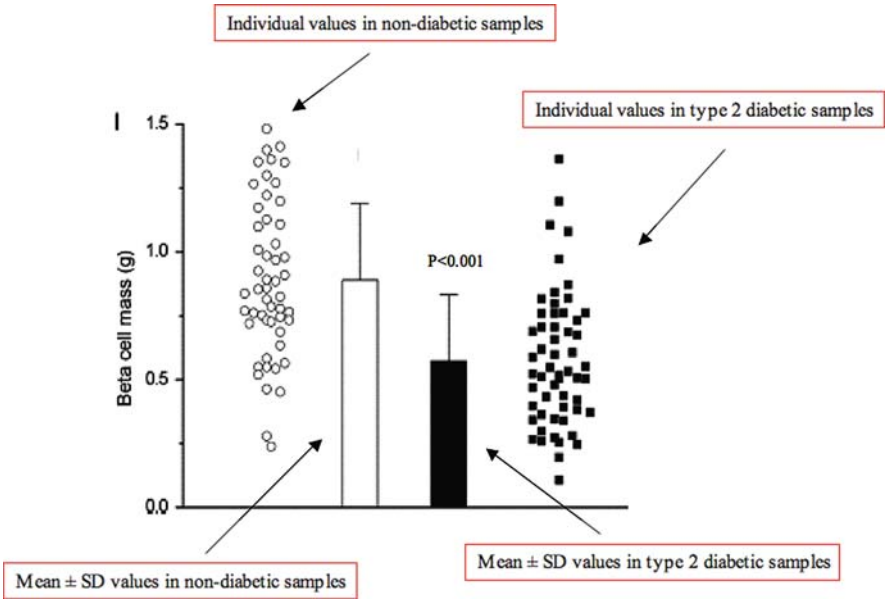

Fig. 22.1 β-cell mass is reduced in type 2 diabetic patients, as compared to non-diabetic controls, although there is a marked inter-subject variability and clear overlap between the two groups (adapted from [21])

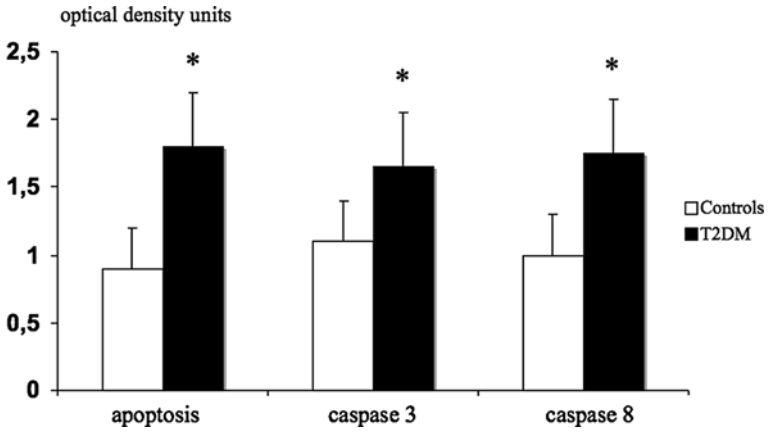

Fig. 22.2 Isolated type 2 diabetic (T2DM) islets show increased apoptosis and enhanced caspase-3 and caspase-8 activities, as compared to non-diabetic controls. Death was measured by ELISA methods evaluating cytoplasmic histone-associated DNA fragments, and caspase activity was determined using a colorimetric assay. * $p < 0.05$ vs. controls (adapted from [22])

microscopy analysis [23]. In addition, by assessing cytoplasmic histone-associated DNA fragments, it has been observed that there is a twofold higher amount of islet cell death with isolated diabetic islets, as compared to non-diabetic islets [22] (Fig. 22.2). This was accompanied by a significant increase in the activity of caspase-3 and caspase-8, key molecules in the apoptotic pathway [22] (Fig. 22.2). Several factors can contribute to cause β-cell apoptosis (see below), and intracellular organelles, including the endoplasmic reticulum, are likely to be actively involved [23]. On the other hand, the enhanced β-cell death rate does not seem to be adequately compensated for by regenerative phenomena in diabetic islets. In autoptic specimens, it has been reported that the relative rate of new islet formation, estimated by fraction of duct cells positive for insulin, and the frequency of β-cell replication, assessed by Ki67 staining, were substantially similar in type 2 diabetic and control pancreata [20].

Therefore, current evidence shows a reduced β-cell amount in human type 2 diabetes, possibly due to increased apoptosis without adequate regeneration. However, the loss of β-cell appears to be 30% on average, which is unlikely to lead to overt diabetes, unless a defect in β-cell function is present as well.

22.3 β-Cell Functional Defects

Several functional properties of the pancreatic β-cells in type 2 diabetes have been directly evaluated ex vivo following islet isolation from the human pancreas. Earlier work showed that the release of insulin evoked by glucose was lower in type 2 diabetic than in non-diabetic islets [24]. However, the secretory response to the

combination of L-leucine and L-glutamine appeared less severely altered [24]. In a detailed study by Deng and colleagues, islets isolated from eight diabetic and nine normal donors were evaluated by in vitro islet perifusion experiments [25]. Basal insulin secretion was similar for both normal and diabetic islets. However, the islets from diabetic donors released less total insulin in response to glucose and also exhibited an elevated threshold for insulin secretion triggering. In addition, it was observed that in comparison with normal islets, an equivalent amount of type 2 diabetic islets did not fully reverse the hyperglycaemic condition when transplanted into diabetic mice [25]. In another study, when insulin secretion was measured in response to glucose, arginine, and glibenclamide in isolated non-diabetic and type 2 diabetic islets, again no significant difference as for insulin release in response to 3.3 mmol/l glucose was observed [26]. However, when challenged with 16.7 mmol/l glucose, diabetic islets secreted significantly less insulin than did non-diabetic cells. Insulin secretion during arginine and glibenclamide stimulation was also lower from diabetic islets than from control islets; however, type 2 diabetic islets released a significantly higher amount of insulin in response to arginine and glibenclamide than in response to glucose. In addition, when perifusion experiments were performed, glucose stimulation did not elicit any apparent increase in the early insulin

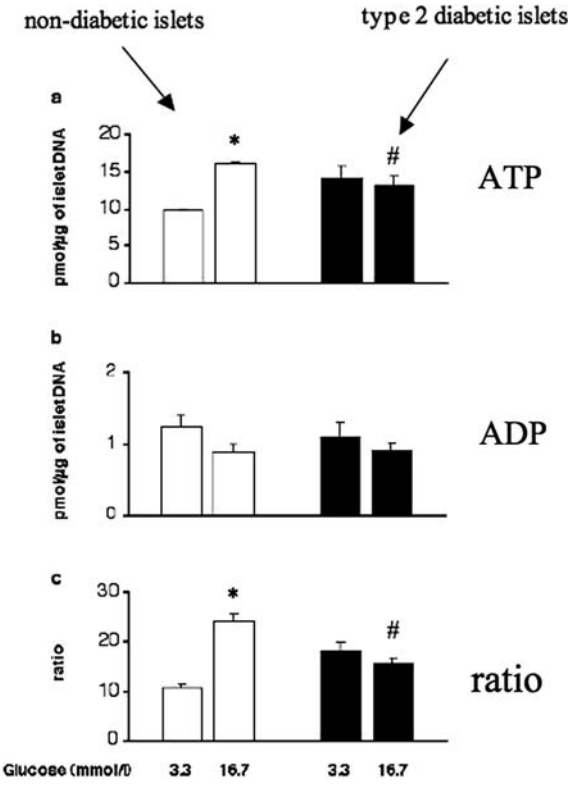

Fig. 22.3 ATP production and ATP/ADP ratio increase in non-diabetic but not in type 2 diabetic islets following exposure to 3.3–16.7 mmol/l glucose concentration. *: significantly higher vs. 3.3 mmol/l glucose; #: significantly lower vs. non-diabetic islets at 16.7 mmol/l glucose (adapted from [27])

secretion phase from diabetic islets, which however promptly released insulin when challenged with arginine or sulfonylurea [26]. Consistent with the observation that β-cell insulin secretion defects in type 2 diabetes β-cells are more selective for glucose-induced stimulation, it has been observed that in type 2 diabetic islets glucose oxidation is reduced, as compared to non-diabetic islets [24, 26]. This has led to the speculation that mitochondria might be involved in causing β-cell dysfunction in type 2 diabetes. In this regard, the morphology and the function of mitochondria in human type 2 diabetic β-cells have been studied [27]. By electron microscopy, mitochondria in type 2 diabetes β-cells appeared round-shaped, hypertrophic, and with higher density volume when compared to control β-cells. When adenine nucleotide content was measured, it was found that islets from diabetic subjects were not able to increase their ATP content in the presence of acute glucose stimulation (Fig 22.3). As a consequence, the ATP/ADP ratio was approximately 40% lower in diabetic than in control islets, which could contribute to the blunted or absent glucose-stimulated insulin release in the former [27] (Fig. 22.3).

In summary, insulin secretion defects in human type 2 diabetic islets have been described by several authors, and data show more marked changes in insulin release in response to glucose, as compared to other fuel and non-fuel stimuli. This suggests that type 2 diabetic β-cells may have alterations in some steps of glucose metabolism, including those at the mitochondria level, leading to reduced ATP production.

22.4 Molecular Changes

Changes at the gene and protein expression levels have been reported in type 2 diabetic pancreatic islets by several authors. Using oligonucleotide microarrays of pancreatic islets isolated from humans with type 2 diabetes versus normal glucose-tolerant controls, Gunton et al. found that 370 genes were differently expressed in the two groups (243 upregulated and 137 downregulated) [28]. Quantitative RT-PCR studies were performed on selected genes, which confirmed changes in the expression of genes known to be important in β-cell function, including major decreases in the expression of *HNF4alpha*, insulin receptor, *IRS2*, *Akt2*, and several glucose-metabolic-pathway genes. There was also a 90% decrease in the expression of the transcription factor ARNT/HIF1beta (hydrocarbon nuclear receptor translocator/hypoxia-inducible factor 1β) [28]. Successively, several genes encoding for the following proteins were found to be downregulated in type 2 diabetic islets by real-time RT-PCR: insulin, glucose transporter 1, glucose transporter 2, glucokinase, and molecules involved in insulin granules exocytosis [26, 29]. Conversely, several genes implicated in differentiation and proliferation pathways have been reported to be increased in diabetic islets, including PDX-1, Foxo-1, Pax-4, and TCF7L2 [26, 30, 31]. Furthermore, changes at the level of the expression of genes involved in regulating cell redox balance have been shown [22]. As a matter of fact, mRNA expression of NADPH oxidase has been found to be increased and that

of manganese- and copper/zinc superoxide dismutases to be decreased in diabetic islets, together with enhanced expression of catalase and GSH peroxidase [22]. In a recent paper, the expression of several genes associated with the function of the endoplasmic reticulum (in particular, those encoding for immunoglobulin heavy chain binding protein, *BiP*, and X-box binding protein 1, *XBP-1*) has been described to be induced by exposure to high glucose in type 2 diabetic islets, but not in control islets [23]. When β-cell-enriched preparations obtained by the laser capture microdissection technique were studied [32], transcriptosome analysis preliminarily performed on four type 2 diabetic and four samples showed that in diabetic samples, there were 1,532 upregulated and 528 downregulated genes [32].

Some information is also available as for protein expression in type 2 diabetic islets. The amount of insulin has been reported to be decreased 30–40% in diabetic islet cells [22, 29]. The expression of AMP-activated kinase, IRS-2, PDX-1 (this latter at odds with gene expression data), and that of proteins involved in exocytosis was also found to be decreased in type 2 diabetic islets in comparison to non-diabetic samples [22, 29]. Preliminary data on type 2 diabetic islet protein profiling have been reported recently [33]. The results showed that although considerable variability existed within the individuals, 31 differentially expressed peaks were detected, and the intensities of some of them were significantly correlated with ex vivo islet insulin release [33].

Whereas many defects at the gene and protein expression level have been described in islet cells from type 2 diabetic subjects, at present it is not possible to distinguish between primary β-cell molecular changes (leading to diabetes) and those occurring as a consequence of the unfavourable microenvironment associated with the diabetic conditions (see below). Since prospective studies in this regard are not feasible for obvious reasons, it would be of interest to compare the molecular properties of β-cells from individuals at different stages of disease.

22.5 The Role of Genetic and Acquired Factors

Type 2 diabetes is a polygenic disease, and in the past few years, linkage studies, candidate-gene approaches, and genome-wide association studies have identified several gene variants which associate with this form of diabetes [34–39]. The majority of these genes are involved in β-cell function and survival, and for some of them the description is available as for their direct effects on some β-cell features in humans. The common Gly(972) → Arg amino acid polymorphism of insulin receptor substrate 1, Arg(972) IRS-1, has been found to be associated with functional and morphological alterations of isolated human islets, including increased susceptibility to apoptosis, diminished glucose-stimulated insulin secretion, and lower amount of insulin granules [40, 41]. Similarly, the E23K variant of *KCNJ11* gene, encoding the pancreatic β-cell adenosine 5′-triphosphate-sensitive potassium channel subunit Kir6.2 and associated with an increased risk of secondary failure to sulfonylurea in patients with type 2 diabetes [42], has been shown to be associated

with impairment of glibenclamide-induced insulin release following 24-hour exposure to high glucose concentration. However, those studies were performed on islets isolated from non-diabetic subjects. More recently, genetic variants in the gene encoding for transcription factor-7-like 2 (TCF7L2) have been associated with type 2 diabetes and impaired β-cell function [43]. It has been shown that the CT/TT genotypes of SNP rs7903146 strongly predicted future diabetes in independent cohorts of patients and that TCF7L2 expression in human islets was increased fivefold in type 2 diabetes, particularly in carriers of the TT genotype [31]. In this study, overexpression of TCF7L2 in human islets reduced glucose-stimulated insulin secretion. However, in another report, depleting TCF7L2 by siRNA resulted in decreased glucose-stimulated insulin release, increased β-cell apoptosis, and decreased β-cell proliferation in human islets [44]. In contrast, overexpression of TCF7L2 protected islets from glucose and cytokine-induced apoptosis and impaired function [44]. It cannot be excluded that in the presence of diabetes, phenotypic changes occurring independent of the genotype may render the overall picture less clear.

Several acquired factors can affect β-cell survival and function [2–6]. In particular, the effects of glucotoxicity and lipotoxicity (terms used to indicate the deleterious effects induced on tissues and cells by prolonged exposure to increased glucose or free fatty acid concentrations) have been studied with isolated islets. Both conditions can lead to increased apoptosis, reduced glucose-stimulated insulin release, and molecular changes [4]. Unfortunately, very little information is available on gluco- and/or lipotoxicity on human type 2 diabetic islets. In a recently published study [23], several features of β-cell endoplasmic reticulum were investigated in islets from non-diabetic and type 2 diabetic subjects. Whereas signs of endoplasmic reticulum stress were found in diabetic β-cells, it was also reported that when the islets were cultured for 24 hours in 11.1 mmol/l glucose, there was the induction of immunoglobulin heavy chain binding protein (*BiP*) and X-box binding protein 1 (*XBP-1*) in the type 2 diabetic islets [23] (Fig 22.4). Obviously, more work is needed on these issues.

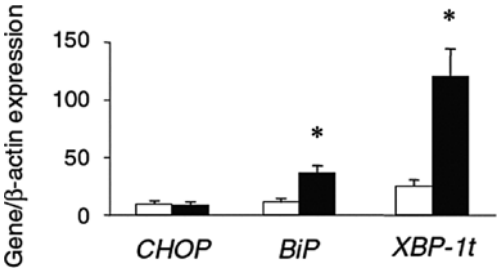

Fig. 22.4 When isolated type 2 diabetic islets were exposed for 24 hours at increased glucose concentration (see text for details), a significant induction of genes involved in endoplasmic reticulum stress (*BiP* and *XBP-1t*) was observed, as measured by quantitative RT-PCR. The expression of another gene (*CHOP*) did not change (adapted from [23])

The mechanisms mediating the deleterious effects of acquired factors are being actively investigated, with increased oxidative stress probably playing an important role [45]. As a matter of fact, when the presence of 8-hydroxy-2'-deoxyguanosine (a marker of oxidative stress-induced DNA damage) and 4-hydroxy-2-nonenal modified proteins (a marker of lipid peroxidation products) was determined by immunostaining in islets of type 2 diabetic patients, both markers resulted significantly increased as compared with non-diabetic individuals [18]. In addition, reduced staining of Cu/Zn superoxide dismutase was observed in the diabetic islet cells [18]. Similar findings were reported in a study performed with isolated type 2 diabetic islets [22], which showed increased content of nitrotyrosine and 8-hydroxy-2'-deoxyguanosine, and reduced expression of Cu/Zn- and Mn superoxide dismutase. All this may contribute to produce a proinflammatory soil, which has been proposed to lead to β-cell damage in type 2 diabetes [46, 47]. Pancreatic islets may respond to metabolic stress by producing inflammatory factors, such as IL-1, and macrophage infiltration has been found in human type 2 diabetic islets. It is however possible that some of these pathways may be activated in subgroups of patients [48].

Dealing with all the information continuously and rapidly coming from genetic studies is not an easy task, but the assessment of the relationships between β-cell genotype and phenotype is crucial to understand why the β-cell fails in type 2 diabetes and in which way it is affected by acquired factors.

22.6 Reversal of β-Cell Damage in Type 2 Diabetes

The possibility that pancreatic β-cell damage induced by acquired factors can be prevented has been demonstrated in isolated non-diabetic islets exposed to different metabolic perturbations [4]. More importantly, a few studies have shown that β-cell dysfunction in type 2 diabetes may be reversible. Exposure of isolated type 2 diabetic islets to antioxidants has led to improved glucose-stimulated insulin secretion and normalized expression of a few ROS scavenging enzymes [26, 49]. As mentioned above, a study showed that isolated type 2 diabetic islets were characterized by reduced insulin content, decreased amount of mature insulin granules, impaired glucose-induced insulin secretion, reduced insulin mRNA expression, and increased apoptosis with enhanced caspase-3 and -8 activities [22]. These alterations were associated with increased oxidative stress, as shown by higher nitrotyrosine concentrations, increased expression of protein kinase C-beta2 and NADH oxidase, and changes in mRNA expression of Mn superoxide dismutase, Cu/Zn superoxide dismutase, catalase, and glutathione peroxidase [22]. When these islets were incubated for 24 hours in the presence of therapeutic concentration of metformin, insulin content and the number of mature insulin granules increased (Fig. 22.5) and glucose-induced insulin release improved, with induction of insulin mRNA expression. Moreover, apoptosis was reduced, with concomitant decrease of caspase-3 and -8 activities. These changes were accompanied

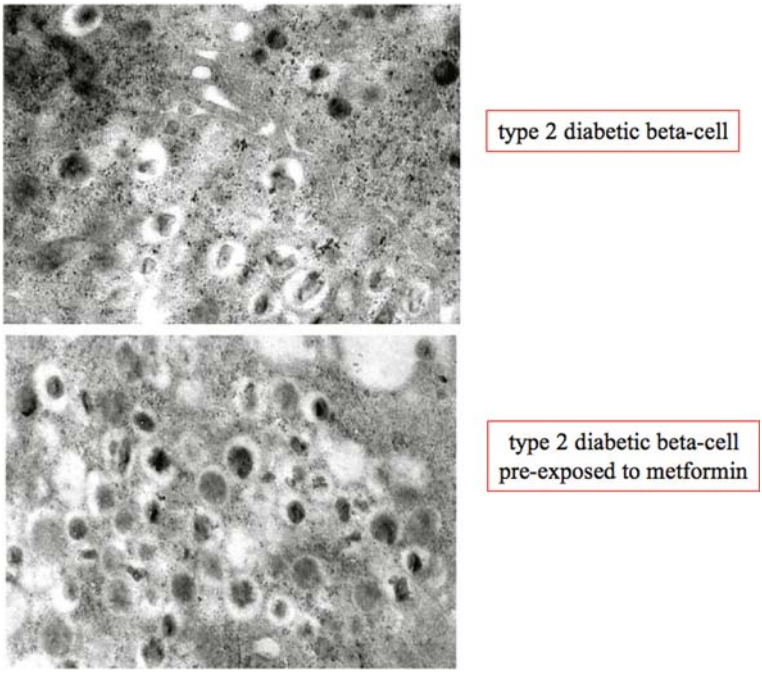

Fig. 22.5 The amount of insulin granules in type 2 diabetic β-cells increases following pre- exposure for 24 hours with therapeutic concentration of metformin. Electron microscopy evaluation, magnification ×160,000 (reproduced with modifications from [22])

by reduction or normalization of markers of oxidative stress [22]. Recently, the role of incretins [GLP-1, glucose-dependent insulinotropic polypeptide [GIP] and some of their analogs] in the therapy of diabetes has received much attention, mainly because of the beneficial actions of these molecules (GLP-1 in particular) on the β-cell [50]. In a recent study [51], pancreatic islets were prepared from the pancreas of non-diabetic and type 2 diabetic donors, and then incubated in the presence of 5.5 mmol/l glucose, with or without the addition of exendin-4 (a long-acting GLP-1 mimetic). Insulin secretion from the type 2 diabetic islets improved after incubation with exendin-4, which also induced a significantly higher expression of insulin, glucose transporter 2, glucokinase, and some β-cell regeneration and differentiation factors, including pancreas duodenum homeobox-1 (Pdx-1).

Therefore, acting directly at the β-cell level to prevent damage or restore functional and survival competence is feasible in vitro. Strategies need to be developed to deliver the appropriate treatment to the β-cell in vivo, to be combined with therapies aiming to limit the negative impact on the islets of acquired conditions such as glucotoxicity and lipotoxicity (see above).

22.7 Conclusions

Pancreatic β-cells in type 2 diabetes have several defects (Table 22.1). Decreased β-cell mass is due to increased apoptosis not compensated for by adequate β-cell regeneration. Insulin secretion defects are more marked in response to glucose, suggesting that handling of this fuel by the β-cell is defective somewhere along the road leading to ATP production. These alterations are accompanied by several molecular defects, possibly due, at least in part, to genetic variations and acquired factors, which still need to be set in a more comprehensive picture. The observation that β-cell defects may be reversible supports the concept that β-cell dysfunction in human type 2 diabetes could not be relentless.

Table 22.1 Main defects of pancreatic β-cells in human type 2 diabetes

β-cell mass
Increased apoptosis
Not sufficient proliferation
Not sufficient neogenesis
β-cell function
Reduced glucose-stimulated insulin secretion
Blunted or absent early phase insulin secretion
Increased proinsulin/insulin ratio
Altered pulsatility of insulin release
Molecular features
Altered expression of genes involved in β-cell function and survival
Altered expression of proteins involved in β-cell function and survival
Increased production of reactive oxygen and nitrogen species

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Chapter 23
Clinical Approaches to Preserve β-Cell Function in Diabetes

Bernardo Léo Wajchenberg

Abstract In type 2 diabetes (DM2) there is progressive deterioration in β-cell function and mass. It was found that islet function was about 50% of normal at the time of diagnosis and reduction in β-cell mass of about 60% at necropsy (accelerated apoptosis). Among the interventions to preserve the β-cells, those to lead to short-term improvement of β-cell secretion are weight loss, metformin, sulfonylureas, and insulin. The long-term improvement was demonstrated with short-term intensive insulin therapy of newly diagnosed DM2, the use of antiapoptotic drugs such as glitazones, and the use of glucagon-like peptide-1 receptor agonists (GLP-1 mimetics), not inactivated by the enzyme dipeptidyl peptidase 4 and/or to inhibit that enzyme (GLP-1 enhancers). The incretin hormones are released from the gastrointestinal tract in response to nutrient ingestion to enhance glucose-dependent insulin secretion from the pancreas and overall maintenance of glucose homeostasis. From the two major incretins, GLP-1 and GIP (glucose-dependent insulinotropic polypeptide), only the first one or its mimetics or enhancers can be used for treatment. The GLP-1 mimetics exenatide and liraglutide as well as the DPP 4 inhibitors (sitagliptin and vildagliptin) were approved for treatment of DM2.

Keywords Type 2 diabetes · β-cell function · Preservation β-cells · Glitazones · GLP-1 mimetics and enhancers

Abbreviations

AST	Aspartate amino transferase
ALT	alanine amino transferase
BMI	body mass index
DM2	type 2 diabetes mellitus
DPP 4	dipeptidyl peptidase 4

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ER	endoplasmic reticulum
FA	fatty acid
FFA	free fatty acid
GIP	glucose-dependent insulinotropic polypeptide
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1 receptor
GLP-2	glucagon-like peptide-2
HbA1c	glycated hemoglobin
HOMA	homeostasis model assessment
HOMA - β or B	HOMA of β-cell function
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
PI/IRI ratio	proinsulin to total immunoreactive insulin ratio
PPARγ	peroxisome proliferator-activated receptor γ
ROS	reactive oxygen species

Type 2 diabetes (DM2) is caused by an insufficient insulin secretion usually in the context of resistance of the peripheral tissues to the action of the hormone and characterized by progressive deterioration of the β-cell function over time. The deterioration occurs regardless of therapy allocation, albeit conventional (mainly diet), insulin, sulfonylureas, or sensitizers such as glitazones and metformin [1, 2]. DM2 subjects show both quantitative and qualitative disturbances in plasma insulin levels (loss of acute insulin response to glucose – loss of the first phase; impaired insulin oscillations during sustained second phase of glucose-induced insulin secretion and defects in proinsulin processing at the β-cell level, resulting in an increased in proinsulin to insulin ratio [3]). Associated with reduced β-cell function found to be about 50% normal level at the time of diagnosis, independent of the degree of insulin resistance and probably commencing 10–12 years before diagnosis and aggravated by increasing fasting plasma glucose levels [4], a reduction in β-cell mass of about 60% has been observed at necropsy. The underlying mechanism was found to be increased β-cell apoptosis, while new islet formation and β-cell replication (normalized to relative β-cell volume) remained normal or increased [5].

While there is consensus that hyperglycemia develops in the context of insulin resistance only if insulin secretion is insufficient, the question remains as to whether this insufficiency reflects functional abnormalities in each β-cell or too low a number of appropriately functioning β-cells, usually referred to as a low β-cell mass [6]. As indicated by Rahier et al. [7], sub-optimal β-cell function leads to a higher risk of developing DM2 if there is also low β-cell mass, while the slow decrease in β-cell mass with duration of diabetes could, at least in part, be a secondary phenomenon caused by exposure to a metabolically abnormal environment: glucolipotoxicity [8].

Paradoxically, it has also been proposed that an important mechanism contributing to β-cell failure in DM2 is the ability to hypersecrete insulin [9]. Hypersecretion, a characteristic in the early stages of the disease, is beneficial in maintaining normal glucose tolerance which may also be an important factor in the progression of β-cell failure [9]. A state of hyperinsulinemia can be caused by increased insulin demand (insulin resistance), a genetic abnormality leading to hypersecretion (as in persistent hyperinsulinemic hypoglycemia of infancy) or the use of insulin secretory drugs (sulfonylureas such as glibenclamide). The increased demands for insulin production could overload the ER, resulting in ER stress and inducing the unfolded protein response. Furthermore, apoptosis of the β-cells has recently been shown to be the result of activation of an ER stress response [10]. Alternatively, the increased glycolytic flux required for increased insulin secretion could result in oxidative stress. In individuals with a genetic predisposition, the increased ER stress could lead to β-cell failure and subsequent diabetes. The treatment of diabetes with insulin secretory drugs could further promote insulin hypersecretion, leading to worsening of β-cell function.

Besides glucotoxicity, lipotoxicity, and glucolipotoxicity, which are secondary phenomena playing a role in β-cell dysfunction, other factors could contribute to the progressive loss of β-cell function in DM2 [3].

In conclusion, drawing on all the information available, it can be suggested that the link between reduced β-cell mass and impaired function could be due to an increased demand on residual β-cells per se leading to changes in function (ER stress or other mechanisms) or related to the hyperglycemia resulting from decreased β-cell mass, driving the impairment in β-cell function. In vitro and in vivo studies in rodents (not in humans, as shown previously) have indicated that persistently high glucose levels play a central role among those factors (FFAs, lipoproteins, leptin, and cytokines) contributing to β-cell demise.

Understanding the mechanisms of β-cell death and thus decreased β-cell mass, at least in rodents, and impaired function has provided the basis of β-cell preservation, especially when one considers that the impaired β-cell function and possibly β-cell mass appear to be reversible to a certain degree, particularly at early stages of the disease where the threshold for reversibility of decreased β-cell mass has probably not been passed. Therefore, any therapeutic intervention aimed at preserving β-cell activity should improve function and prevent further reduction in mass.

23.1 Clinical Impact of Therapies Aimed at β-Cell Preservation

23.1.1 Short-Term Improvement of β-Cell Insulin Secretion

The current diabetes treatment options which lead to short-term improvement of β-cell secretion include weight loss and antidiabetic medications: oral insulin secretagogues and insulin:

Weight loss improves insulin secretion in obese DM2 [11]. Among the oral antidiabetic drugs, metformin improves glucose levels before and after meals without significant changes in insulin levels, indicating improved glucose sensitivity, without changes in insulin secretion [12]. The sulfonylureas and glinides are commonly been used to stimulate insulin secretion in DM2 patients, enhancing β-cell responsiveness to glucose [13]. Several studies have shown that treatment with sulfonylureas is not associated with any change in the decay curve of β-cell function with time [14, 15]. Moreover, these compounds have been shown to cause apoptosis and therefore loss of β-cell mass [16]. Finally, short-term intensive insulin therapy in patients with DM2 has been shown to improve endogenous β-cell function and insulin resistance [17, 18]. However, prolonged benefit has rarely been demonstrated with virtually all patients becoming hyperglycemic again after a few weeks [19]. Until recently, it was unknown whether such outcomes pertained to new-onset DM2, although patients having failed diet therapy can show a good response to a short period of intensive insulin therapy by continuous subcutaneous insulin infusion (CSII), as initially demonstrated by Ilkova et al. [20].

23.1.2 Long-Term Improvement of β-Cell Insulin Secretion

Treatments that may lead to long-term improvement in β-cell insulin secretion include short-term intensive insulin therapy of newly diagnosed DM2 and the use of oral insulin sensitizers, glitazones and incretin mimetics (GLP-1 mimetics and enhancers) which have shown clinical evidence of effects on human β-cell function, the latter drugs having demonstrated, at least in rodents, to be associated with expansion of β-cell mass via stimulation of β-cell proliferation, promotion of islet cell neogenesis and inhibition of β-cell apoptosis [3, 21–23].

23.2 Short-Term Intensive Insulin Therapy of Newly Diagnosed DM2

Insulin therapy is the most effective antidiabetic therapy and has a variety of effects that may protect against the progression of β-cell dysfunction as suggested by the clinical studies to be outlined later. First, correcting hyperglycemia with insulin may alleviate glucolipotoxicity. Preclinical studies also suggest that insulin has anti-apoptotic effects via its action on IRS (insulin receptor substrate) proteins and may promote β-cell growth [24]. Numerous in vitro and clinical studies have also demonstrated that insulin therapy has potential anti-inflammatory benefits independent of its ability to lower blood glucose levels [25]. Further investigation is needed to determine the clinical implications of insulin's anti-inflammatory properties in the progression of DM2.

Optimal metabolic control, especially early intensive glycemic control, plays a role in the prevention of progressive β-cell dysfunction and possibly destruction of the β-cells with worsening of diabetes, as it will be presented below.

Table 23.1 Intensive insulin therapy in newly diagnosed type 2 diabetes

Author	*n*	Mean age (years)	Mean BMI (kg/m^2)	Baseline HbA_1c(%)	Therapy		Patients c/ euglycemia c/ therapy (%)	Patients c/euglycemia	
					Type	Duration (days)		At 6 months (%)	At 12 months (%)
Ilkova et al. [20]	13	50	26.9	11.0	CSII	14	92	69	N/A
Ryan et al. [27]	16	52	30.8	11.8	MDI	14–21	88	N/A	44
Li et al. [28]	138	49	25.0	10.9	CSII	14	91	67	47
	133	50	25.1	9.8	CSII	14–35	97	N/A	51
Weng et al. [29]	118	51	24.4	9.7	MDI	14–35	95	N/A	45
	101	52	25.1	9.5	OHA	14–35	84	N/A	27
Chen et al. [30]	22	59	27.7	11.7	MDI∗	1 year	N/A	65∗∗	55∗∗
	8	56	26.6	11.3	OHA	1 year	N/A	35∗∗	32∗∗

Modified, with permission from Retnakaran and Drucker [26].
N/A, not available; CSII, continuous subcutaneous insulin infusion; MDI, multiple day injections; OHA, oral hypoglycemic agents.
∗After 10–14 days of intensive (MDI) therapy.
∗∗Patients (%) with $HbA_1c \leq 6.5$.

Table 23.1 shows that in the available studies, early implementation of a short course of intensive insulin therapy either by continuous subcutaneous insulin infusion or by multiple daily injections can induce sustained euglycemia, in patients with DM2 [27–30], while off any antidiabetic therapy. The remission of DM2 achieved in these studies persisted for 1 year after cessation of insulin therapy in about 46% of patients. In the small series of patients treated for 1 year, after a short-term intensive insulin therapy, accompanied by Chen et al. [30], HbA1c levels were significantly lower in the insulin group than in the oral hypoglycemic agent(s) group at the sixth month and after 1 year the glycated hemoglobin level remained lower in the insulin group. Furthermore, Li and colleagues [28], as well as Weng et al. [29] and Chen et al. [30], reported that patients who maintained euglycemia while off oral antidiabetic therapy for 1 year showed greater recovery of β-cell function than their counterparts. It was suggested that an improvement in β-cell function, especially restoration of the first-phase insulin secretion, might be responsible for the ability of intensive insulin therapy to induce sustained euglycemia. Furthermore, proinsulin decreased highly significantly as did the PI/IRI ratio, indicating an improvement in the quality of insulin secretion [28, 29].

It should be noticed that in all series of patients the mean BMI, except in that from Ryan et al. [27], was within or slightly above the normal range, what is infrequent in the western countries where the majority of the patients are obese at admission. It could be suggested, at least for the Asian patients, that they presented a different phenotype of the disease with predominant β-cell failure and much less insulin resistance.

23.3 Glitazones

23.3.1 Indirect Effects by Amelioration of Insulin Sensitivity

The glitazones are agonists of PPARγ, a nuclear receptor that regulates transcription genes involved in lipid and glucose metabolism. Although predominantly expressed in adipose tissue, PPARγ is present in other insulin-sensitive tissues, including the pancreatic islet cells [31]. The development of small, insulin-sensitive adipocytes enhances glucose uptake and decreases hepatic glucose output, improving glycemic control as well as lowering plasma FFAs in DM2. Improving insulin sensitivity in the periphery may improve the glucose sensing ability of β-cells and preserve β-cell function by reducing the demand on these cells. It has been postulated that the improvement in β-cell function, particularly the normalization of the asynchronous insulin secretion that characterizes β-cell failure, could be related to a reduction in glucolipotoxicity due to improved glycemic control and/or improved insulin sensitivity seen with glitazones. This could suggest an increased ability of the β-cell to sense and respond to glucose changes within the physiological range after glitazone treatment [3].

23.3.2 Direct Effects via PPARγ Activation in Pancreatic Islands

Preclinical data in rodents has suggested that glitazones decrease β-cell apoptosis, maintaining β-cell neogenesis and prevent islet amyloidosis. Various mechanisms of action have been proposed to explain these effects [3].

In humans, as a class effect, glitazones consistently improve basal β-cell function, as measured by the HOMA model and observed during glitazone monotherapy and combination therapy. Further evidence of the beneficial effects on β-cells originates from other studies in which treatment with glitazones alone or added to maximal doses of a sulfonylurea and metformin or an insulin restored the first-phase insulin response to an IV glucose tolerance test [32]. In all studies, the beneficial effect of glitazones on β-cell function was independent of glucose control (as suggested by a similar reduction in HbA1c with no improvement in β-cell function found in the insulin-treated group), indicating that glitazones can promote recovery of β-cell function independently of the amelioration of insulin sensitivity [3].

Furthermore, extension studies with glitazones indicate that improvements in β-cell function are sustained over time in some individuals, both as monotherapy and in combination with metformin and/or sulfonylurea [33, 34]. Another study evaluated the durability of efficacy of rosiglitazone, metformin, and glyburide (glibenclamide) treatment for recently diagnosed DM2 in maintaining long-term glycemic control along with their effects on insulin sensitivity and β-cell function in 4,360 patients [15]. In this study, the cumulative incidence of monotherapy failure at

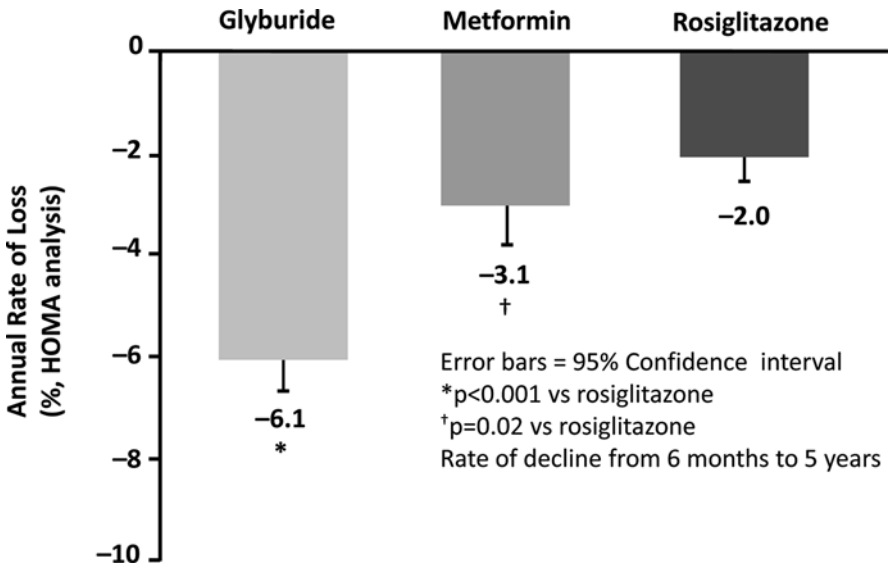

Fig. 23.1 ADOPT: Rosiglitazone reduces rate of loss of β-cell function. Analysis includes only patients continuing on monotherapy. Adapted from Kahn SE et al. [15]

5 years was 15% with rosiglitazone, 21% with metformin, and 34% with glyburide ($p < 0.001$; for both comparisons with rosiglitazone). During the first 6 months, levels of β-cell function (as evaluated by HOMA) increased more in the glyburide group than in either the rosiglitazone or the metformin groups. Thereafter, levels of β-cell function declined in all three groups. The annual rate of decline after 6 months was 6.1% with glyburide, 3.1% with metformin, and 2% with rosiglitazone ($p < 0.001$ vs. glyburide and $p = 0.02$ vs. metformin) – Fig. 23.1. In conclusion, the study showed the efficacy of glitazones, as compared with other oral glucose-lowering medications, in maintaining long-term glycemic control in DM2.

23.4 Incretin Mimetics

Incretin hormones are released by gastrointestinal tract in response to nutrient ingestion and enhance insulin secretion and aid in the maintenance of glucose homeostasis. The two major incretins are GLP-1 and GIP, which are released by enteroendocrine L cells located in the distal ileum and the colon and by the K cells in the duodenum, respectively [35]. They provide the additional stimulus to insulin secretion during oral ingestion that it not provided with IV glucose infusion. These incretins increase insulin secretion in a glucose-dependent manner through activation of their specific receptors in β-cells. In newly diagnosed DM2 with relatively good glycemic control) (HbA1c ~6.9%), both GIP and GLP-1 secretion in response to glucose and mixed meal challenges are the same or even increased when compared with healthy subjects [36, 37]. However, in long-standing DM2 with poor glycemic control (HbA1c ~8–9%) the GLP-1 response is decreased, whereas GIP secretion is unchanged [38]. In addition, insulin response to exogenous GLP-1 is 3- to 5-fold lower in DM2. However, acute GLP-1 administration is able to increase insulin secretion to normal levels and to lower plasma glucose effectively [39]. In contrast, exogenous GIP, even at supraphysiological doses, has markedly reduced insulinotropic action with little or no glucose-lowering effects in DM2 [40].

Thus, deterioration of glucose homeostasis can develop in the absence of any impairment in GLP-1 levels. This could suggest that the defects in GLP-1 concentrations previously described in patients with long-standing DM2 are likely to be secondary to other hormonal and metabolic alterations, such as fasting hyperglucagonemia and body weight which were negatively associated with GLP-1 levels, as assessed by the incremental areas under the curves, after oral glucose and meal ingestion [37]. Conversely, there is a positive relationship between GLP-1 and increasing age and a negative association with higher BMI levels. These associations were however stronger after oral glucose ingestion than after mixed meal ingestion. Accordingly, another study found that obesity and glucose tolerance each attenuate the incretin effect (i.e., the gain in β-cell function after oral glucose vs. intravenous glucose) on β-cell function and GLP-1 response [41]. In both studies it was concluded that GIP and GLP-1 appeared to be regulated by different factors and are independent of each other [37, 41].

Therefore, therapeutic strategies for DM2 within the incretin field focused on the use of GLP-1, GLP-1 analogues [GLP-1 receptor (GLP-1 R) agonists or GLP-1 mimetics], and GLP-1 enhancers and not GIP.

GLP-1 at pharmacological doses also has other non-insulinotropic effects beneficial for treating DM2: suppression of glucagon secretion in the presence of hyperglycemia and euglycemia, but no hypoglycemia, leading to improved hepatic insulin resistance and glycemic control; slowing of gastric emptying and gut motility, causing delayed nutrition absorption and dampened postprandial glucose excursion; and increasing the duration of postprandial satiety, leading to lower food intake, weight loss, and improved insulin resistance [35]. More importantly, acute GLP-1 infusion normalized fasting plasma glucose in patients with long-standing uncontrolled DM2 who were no longer responsive to sulfonylureas or metformin [42]. One major drawback of GLP-1 treatment is its short half-life (2 min), since it is rapidly degraded by dipeptidyl peptidase (DPP 4), which cleaves the N-terminal dipeptides (His 7-Ala 8) from GLP-1 (7–36) and the generation of the inactive metabolite GLP-1 [9–36, 43].

Modifications in the GLP-1 molecule to prevent degradation by DPP 4 have resulted in two compounds namely, the GLP-1R agonists, exenatide and liraglutide. Exenatide (synthetic exendin-4) is a 39 amino acid peptide produced in the salivary glands of the lizard "Gila monster" with 53% homology to full-length GLP-1. It binds more avidly to GLP-1R than GLP-1 and exendin-4 is not a substrate for DPP 4 because it has a Gly8 in place of an Ala8. Because exenatide is a peptide, it must be injected sc. Liraglutide is a long-acting GLP-1 analogue having a 97% homology with GLP-1 and resists DPP 4 degradation by fatty acid acylation and albumin binding, with a half-life of 12–14 h, allowing for a single daily dose administration while exenatide with a much shorter half-life (~2–4 h) has to be given in at least twice daily [44] (Table 23.2).

Table 23.2 Incretins mimetics: exenatide vs. liraglutide

	Exenatide	Liraglutide
Administration	Injection	Injection
Half-life [h]	≈ 2–4	≈ 12–14
Frequency of injection	Twice daily	Once daily
Dose per injection	5–10 μg	Up to 2 mg
DPP-4 substrate?	No	No
Insulin secretion^{+}	↑	↑
Glucagon secretion^{+}	↓	↓
Fasting glucose	↓	↓↓↓
Weight reduction	Yes	Yes
Gastric emptying	↓	(↓)
Antibody production	Yes (≈ 45%)	No

Reproduced with permission from Wajchenberg [3].
$^{+}$Glucose-dependent.

The acute effect of exogenous GLP-1 or GLP-1 R agonists on β-cells in rodent models of diabetes and in cultured β-cells is stimulation of glucose-dependent insulin release, whereas the subacute effect is enhancing insulin biosynthesis and stimulation of insulin gene transcription. Their chronic action is stimulation of β-cell proliferation, induction of islet neogenesis from precursor ductal cells, and inhibition of β-cell apoptosis, thus promoting an expansion of β-cell mass. This was also demonstrated in human islets freshly isolated from three cadaveric donors treated with liraglutide [3]. These effects have major implications in the treatment of DM2 because they directly address one of the fundamental defects in DM2, i.e., β-cell failure.

23.4.1 Exenatide

Clinical trials in DM2 patients who have not achieved adequate glycemic control on metformin and/or sulfonylurea, metformin, and/or TZD, as well as comparative trials with insulin glargine and biphasic insulin aspart, are available in the literature [45, 46]. With exenatide, 10 μg twice daily as adjuvant therapy to oral hypoglycemic agents, a significant number of patients (32–62%) achieved HbA1c of 7% or less when compared to placebo (7–13%), glargine (48%), and biphasic insulin aspart (24%). HbA1c reductions of 0.8–1.1% were sustained up to 3 years. Progressive weight loss ranging from 1.6–2.8 kg noted at 10 weeks to 5.3 kg at 3 years was also observed. Anti-exenatide antibodies were detected in 41–49% of patients in those treated with the drug but were not associated with glycemic control [38].

Regarding the side effects, severe hypoglycemia was rare while mild to moderate hypoglycemia was seen in 16 vs. 7% (exenatide vs. placebo) and more commonly in coadministration with a sulfonylurea. The most common side effects of exenatide were nausea (57%) – usually mild to moderate and being most common during the initial 8-week therapy and declining thereafter – and vomiting (17%). Overall, 4% of patients withdrew from the studies because of gastrointestinal side effects (46).

There have been reports of pancreatitis in the exenatide development program. Seven exenatide-treated subjects experienced pancreatitis compared with two in placebo/comparator cohorts, the corresponding incidence rate of pancreatitis across the entire development program being lower with exenatide than placebo. Of the exenatide-treated subjects who developed pancreatitis all had at least one independent risk factor for pancreatitis [47].

In the postmarketing period (from its approval by the FDA in April 2005 until December 2006) there were reported to the FDA 30 cases of pancreatitis in patients receiving exenatide, confirmed by computed tomography or ultrasound in 37% of the patients and 90% reported one or more possible contributory factors, including concomitant use of medications that list pancreatitis among reported adverse effects in product labeling or confounding conditions, such as obesity, gallstones, severe hypertriglyceridemia, and alcohol use. In August 2008, the FDA posted two fatal cases of hemorrhagic or necrotizing pancreatitis possibly related to the use

of exenatide. Given the number of patients on the drug, this figure is not alarming but remains a concern. In four other fatal cases, the causes of death did not appear directly attributable to pancreatitis [48]. It should be mentioned that a recent retrospective cohort study with a large US health care claims data base found that the cohort with DM2 had a 2.8-fold greater risk for acute pancreatitis compared with the cohort without diabetes [49].

23.4.2 Liraglutide

The results from phase three trials have now started to be published in peer review journals. In a 5-week dose escalation study (up to the maximum tolerated dose of 2 mg), the liraglutide/metformin combination was associated with a 0.8% reduction in HbA1c and a 70 mg/dl reduction in fasting glucose when compared with metformin alone. Furthermore, liraglutide/metformin significantly reduced fasting glucose (21.6 mg/dl) and body weight (2.9 kg) compared with the metformin/glimepiride group and liraglutide/placebo significantly reduced fasting glucose (25.2 mg/dl) when compared with metformin/placebo. [50]. In a 14-week study of liraglutide vs. placebo, liraglutide at the highest dose (1.9 mg) significantly reduced HbA1c by 1.74% from an average A1c of 8.5% when compared to the placebo group in which HbA1c increased by 0.29%. Besides, a dose-dependent decrease in body weight was seen. The percentage of patients achieving HbA1c of 7% or less was 46% with the highest dose and 38% at the lowest (0.65 mg) and 5% on placebo, respectively [51].

In phase three trials, liraglutide was evaluated as both monotherapy and as add-on to either one or two oral antidiabetic drugs. As monotherapy, liraglutide was investigated vs. the sulfonylurea glimepiride [Liraglutide Effect and Action in Diabetes (LEAD) trial 3] and as an add-on to one oral agent, in combination with metformin (LEAD-2) or glimepiride (LEAD-1) and as an add-on to two oral agents, in combination with metformin and rosiglitazone (LEAD-4) or metformin and glimepiride (LEAD-5). In the 52-week monotherapy trial (LEAD-3), HbA1c reduction of 1.1% and 0.8% was reported with 1.8 and 1.2 mg doses of liraglutide, respectively – each significantly more than the 0.5% reduction with glimepiride 8 mg dose. HbA1c reduced by 1.6% with liraglutide in the treatment naive patients. Both fasting (−15, −26, and −5 mg/dl from baseline in the liraglutide 1.2 and 1.8 mg and in the glimepiride groups, respectively) and postprandial values were reduced significantly on liraglutide (−31, −37, and −23 mg/dl from baseline in the liraglutide 1.2 and 1.8 mg and in the glimepiride groups, respectively]. Participants in the liraglutide groups lost weight, independent of the presence of nausea, up to 2.45 kg by the end of the study, compared with the weight gain of about 1.12 kg on glimepiride. The rates of minor hypoglycemic episodes (<56 mg/dl) were significantly lower for the liraglutide groups vs. glimepiride and no subjects reported major hypoglycemic events; HOMA-IR and fasting plasma glucagon showed significant decreases with liraglutide but mean increases with glimepiride. The proinsulin to insulin ratio and HOMA-B showed no significant differences between treatments [52].

In the combination trials (LEAD-1, 2, 4, and 5), liraglutide provided mean HbA1c reductions up to 1.5%, again with significantly more patients achieving HbA1c targets with liraglutide than with comparator treatments. Liraglutide improved the glucose profile with a similar effect on fasting and postprandial plasma glucose of around 36–54 mg/dl. Significant improvements were also demonstrated in β-cell function as measured by HOMA-B and proinsulin to insulin ratio. As with the monotherapy trial, liraglutide resulted in substantial weight loss in the combination trials – up to 2.8 kg after 24 weeks (LEAD-2) – and also demonstrated clinically meaningful reductions in systolic blood pressure (up to an average of 6.7 mmHg in LEAD-4). Again, liraglutide was associated with a low rate of hypoglycemic events [53–56].

Liraglutide presenting greater homology to native human GLP-1 induces less antibody formation than exenatide. In effect, LEAD-1, 2, 3, 4, 5 meta-analysis of antibody formation, with study duration of 26 weeks [57] compared 30 weeks treatment of DM2 patients on exenatide added to metformin [58], indicated that 8.6% of patients on liraglutide had an increase in antibodies against 43% when on exenatide + metformin.

Regarding liraglutide and pancreatitis, from the available data, in LEAD-3, a monotherapy study, two participants (from a total of 488 patients randomized for liraglutide) developed pancreatitis: one after 197 days of treatment and the other after 333 days of treatment. Both patients recovered, one continued in the study and thus had a rechallenge without recurrence of pancreatitis. In LEAD-2 (300 patients randomized for liraglutide and 100 for glimepiride), two cases of pancreatitis were noted, one in a subject randomized to liraglutide and one in a subject randomized to glimepiride [48].

Most frequently reported adverse effects were transient nausea and vomiting, especially at the higher doses.

Regarding the important question over whether GLP-1 and GLP-1 mimetics have an effect on β-cell mass in humans even though they present favorable effects on β-cell function, such as first-phase insulin secretion and homeostasis assessment β-cell index (HOMA β) as seen with chronic exenatide use up to 3 years [59]. This improvement in function may be due to the restoration of glucose competence to β-cells and the insulinotropic glucose-lowering and weight loss effects of exenatide, and perhaps not because of any direct effect of exenatide on β-cell mass, as previously indicated. At present there is no strong evidence that incretin mimetics and DTT 4 inhibitors can expand or at least maintain β-cell mass, in humans, and as such be able to delay the progression of the disease [60].

Another question yet to be elucidated is the mechanism by which GLP-1 and GLP-1 mimetics lower glucagon secretion from α-cells. The ability of GLP-1 and incretin mimetics to lower glucagon levels in DM2, in whom they are high throughout the day, contributes to the overall glucose-lowering effect. By enhancing endogenous insulin secretion with suppressing glucagon secretion, a more physiological insulin to glucagon ratio in the portal vein should be established, resulting in better suppression of hepatic glucose output. The mechanism(s) by which GLP-1 and GLP-1 mimetics lower glucagon secretion remains unclear and discussion of

this issue is beyond the scope of this publication. According to Dunning and Gerich [61] in a review of published studies, the defect(s) in α-cell function that occurs in type 2 diabetes reflects impaired glucose sensing. Because local insulin is a key regulator of glucagon secretion and defective β-cell glucose sensing in DM2 is indisputable, many, if not all, of the characteristic defects in the α-cell function may be secondary to β-cell dysfunction. It is interesting to note that attenuated and delayed glucagon suppression in DM2 occurs after oral ingestion of glucose, while isoglycemic intravenous administration of glucose results in normal suppression of glucagon, possibly due to the glucanotropic action of GIP and GLP-2 after oral glucose. This phenomenon contributes both to the glucose intolerance and to the reduced incretin effect observed in DM2 patients [62, 63].

23.5 Incretin Enhancers (DPP 4 Inhibitors)

Preclinical studies have demonstrated that DPP 4 inhibitors, which prevent the degradation of native GLP-1 by inhibiting the activity of the DPP 4 enzyme and thus increasing endogenous GLP-1 (and GIP) levels, may promote β-cell proliferation and neogenesis and inhibit apoptosis [3]. Thus, they have emerged as a therapeutic strategy for enhancing GLP-1 action in vivo. However, there are suggestions that mediators other than GLP-1 may contribute to the therapeutic effect of DPP 4 inhibition [64]. Alternatively, there are indications that GLP-1 may work indirectly through activation of the autonomic nervous system [65] Table 23.3 summarizes the overlapping and distinct properties of GLP-1R agonists vs. DPP inhibitors for the treatment of DM2.

DPP 4 inhibitors were developed to augment biologically active, endogenously secreted plasma GLP-1. In humans, sitagliptin, a DPP 4 inhibitor, both after a single

Table 23.3 GLP-1 agonists vs. DPP 4 inhibitors

	GLP-1R	DPP-4 inhibitors
Administration	Injection	Orally available
GLP-1 concentrations	Pharmacological	Physiological
Mechanism of actions	GLP-1	GLP-1 + GIP
Activation of Portal glucose sensor	No	Yes
↑ Insulin secretion	+++	+
↓ Glucagon secretion	++	++
Gastric emptying	Inhibited	+/−
Weight loss	Yes	No
Expansion of β-cell mass in preclinical studies	Yes	Yes
Nausea and vomiting	Yes	No
Potential immunogenicity	Yes	No

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dose and a once-daily dose for 10 days, resulted in about a 2-fold increase in active GLP-1 (aGLP-1) after meals. Besides, sitagliptin decreased total GLP-1 (tGLP-1) in the presence of increased aGLP-1 [38]. Whether the 2-fold increase in aGLP-1 is sufficient to explain the glucose-lowering effect with a reduction of HbA1c remains a matter of controversy.

If DPP 4 inhibitors lower blood glucose as a direct consequence of increased aGLP-1 levels, plasma insulin should also increase. However, fasting and postprandial plasma insulin and C-peptide levels did not differ before and after 10 days DPP 4 inhibition in both healthy and DM2 subjects [66, 67]. Indeed, infusions of GLP-1 that result in comparable plasma aGLP-1 attained by DPP 4 inhibition do not induce insulin secretion, but the same amount of insulin is secreted at a lower glucose level, or insulinogenic index is improved. Furthermore, the degree of aGLP-1 increase after DPP 4 inhibition is not of sufficient magnitude to inhibit gastric emptying.

DPP 4 inhibition results in lower postprandial plasma glucagon levels [45]. However, the reduced glucagon secretion is not evident in the fasting state when it would be most beneficial to decrease nocturnal hepatic glucose output. The postprandial glucagon suppressive effects of DPP 4 inhibitors, although significantly different from placebo, are weak and short-lived while levels are much higher than in nondiabetic subjects, and therefore are unlikely to account for the full antihyperglycemic effect [38].

There are presently two DPP 4 inhibitors approved for treatment of DM2, sitagliptin and vildagliptin. Other DPP 4 inhibitors are undergoing clinical development, namely alogliptin and saxagliptin.

23.5.1 Sitagliptin

In clinical trials, sitagliptin used as monotherapy or in combination with other oral hypoglycemic agents has been shown to be effective in decreasing HbA1c and lowering both fasting and postprandial glucose levels. HOMA of β-cell function and fasting PI:IRI ratios were significantly improved with sitagliptin therapy. As initial therapy, sitagliptin/metformin combination therapy has proven more effective than either sitagliptin or metformin monotherapy with an HbA1c reduction of 1.9% compared to 0.6–0.7 and 1.13%, respectively, after 24 weeks. As adjuvant therapy, sitagliptin in combination with metformin, glipizide, or pioglitazone yielded an HbA1c reduction of 0.6–0.7% when compared with placebo. From a 30 week extension trials on sitagliptin monotherapy, initial sitagliptin combination therapy with or without metformin, and sitagliptin as adjuvant therapy to metformin showed that the reduction in HbA1c was sustained at week 54 [46, 38].

A clinical analysis of 5,141 patients in clinical trials for up to 2 years (from 24 to up to 104 weeks) showed that sitagliptin monotherapy or combination therapy (metformin, pioglitazone, sulfonylurea, or sulfonylurea and metformin) was well tolerated, and hypoglycemia occurred in the setting of combination therapy [69]. In another 24 week study, Goldstein et al. [70] evaluated initial combination therapy

with sitagliptin plus metformin in DM2 patients whose initial A1c was 7.5–11%. The patients were randomized to sitagliptin (100 mg/day), metformin (two doses), and sitagliptin associated with either one of the metformin doses or placebo. The best results were obtained in the cohort that received sitagliptin 100 mg plus metformin 2000 mg/day (the highest dose) with 66% of patients achieving an HbA1c of <7% and 44% an A1c <6.5%. The incidence of hypoglycemia was low and gastrointestinal side effects were no greater during combination therapy than with metformin alone.

Because sitagliptin is cleared primarily by the kidneys, a dosage adjustment is recommended in patients with moderate or severe renal insufficiency and in those patients with end-stage renal disease requiring hemodialysis or peritoneal dialysis [71].

23.5.2 Vildagliptin

Preclinical studies on streptozotocin-induced β-cell injury in mice have demonstrated that vildagliptin and injectable exenatide presented a protective effect against β-cell injury as well as promoting early differentiation of pancreatic progenitor cells, increasing formation of ductal β-cells and improving glucose tolerance to a similar extent in streptozotocin-diabetic mice. Furthermore, the effect was maintained after the treatment washout. Neonatal rats treated with vildagliptin presented an increased number of replicating islet cells and reduced number of apoptotic islet cells [3].

Vildagliptin, as a potent and selective DPP 4 inhibitor, improves glycemic control in DM2 patients. Studies involving oral hypoglycemic agents-naïve patients, 24 weeks treatment with vildagliptin (50–100 mg daily) were reported to decrease HbA1c by 0.9–1.1% [71]. In another study, in which the initial HbA1c value was >9%, the mean decrease was 1.8%. When the recommended daily dose is 100 mg, 50 mg is administered in the morning and the remaining dose in the evening. Doses higher than 100 mg are not recommended.

In patients with DM2 and mild hyperglycemia (HbA1c of 6.2–7.5%), 52-week treatment with vildagliptin (50 mg qd) significantly decreased HbA1c, fasting plasma, and postprandial glucose levels and improved β-cell function, namely insulin secretory tone, glucose sensitivity, and rate sensitivity. However, none of the effects of vildagliptin were maintained after a 4-week washout period, suggesting that long-term studies are necessary to determine whether DPP 4 inhibition modifies disease progression [73].

To assess the 2-year efficacy and tolerability of vildagliptin (50 mg once daily) in patients with drug-naïve DM2 subjects with mild hyperglycemia, a trial comprising a 52-week core study with a four week treatment-free washout was conducted, followed by a 52-week extension with further washout period. The 2-year treatment with vildagliptin attenuated the progressive loss of glycemic control seen in patients receiving placebo only. This appeared to be the result of a corresponding

attenuation of the deterioration of β-cell function as assessed by the insulin secretory rate relative to glucose [74].

As monotherapy, vildagliptin 50 mg bid was effective as rosiglitazone 8 mg once daily and acarbose 100 mg tid in lowering HbA1c but not as effective as metformin 1000 mg bid. In combination therapy, using metformin or a glitazone, the recommended dose is 50 mg bid. When used in dual combination with a sulfonylurea, the indicated dose is 50 mg once daily administered in the morning, since vildagliptin 100 mg daily was no more effective than 50 mg once daily. Vildagliptin is effective as adjuvant therapy when administered in patients inadequately controlled with sulfonylurea, metformin, glitazone, or insulin therapy with HbA1c reduction of 0.6, 0.9, 1.0, and 0.5%, respectively. In addition, vildagliptin and pioglitazone were equally effective as adjuvant therapy for patients inadequately controlled on metformin, producing HbA1c reductions of 0.9 and 1.0%, respectively [38].

No vildagliptin dose adjustment is required in patients with mild renal impairment (creatinine clearance $\geq$50 ml/min) and the drug is not recommended in those with moderate to severe renal impairment or in hemodialysis patients with end-stage renal disease. Moreover, vildagliptin should not be used in patients with hepatic impairment, including patients with pre-treatment ALT or AST > 3× upper limit of normal [75].

The side effects of vildagliptin are comparable to those of sitagliptin. In a systematic review and meta-analysis of incretin therapies, vildagliptin presented no risk of gastrointestinal adverse events.

To compare the effects of vildagliptin 50 mg bid vs. sitagliptin 100 mg once a day on 24 h acute glucose fluctuations in DM2 patients inadequately controlled on metformin, which was maintained, it was demonstrated that the efficacy of both DPP 4 inhibitors was comparable on the main glucose parameters: HbA1c, fasting and postprandial glucose reductions over a 3-month study period. However, the effects on glucose fluctuations over a day, estimated by the mean amplitude of glycemic excursions during 48 h continuous subcutaneous monitoring (CSGM), were more pronounced in the vildagliptin than in the sitagliptin group, which could be due to different mode of administration of both drugs. Furthermore, while the increase in circulating GLP-1 after food intake was substantially identical in the two groups, a significant and sustained increase during interprandial period of active GLP-1 in vildagliptin bid-treated toward sitagliptin once a day occurred, probably related to differences in pharmacokinetic profiles in DPP 4 inhibition, a potential mechanism for the different effects on glucose fluctuations over a daily period [76]. From a practical point of view, since glucose variations over time, linked to daily fluctuations of glycemia, are associated with an activation of oxidative stress, which can lead to chronic diabetic complications, DPP 4 inhibition therapy should target not only reducing HbA1c but also flattening acute glucose fluctuations over a daily period.

The first head-to-head study comparing a GLP-1 analogue (exenatide: 5 μg twice daily for the first week followed by 10 μg twice daily for the second week) to a DPP 4 inhibitor (sitagliptin: 100 mg daily), in 61 DM2 patients who were on a stable regimen of metformin, was a 4-week study (patients were randomly assigned to either exenatide or sitagliptin for 2 weeks and then switched to the alternate therapy for the

remaining 2 weeks) with HbA1c 7.0–10%, fasting plasma glucose <280 mg/dl, and body mass index: 25–45 kg/m^2. In response to a standard meal, patients treated with exenatide had significantly improved 2 h post-meal glucose compared to sitagliptin-treated subjects (133 vs. 208 mg/dl, respectively, baseline 245 mg/dl; $p < 0.0001$). Patients who were switched from sitagliptin to exenatide after 2 weeks showed further improvements in post-meal glucose (−76 mg/dl), while patients who switched from exenatide to sitagliptin partially lost the post-meal glucose (+73 mg/dl) control achieved with exenatide. The study also showed that after 2 weeks of treatment both exenatide and sitagliptin improved fasting plasma glucose. Additionally, exenatide also significantly improved an indicator of β-cell function, the insulinogenic index of insulin secretion compared to sitagliptin. Exenatide also reduced high post-meal glucagon to a greater extent than sitagliptin and slowed gastric emptying. Exenatide reduced food intake compared to sitagliptin during buffet-style meals. The most common adverse effects for both exenatide and sitagliptin were mild to moderate nausea (exenatide: 34 vs. sitagliptin: 12%) and vomiting (exenatide: 24 vs. sitagliptin: 3%). There were no major hypoglycemic events; a single event of minor hypoglycemia was reported in a patient treated with exenatide [77]. The differences between exenatide and the DPP 4 inhibitors are explained by the 5-fold greater plasma exenatide concentrations [50–60 pM] following sc injection compared to plasma GLP-1 concentrations (10–15 pM) achieved following oral DPP 4 inhibitor administration.

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Chapter 24
Immunology of β-Cell Destruction

Daria La Torre and Åke Lernmark

Abstract The pancreatic islet β-cells are the target for an autoimmune process that eventually results in an inability to control blood glucose due to the lack of insulin. The different steps that eventually lead to the complete loss of the β-cells are reviewed to include the very first step of a triggering event that initiates the development of β-cell autoimmunity to the last step of appearance of islet-cell autoantibodies, which may mark that insulitis is about to form. The observations that the initial β-cell destruction by virus or other environmental factors triggers islet autoimmunity not in the islets but in the draining pancreatic lymph nodes are reviewed along with possible basic mechanisms of loss of tolerance to islet autoantigens. Once islet autoimmunity is established the question is how β-cells are progressively killed by autoreactive lymphocytes which eventually results in chronic insulitis. Many of these series of events have been dissected in spontaneously diabetic mice or rats, but controlled clinical trials have shown that rodent observations are not always translated into mechanisms in humans. Attempts are therefore needed to clarify the step 1 triggering mechanisms and the step to chronic autoimmune insulitis to develop evidence-based treatment approaches to prevent type 1 diabetes.

Keywords Islet autoimmunity · Autoantigens · Prediction · Prevention · Insulitis · Islet autoantibodies · CD4+ T cells · CD8+ T cells · T regulatory cells · Antigen-presenting cells · Dendritic cells

Abbreviations

APC	Antigen-presenting cells
BB	Bio breeding
BCR	B-cell receptor

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CTL	Cytotoxic T lymphocytes
CTLA-4	Cytolytic T lymphocyte-associated antigen
cTreg	Conventional regulatory T
DC	Dendritic cells
Fas-L	Fas-Ligand
FoxP3	Forkhead–winged helix
GABA	Gamma-amino-butyric acid
GAD	Glutamic acid decarboxylase
HLA	Histocompatibility antigens
HSP	Heat-shock protein
IA-2	Insulinoma-associated antigen-2
IAA	Insulin autoantibodies
ICAM	Intercellular adhesion molecule
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
ICA	Islet cell antibodies
ICSA	Islet cell surface antibodies
IL	Interleukin
iVEC	Islet vascular endothelial cells
LFA-1	Leukocyte function-associated antigen-1
NF	Nuclear factor
NK	Natural killer lymphocyte
NKT	Natural killer T
NO	Nitric oxide
NOD	Non obese diabetic
nTreg	Natural regulatory T
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed death-1
pDC	Plasmacytoid dendritic cell
pLN	Pancreatic lymph node
pMHC	Peptide-MHC
PRR	Pattern recognition receptors
TCR	T-cell receptor
TEDDY study	The environmental determinants of diabetes in the young
TF	Transcription factor
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSA	Tissue-specific antigen
VNTR	Variable nucleotide tandem repeat
ZnT8t	Zinc transporter isoform-8

24.1 Background and Historical Perspectives

Immune-mediated selective destruction of the pancreatic islet β-cells is the hallmark of type 1 diabetes mellitus (T1D), formerly known as insulin-dependent diabetes mellitus [1–4]. The immunogenetic feature of the disease is a polygenic inheritance of susceptibility, which is reflected in a highly polyclonal autoimmune response targeting several β-cell antigens. The autoimmune response is associated with progressive β-cell destruction that eventually leads to overt clinical disease. As attested by prospective studies of children at genetic risk for T1D (DIPP, DAISY, and BabyDIAB), the appearance of specific islet autoantibodies marks the initiation of islet autoimmunity and may be detectable for months to years [5, 6] during which time β-cell dysfunction proceeds asymptomatically. T1D may therefore be viewed as a two-step disease. The first step is the initiation of islet autoimmunity, the second step is precipitation of diabetes when islet autoimmunity has caused a major β-cell loss (>80%) [6], and insulin deficiency becomes clinically manifest.

At diagnosis, the typical histological finding of affected islets, first described in short-duration diabetes patients at the beginning of last century [7], and termed 'insulitis' [8, 9], consists of an infiltrate of inflammatory cells associated with a loss of the β-cell endocrine subpopulation. The infiltrate consists of mononuclear cells [10, 11] and T and B lymphocytes [12]. Little is known about insulitis during the first step of the disease when subjects have preclinical islet autoimmunity. Recent studies suggest that the mere presence of an islet autoantibody does not predict insulitis [13].

The understanding of T1D etiology and pathogenesis is complicated by the lack of epidemiological data on the first step of the disease. In contrast, the epidemiology of T1D is developing rapidly through registers in many different countries. The incidence is different among age groups, highest among children [14–16], but the disease may occur at any age [17].

Annual incidence shows geographical variation among different countries and ethnic groups, from 0.1 per 100,000 children in parts of Asia and South America to the highest rate in Finland (64.2 per 100,000) [16, 18]. The mode of inheritance is complex as 80–85% of T1D is occurring sporadically [19] and the risk of becoming diabetic is approximately 7% for a sibling and 6% for the children of T1D parents [20].

An autoimmune etiology for T1D was suspected approximately 40 years ago from the association between diabetes and other autoimmune diseases [21–23]. The first attempt to identify an autoimmune reaction toward the endocrine pancreas dates back to 1973, when testing for leukocyte migration inhibition to islet antigens suggested that T1D patients might be sensitized to pancreatic antigens [24]. Nearly concomitantly, T1D was reported to be correlated to histocompatibility antigens (HLA) [25] that govern antigen recognition by immune effectors. Association studies have proved that the greatest contribution to genetic susceptibility to T1D is exerted by *HLA* class II alleles on chromosome 6, the HLA-DQ haplotypes DQ2 and DQ8, and DQ6.2 conferring the highest risk or protection [26–28]. The detailed

mechanisms by which different HLA molecules provide either risk or resistance to T1D is not fully understood [29]. It is possible that different conformations of the MHC molecules pocket yield different peptide-binding properties and influence antigen presentation by antigen-presenting cells (APC) to effector T cells [30–32].

If genetic background appears to be a prerequisite for the development of β-cell autoimmune destruction, a major role in the penetrance of a susceptible genotype is played by environmental factors. Virus infections have figured prominently in T1D epidemiological investigations [33–35]. The possible contribution of a virus infection to trigger islet autoimmunity (step 1 of the disease) or to the progression to clinical onset in islet autoantibody-positive individuals needs to be sorted out. The contribution of dietary factors is equally controversial [36–40]. Maternal factors [41], vaccinations [36, 42, 43], or toxins have also been considered [44]. Environmental factors likely account for the low concordance rate for T1D among monozygotic twins (30%) [45–47]. Similarly, the geographic distribution underscores the importance of the environment [3, 48, 49]. The multifactorial etiopathogenesis is also evident in the spontaneous diabetes in the nonobese diabetic (NOD) mouse and the bio breeding (BB) rat employed over the past three decades. These animals have given insights in the immunogenetics of T1D [50, 51], though the utility of these animals in preclinical trials to guide human research has been limited [52].

Although the event that initiates the autoimmune process (step 1) is not yet understood, the fact that it specifically targets the β-cells promoted the attempt to find which β-cell-specific antigens could give rise to the abnormal immunological recognition. The interest was initially focused on autoantibodies as useful tools in attempts to identify autoantigenic molecules (Table 24.1) and clarify the pathological immune response. The first description of pancreatic islet autoantibodies was in 1974, when indirect immunofluorescence on frozen human pancreas sections revealed circulating islet cell antibodies (ICA) in the serum of T1D patients with polyendocrine disease [53]. A few years later islet cell surface antibodies (ICSA) were demonstrated in newly diagnosed T1D patients using dispersed cell preparations of rodent pancreatic islets [54]. The molecular characteristics of islet autoantigens remained unknown until the demonstration in 1982 that sera from new-onset T1D patients had autoantibodies immunoprecipitating a 64 kDa protein in isolated human islets [55]. The 64 kDa immunoprecipitate proved in 1990

Table 24.1 β-cell autoantigens

Antigen	Mol weight Da	Autoantibody abbreviation	References
Glutamic acid decarboxylase	65,000	GAD65Ab	[83]
Insulin	5800	IAA	[387]
IA-2	4000	IA-2Ab	[61]
IA-2-β (Phogrin)	3700	IA-2βAb	[62]
Zinc transporter ZnT8 R/W/Q variants	4100	ZnT8Ab	[64]

to have gamma-amino-butyric acid (GABA)-synthesizing enzymatic activity [56]. Molecular cloning of human islet glutamic acid decarboxylase (GAD) showed that the β-cells expressed the unique human isoenzyme GAD65 [57]. GAD65 is expressed in several cell types but, apart from some brain neurons, it is mainly localized to synaptic-like microvesicles in the β-cells. GAD65 is in part responsible for the β-cell-specific pattern of ICA [58]. Antigenic properties of insulin A and B chains and of the precursor proinsulin were postulated, as this autoantigen would explain β-cell specificity and have possible physiopathological involvement. In 1983 autoantibodies reacting with insulin (insulin autoantibodies IAA) were demonstrated in T1D patients, uncorrelated to insulin administration [59]. In 1994 trypsin digestion of the 64 kDa immunoprecipitate revealed a 37/40 kDa autoantigen pair, recognized by sera of T1D patients [60]. This observation eventually led to the identification of two members of the tyrosine phosphatase family, sharing 74% of intracellular domain, insulinoma-associated antigen-2 (IA-2) and IA-2 beta (or phogrin) [61], which is probably less involved in T1D autoimmunity [62]. IA-2 is a transmembrane molecule of islet secretory granules and may be physiologically implicated in insulin secretion [63]. More recently, in 2007, autoantibodies to the zinc transporter isoform-8 (ZnT8t) were reported [64]. The ZnT8 protein mediates Zn^{2+} cation transport into the insulin granules, facilitating the formation of insulin crystals [64]. ZnT8 polymorphic variants [65] represent not only targets of islet autoimmunity but also a genetic marker for type 2 diabetes [65].

Continued study of serum samples from T1D patients identified additional candidate targets of the humoral immune response. Autoantigens reported so far have different tissue expression patterns and subcellular localizations, as DNA topoisomerase II [66], heat-shock protein 60 (HSP60) [67], HSP-70 [68], HSP-90 [69], vesicle-associated membrane protein-2 (VAMP2) and inhibitory neuropeptide Y (NPY) [70], carboxypeptidase H (CPH) [71], and others [72–80]. Further definition of this wide array of islet antigens is needed to define autoantigens with a pathogenetic role in islet destruction (step 1) from those that become secondarily available to immune system due to ongoing tissue damage. Thus, their relevance for the prediction of T1D is unclear. Many assays have been used to detect islet autoantibodies, but it was only from the mid-1990s, when recombinant human GAD65 cDNA became available, that simple and reproducible immunoprecipitation assays were developed with in vitro transcribed, translated, radiolabeled antigen [57, 81]. The novel in vitro labeling made large screenings and standardization workshops applicable [82–84]. Their high sensitivity and specificity and early appearance during the autoimmune process made autoantibodies useful clinical markers not only for diagnosis [85] but also for disease prediction [2, 86, 87]. Despite the attested association with T1D, there is no evidence that islet autoantibodies directly contribute to β-cell damage, though the B lymphocytes producing islet autoantibodies may contribute as APC [88].

Adoptive transfer studies on the NOD mouse suggest that islet damage in T1D is mainly mediated by T-cell effectors [89]. The pivotal role in these mice of cytotoxic CD8+ T cells in the initiation and progression of destructive insulitis [51] and of CD4+ T cells that act as ‘helper’ cells [90] is well recognized. Studies of the

cellular arm in human T1D have detected CD4+ and CD8+ T cells that recognize the same autoantigens as targeted by the humoral arm [91, 92], sometimes with epitope overlapping [68, 93]. As expected, T-cell reactivity to minor autoantigens has also been reported [68, 94, 95]. However, cellular immunoreactivity to islet autoantigens is less easily assessed than the autoantibody response and is not yet applicable in the clinic. Most of the studies performed in the last decade to identify self-reactive T cells in the peripheral blood of T1D patients are based on the indirect detection of T-cell presence, through antigen-induced proliferation assays [96] or cytokine release (ELISPOT) analysis [97]. The latter analysis has limitations, as it does not allow precise enumeration of the cytokine-producing cells and may yield false-negative results if T cells are producing other cytokines than detected in the actual assay. In the last 5 years, the MHC tetramer technique has provided a novel insight into specific T cells and their precursor frequencies. The tetramer resembles the physiological MHC peptide/TCR interaction and offers phenotyping and selective isolation of antigen-specific T cells, upon a stimulation with the tetramer itself [98]. The technique is highly specific for the HLA type and the peptide lodged in the MHC peptide-binding groove. Attempts have been made in addition to further study the structural requirements in transgenic mice expressing the T1D-associated HLA-DR4 and DQ8 to identify peptides recognized by autoantigen-specific T cells [99]. As pointed out in a recent T-cell workshop, traditional in vitro proliferation assays suffer from methodological limitations [100] related to peculiarities of autoreactive T cells as low peripheral frequency [101], rapid number reduction [102, 103], ongoing modification of immunodominant specificity [103], and low TCR avidity [101]. As will be discussed later, these studies have produced inconsistent results [100] and have globally failed to detect marked differences on T cells from T1D patients and controls. The presence of autoreactive T cells in healthy subjects suggests that central tolerance is physiologically incomplete and that T-cell peripheral regulatory phenomena may be strongly involved in the development of the autoimmune process. From the early 1990s, immunoregulation of autoreactive T cells was viewed within the oversimplified model of Th1 and Th2 phenotypes [3]. The simplified notion was that progression of tissue-specific autoimmunity results from a functional imbalance between pathogenic Th1 cells and immunoregulatory Th2 cells. During the past several years it has become clear that a larger number of subsets of immunomodulating regulatory T cells (Treg) exists and contributes to the maintenance of peripheral tolerance [104, 105]. Recent advances point out at a crucial immunomodulating role of a subset of APC called dendritic cells (DC) [106]. It has been hypothesized that DC may be involved in the early breakdown of tolerance, as well as in the maintenance of β-cell destruction [107].

The understanding of T1D has improved over the years since the rediscovery of insulitis in 1965 [108]. The recognition that T1D is a two-step disease characterized by a long prodrome of islet autoimmunity prior to clinical onset has allowed new hypotheses to be developed, as to the initiation of the β-cell destructive process. The transition from islet autoimmunity to clinical T1D will also require a redefinition of the role of environmental factors triggering the disease. In this chapter we will review possible mechanisms of induction of β-cell autoimmunity and the role of

environmental factors in this process. The reader is referred to other recent reviews on this or similar topics [2, 109–111]

24.2 Autoimmune β-Cell Destruction

24.2.1 Genetic Etiology

The major genetic factor for T1D is HLA-DQ on chromosome 6 [112–114]. Both sib-pair analyses and association studies have indicated in Caucasians that the HLA-DQ A1-B1 haplotypes A1*0301-B1*0302 (DQ8) and A1*0501-B1*0201 (DQ2), alone or in combination (DQ2/8), confer the highest risk for T1D. Nearly 90% of newly diagnosed children carry DQ2/8 (about 30%), DQ8, or DQ2 in combination with other haplotypes [115]. Among the many haplotypes there are combinations, in particular with the DQA1*0201-B1*0602 (DQ6.2) haplotype, which is negatively associated (protective) with T1D. However, the effect is attenuated with increasing age [116]. The rising incidence of T1D is, however, puzzling as it is associated with a reduced overall contribution of high-risk HLA types in parallel with an increase in DQ8 and DQ2 combinations which did not confer risk 20 years ago [117–119]. The mechanisms by which DQ8, DQ2, or both increases the risk for T1D are not fully clarified. The function of the DQ heterodimers to present antigenic peptides to the immune system is well understood. It remains to be determined why the DQ2/8 heterozygocity is associated with a young age at onset [120]. It has been speculated that the DQ2 and DQ8 molecules are important to maintain central or peripheral tolerance to the β-cell autoantigens GAD65, IA-2, insulin, or ZnT8. This possibility needs further exploration as it cannot be excluded that the primary association between T1D and HLA is the 'step 1' part of the disease rather than the progression to clinical onset. This hypothesis is supported by the observation that the presence in healthy subjects of GAD65 autoantibodies is associated with DQ2 and IA-2 autoantibodies with DQ8 [86, 121].

Several investigations suggest that HLA contributes about 60% to the genetic risk of T1D [122]. Major efforts have therefore been made to identify non-HLA genetic risk factors for type 1 diabetes [112]. These studies have been highly rewarding as more than 40 genetic factors (see examples in Table 24.2) have been found to contribute [112]. Interestingly enough, many of the genetic factors are important to the function of the immune system. *PTPN22* is a regulator of T-cell function and a genetic polymorphism results in a phosphatase variant that is increasing the risk not only for T1D but also for rheumatoid arthritis, juvenile rheumatoid arthritis, systemic lupus erythematosus, Graves' disease, generalized vitiligo, and other human autoimmune diseases [123]. The *PTPN22* polymorphism seems in particular to affect progression from pre-diabetes to clinical disease [124] also in individuals with lower risk HLA genotypes [125]. The variable nucleotide tandem repeat in the promoter region of the insulin gene *INS VNTR* seems to contribute to T1D by the mechanisms of central tolerance [126]. In newly diagnosed T1D patients the presence of insulin autoantibodies is associated with the *INS VNTR* polymorphism [120].

Table 24.2 Non-HLA genetic factors in type 1 diabetes

Gene (Syno.)	Name	Chromosome	Function	Association with other autoimmune diseases
PTPN22 (PEP, Lyp1, Lyp2, LYP, PTPN8)	Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	1p13	Encodes tyrosine phosphatase may be involved in regulating CBL function in the T-cell receptor-signaling pathway.	T1D and 22 other diseases
CTLA-4 (DDM12, CELIAC3)	Cytotoxic T-lymphocyte-associated protein 4	2q33	Possible involvement in regulating T-cell activation.	T1DM and 99 other diseases
IFH1 (MDA5)	Interferon induced with helicase C domain 1	Chr.2q24	Proposed involvement in innate immune defense against viruses through interferon response.	T1DM association
IL2 (lymphokine, TCGF)	Interleukin 2	Chr.4q27	Encodes a cytokine important for T and B cells proliferation. Stimulate B cells, monocytes, killer and NK cells.	T1DM and 39 other diseases
ITPR3 (IP3R3)	Inositol 1,4,5-triphosphate receptor 3	Chr.6p21.3	A second messenger that mediates the release of intracellular calcium.	Strong T1DM association
BACH2 (BTB and CNC homology 1)	Basic leucine zipper transcription factor 2	Chr.6q15	Important roles in coordinating transcription activation and repression by MAFK (by similarity).	T1DM association
IL2RA (IDDM10, CD25)	Interleukin-2 receptor, alpha (chain)	Chr.10p15	Receptor for interleukin-2.	Strong association with T1DM

Table 24.2 (continued)

Gene (Syno.)	Name	Chromosome	Function	Association with other autoimmune diseases
INS VNTR (proinsulin, ILPR, MODY)	Insulin II; insulin 2; insulin	Chr.11p15	Regulating glucose metabolism through adjusting central tolerance to insulin.	T1DM and 38 other diseases
TH (TYH, The)	Tyrosine hydroxylase	Chr.11p15	Encodes a protein that converts tyrosine to dopamine. Plays a key role in adrenergic neurons physiology.	T1DM and 35 other diseases
ERBB3 (c-erbB3, HER3, LCCS2)	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3	Chr.12p13	Encodes a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases binds and is activated by neuregulins and NTAK.	T1DM and multiple sclerosis
C12orf30 (C12orf51, KIAA0614)	Similar to KIAA0614 protein	Chr.12q24	Not yet determined.	T1DM association
CLEC16A/KIAA0350 (Gop-1)	C-type lectin domain family 16, member A	Chr.16p13	Unknown. Proposed to be related to immune modulation mechanisms.	Strong association with T1DM
PTPN2	Protein tyrosine phosphatase, non-receptor type 2	Chr.18p11	Encode a PTP family protein and may be related to growth factor mediated cell signaling.	T1DM association
BASH3A (TULA, CLIP4)	Ubiquitin-associated and SH3 domain-containing protein A	Chr.21q22	Promotes accumulation of activated target receptors, such as T-cell receptors, EGFR and PDGFRB.	T1DM association

The many other genetic factors listed in Table 24.2 are all shown to be significantly associated with T1D [112]. The function of these genes is understood individually; however, it is not clear how these factors may interact to increase the risk for the development of islet autoimmunity (step 1), T1D (step two), or both. The majority of the genetic factors seem to be associated with the immune system (Table 24.2). It is therefore attractive to speculate that their contribution is related to the ability of the immune system to mount an autoimmune reaction specifically directed toward the islet β-cells.

24.2.2 Immune Cells in Tolerance

Epitope presentation to T and B cells is the key step in the generation of tolerance, in its early failure, and during the maintenance of autoimmunity. The capacity to distinguish between self and nonself, which is the hallmark of a functional immune system, is lost when central and peripheral tolerization fail, leading to the development and expansion of autoreactive pathogenic effector cells. Central tolerance is induced at the site of lymphocyte development (the thymus and bone marrow, respectively, for T and B cells), while peripheral tolerance occurs at sites of antigen recognition, namely in lymphoid and non-lymphoid tissues. Central to the function of tolerance are APC.

24.2.2.1 APC

The recognition by T and B lymphocytes of antigens presented in the context of MHC surface of an APC is the first step of the adaptive immune response. Macrophages and particularly DC are the most efficient APC, as they show constitutive expression of MHC class II molecules, cytokine secretion, and migrating capacity [127]. APC have a dual role: uptake, processing, and presentation of antigens to T cells and regulating T-cell-driven responses through cytokine release. APC are involved in T-cell tolerance mechanisms at both central (clonal deletion) and peripheral level (clonal anergy). Negative selection of autoreactive clonotypes derived by random T-cell receptor (TCR) rearrangement is guided by T-cell affinity for self-peptide–MHC (pMHC) complexes presented in the thymus [128, 129]. An inadequate binding affinity spares self-reactive T cells from apoptosis [130]. The thymic expression of tissue-specific antigens (TSA) is regulated by the autoimmune regulatory (AIRE) transcription factor [131]. Insufficient level of expression and presentation of TSA-derived peptides is observed in subjects with a mutated *AIRE* gene. In mice, β-cell-derived proteins have been found to be expressed on the surface of thymic epithelial (TEC) and medullary (mTEC) cells and DC [132, 128]. It is possible that some β-cell autoantigens are not present in the thymus at sufficient concentrations to induce negative selection. This mechanism may explain the correlation of T1D protection with the ‘long form’ of *INS VNTR* [133, 134]. The number of ‘tandem repeats’ modulates thymic expression of this autoantigen and the ‘long variant’ results in increased insulin mRNA within the thymus

[133, 134]. This higher thymic insulin expression is thought to enhance the deletion of insulin-specific thymocytes and may account for the protective phenotype [135, 136].

Similarly, GAD65 expression in thymus may physiologically contribute to specific central tolerance, but its presence first suggested by immunochemical studies [137] has not been further confirmed [138].

Transcriptional modifications due to alternative splicing have been proposed to explain IA-2 immunogenicity, as IA-2 is not expressed full length in thymus, but in an alternatively spliced transcript derived from the deletion of exon 13 [139]. This may account for the escape of a subset of IA-2-reactive T cells. Interestingly, several B- and T-cell epitopes map to IA-2 exon 13 [140]. So far, it is not clear to what extent central tolerance and thymic expression are important to antigen presentation of the ZnT8 transporter protein. The efficiency of thymic negative selection can also be reduced in case of elevated threshold for clonal deletion, as may result from the single-nucleotide polymorphisms in the *PTPN22* gene associated with T1D [23, 133]. The mutation of this gene, that encodes for a negative regulator of TCR signaling [141], may increase the activation threshold needed for deletion of CD4+ and CD8+ T cells [133, 134, 141].

APC-T-cell interaction in the peripheral lymphoid organs is the key in peripheral tolerization. APC provide costimulatory molecules, such as CD40 [142], and adhesion molecules as leukocyte function antigen 1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) that are necessary to activate naïve T cells [111] and molecules of the B7 family [143]. Without coactivation of B7-CD28 on the T-cell surface, the MHC-TCR signaling induces apoptosis of naïve T cells, mediated by upregulation of 'Activation-Induced Cell Death' [144]. Surviving T cells become anergic, i.e., unresponsive to subsequent antigen stimulation, through an active process involving a number of anergy factors [145]. T-cell reactivity is also controlled by negative regulatory receptors as cytolytic T lymphocyte-associated antigen (CTLA)-4 which binds to CD86 on the APC [146] and attenuates T-cell activation by competing for B7-CD28 ligation [147], and programmed death-1 (PD-1) [148]. The susceptibility to T1D associated to some splice variants of the human *CTLA-4* gene may be due to reduced expression of CTLA-4 and insufficient costimulatory molecule blockade [149].

Among APC, DC are peculiar, highly specialized effectors with ontogenic, morphologic, and functional heterogeneity and can be mainly divided into conventional or myeloid DC (mDC) and plasmacytoid DC (pDC), upon superficial clusters of differentiation and secretive function [150]. pDC are potent productors of IFN-α and are connected to the innate immune system through the expression of toll-like receptors (TLR) specific for the detection of viral infections [150, 151]. Emerging evidence suggests a close relationship between pDC and autoimmune conditions [109]. In healthy subjects, autoantigen-bearing DC are physiologically found in blood, peripheral lymphoid organs, and thymus, where they are important source of TSA [137]. DC are also reported to display proinsulin epitopes through direct transcriptional events in a capture-independent way [152]. DC are thought to be the only APC effective in 'cross-presentation' [153], which is an

unconventional mechanism for processing and presenting exogenous antigens in the context of MHC I molecules directly to cytotoxic T lymphocytes (CTL) [153, 154]. Cross-presentation of parenchymal antigens is thought to be involved mainly in the detection of viral infections [154] but has also been proposed to contribute to peripheral tolerance [155]. The different outcome of the interaction between T cells and DC is ruled by their maturative status. Under homeostatic conditions, DC are in an immature status, which is predominantly tolerogenic and characterized by low expression of costimulatory factors as CD40, CD80, and CD86 [107, 156]. This homeostatic status may be maintained through the downregulation of nuclear factor-κB (NF-κB), which is a critical transcription factor for many genes involved in APC activation in mice [157]. After the activation by an antigen, DC undergo maturation, express pMHC complexes, and promote antigen-specific T-cell clonal expansion. At this mature stage, DC are generally immunogenic and produce costimulatory molecules and several cytokines. Among them, interleukin (IL) 12p70, which promotes differentiation of CD4+ T helper cells (Th0) and CD8+ effectors, IL-1-β, tumor necrosis factor (TNF) alfa, and interferon (IFN)-γ [107]. Under specific conditions, such as transforming growth factor (TGF) beta and IL-10-enriched environment, mature DC can develop tolerogenic properties [158], secrete cytokines as IL-10 [159], which inhibits the activation of other APC, and promote antigen-specific expansion of Treg subsets [158–160]. Tolerogenic DC may also inhibit T-cell proliferation through the enzyme indoleamine 2,3-dioxygenase (IDO) [161] or directly induce T cells apoptosis through PD ligand 1 (PDL-1) [162]. In the NOD mouse, the binding of PDL-1 to the T-cell PD-1 receptor downregulates the priming of diabetogenic T cells in early stages of diabetes and inhibits islet destruction at a later phase [163].

The physiology of B cells as APC indicates that these cells are able to take up antigen at very low concentrations through their antigen-specific membrane-bound immunoglobulin [164] and to present it to T cells. The antigen presentation is enhanced in the presence of specific autoantibodies [165]. In the NOD mouse, antigen presentation by B cells may be important for the initiation of the autoimmune attack [166] and for the spreading of T- and B-cell determinants during the progression of the disease [167, 168]. A recent study on human B cells in T1D showed that B cells may regulate the autoimmune T-cell repertoire by enhancing the presentation of determinants located outside the B-cell immunodominant area [169]. Moreover, the minute amounts of antigen presented by B cells may be important for the maintenance of autoimmune reactivity in the later phase, once most of the target tissue has been destroyed [169]. Of interest, HLA-restricted B- and T-cell epitopes are in close proximity within the GAD65 molecule [170], and recently an overlapping within T and B IA-2 epitopes has been described [171]. These observations suggest that antigen–antibody complexes may influence antigen presentation by APC and thereby T-cell reactivity [170]. The T- and B-cell synapse has been discussed in a recent review [111]. However, there are major gaps in our understanding of the possible importance of the T–B-cell synapse within the human islets of Langerhans. B cells are also the most frequent APC expressing CD1d, the restriction molecule responsible for antigen presentation to natural killer T (NKT) cells, a T-cell subset

linking the innate and adaptive immune system with a still controversial role in β-cell destruction [172].

There is wide evidence from studies on T1D pancreas with insulitis that MHC class II expression is increased on islet vascular endothelial cells (iVEC) [11, 12, 173], as a result of de novo expression induced by the inflammatory cytokine IFN-γ [174]. The possibility that human VEC can act as APC and present exogenous antigens on HLA class II molecules to CD4+ T cells was reported previously [175]. More recent data on iVEC suggest that these cells are capable to internalize, process, and present disease-relevant epitopes from GAD65 [174] and insulin [176]. The in vivo acquisition of these autoantigens by iVEC is not clearly established. Since iVEC are physiologically exposed to very high insulin concentration, it is likely that these cells take up insulin and process it into peptides through endosomal degradation, rather than acquire peptides or pMHC complexes produced by β-cells [176]. The mechanism is even more unclear for non-secreted antigens. Although it is uncertain whether islet vascular endothelium has any prominent role in the priming of autoreactive T cells, given the recognized importance of professional APC, it has been suggested that iVEC may be important for the trafficking of activated T cells providing antigen-driven homing specificity [176].

24.2.2.2 T Cells

Recent progress in studying peripheral tolerance has highlighted the importance of immunoregulation by Treg, co-expressing CD4 and the alfa-chain of the IL-2 receptor complex (CD 25) [177]. Treg are potent suppressors of organ-specific autoimmunity [105]. Natural Treg (nTreg) originate from intrathymic recognition of self-pMHC complexes [177, 178] and are functionally marked by the constitutive expression of forkhead–winged helix transcription factor (FoxP3) [179], while conventional Treg (cTreg) differentiate from naïve CD4+ T cells in the periphery [104]. Although FoxP3 plays a major role in Treg development and activity, as mutations in *FOXP3* gene in humans determine severe multi-organ autoimmunity (IPEX syndrome) [180], Treg function is complex (181) and involves other transcriptional signaling as TGF-β, IL-2, and possibly others [182]. The possible dysregulation of IL-2 signaling in Treg suppressor activity is supported by the association of T1D and polymorphisms within the IL-2 receptor alfa gene region in humans [134, 183]. Immunoregulation by Treg affects T cells, B cells, and APC antigen-specific cellular responses in different manners, including production of anti-inflammatory cytokines (TGF-β, IL-10, and IL-35) and contact-dependent mechanisms [184], possibly involving CTLA-4 and direct cytolysis [178]. The primary site at which nTreg control β-cell autoimmunity is within the islet infiltrate on CTL and inflammatory cells [185]. In the secondary lymphoid tissue, nTreg regulate DC activation inducing DC secretion of IDO [161], by binding CD80/CD86 via CTLA-4, and prevent the priming of naïve autoreactive CD4+ and CD8+ T cells [186]. cTreg are distinguished based on their cytokine secretion pattern [104, 187] and can produce IL-4 (‘Th2-like’ cells), TGF-β (‘Th3-like’ cells), and IL-10 [187, 188]. IL-10 is a potent systemic immune suppressor that regulates activation, proliferation, and

IFN-γ release by effector T cells [189] and indirectly controls DC activity [190]. IL-10 producing cTreg have also been reported to mediate a direct suppression by cell–cell contact, independently of IL-10 secretion [191].

24.2.2.3 B Cells

Little is known about self-tolerance mechanisms for B cells [192]. Immature B cells in the bone marrow are expressing a potentially polyreactive B-cell receptor (BCR), which results from stochastic gene recombination. B peripheral maturation process is thought to involve three checkpoints [193]. Twenty to fifty percent of autoreactive immature B cells undergo rearrangement of immunoglobulin light-chain genes and replacement of self-reactive BCR through a process called 'receptor editing'; the remaining self-reactive B cells undergo peripheral deletion or peripheral anergy [193]. The extent to which deletion and anergy contribute to B-cell tolerance has not yet been determined. Although evidences of aberrant receptor editing have been associated with autoimmunity in mouse and human diseases [194], to what extent these defects participate in the establishment of autoimmunity is still unclear.

24.2.3 What Happens in the Islet?

It is presently unclear whether in humans the initiation of autoimmune β-cell destruction requires autoreactive T cells simultaneously recognizing multiple β-cell antigens, or if T cells primarily target a single antigen. In the mouse, the chronology of appearance of islet T-cell reactivity suggests GAD65 as a triggering antigen [195]. Knock-out mice indicate a key role for insulin or proinsulin [196]. At the time of diagnosis in human T1D, patients exhibit autoimmune responses to a number of islet-cell antigens [17]. These responses representing 'antigen spreading' may be explained with the release of previously sequestered immunogenic proteins during the ongoing β-cell damage, as the clinical onset is manifest when more than 80% of the islets have been destroyed. The variability in reactivity to individual antigens may in turn be due to 'epitope spreading,' which consists of intramolecular shifting of the recognized epitopes with the progression of the autoimmune attack, and subsequent activation of new T-cell clonotypes. These events may provide an explanation for the widely diversified anti-islet immune response in T1D.

Priming of naïve CD4+ T cells by islets antigens presenting APC would be the first event in initiating islet autoimmunity (step 1) and diabetogenesis (step two) (Fig. 24.1). This event most likely takes place in the pancreatic lymph nodes (pLN). Islet antigen presentation in pLN has been demonstrated in the mouse [197, 198], but the detailed mechanisms in humans are unclear. What promotes the earliest event, namely uptake of antigen by APC in the islets, is still a matter of debate. Initial, still not fully characterized, insults (virus infection or other external damage, for example, environmental toxins) may elicit an innate immune response through the generation of exogenous or endogenous ligands for the pattern recognition receptors (PRR) on the β-cell surface. The activation of these receptor triggers

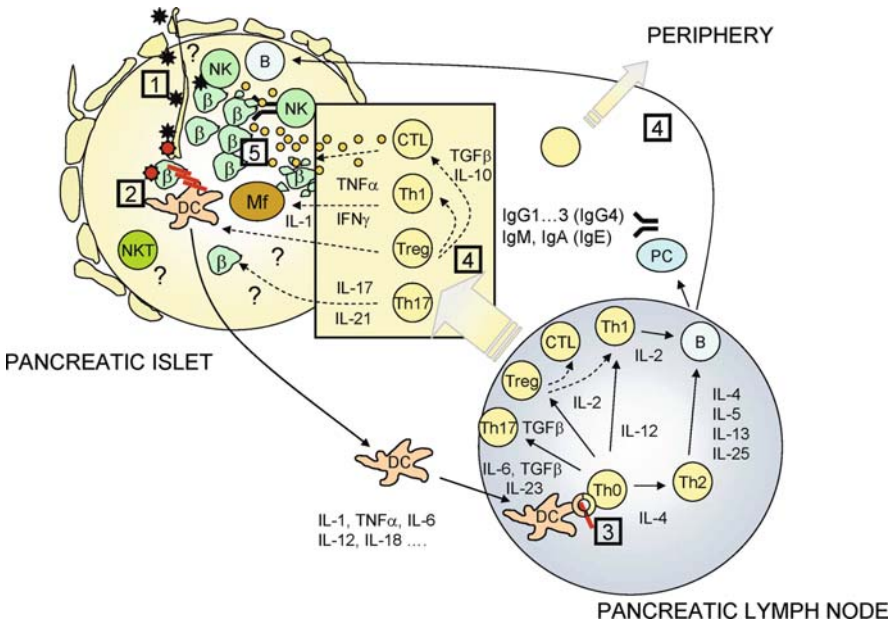

Fig. 24.1 Schematic view on possible immunopathogenesis of β-cell destruction. **Steps of events:** (**1**) Environmental factors are conditioning the relevant milieu by activation of dendritic cells (DC), macrophages (Mf), natural killer (NK) cells, and natural killer T cells (NKT); (**2**) Intake of antigens or cross-reactive peptides by dendritic cells (DC); (**3**) Presentation of peptides to naive T helper (Th0) cells and subsequent activation and proliferation of type 1 (Th1) and type 2 (Th2) helper cells, IL-17-producing helper cells (Th17), regulatory T (Treg) cells, cytotoxic T cells (CTL), B and plasma cells (PC), and activation of different cell subsets by cytokines; (**4**) Migration of activated cells from pancreatic lymph node to the islets, cross talk with periphery; (**5**) β-cells destruction by cytokine- and perforin/granzyme-mediated mechanisms. ✸ – environmental factor (virus etc.); ● – islet antigens; ▬ – islet antigens or cross-reactive peptides; ○ – islet-specific T cell; Ab – autoantibodies

intracellular responses including cytokine production, endoplasmic reticulum stress, and accumulation of misfolded proteins, which result in β-cell apoptosis and promote local inflammation [110]. Notably, mouse experiments suggest that β-cell apoptosis is a required step for T-cell activation [199]. Dying β-cells may release immunostimulatory 'danger' signals, physiologically aimed at eliminating the initial harmful factor. This requires a transfer to adaptive immune response mediated by the enrollment of APC and the establishment of a pro-inflammatory local environment (IFN, IL-1-β, and chemokines) to attract other immune cells. A defective resolution of the early inflammation results in a chronic destructive autoimmune reaction and may be dependent on the individual genetic background. For example, the DR3-DQ2 haplotype seems to be permissive of organ-specific autoimmunity [200, 201].

It is evident in the NOD mouse that DC are the first cells to infiltrate the islets [201]. Similar observations have been made in the BB rat [202] as well as in human

T1D [203]. The scavenging function of DC has been proven also in other mouse strains, in which physiological islet-cell death in pancreas remodeling [204, 205] is followed by DC in situ activation and migration to pLN [204]. Moreover, DC may cross-present peptides derived from apoptotic cells directly onto MHC class I molecules, without processing in the cytosol [206]. Taken together, it is likely that antigens derived from β-cells dying upon external damage may be taken up by APC in the pancreatic islets and transported to pLN (Fig. 24.1). In humans, this has not been possible to fully demonstrate, though expression of the β-cell autoantigens proinsulin, GAD65, and IA-2 has been detected in human peripheral DC [137]. Currently, our understanding on possible mechanisms of the very early events in islet autoimmunity relies on studies in animals.

24.2.3.1 Virus-Induced β-Cell Killing

It has been amply demonstrated that CTL specific to viral antigens are able to kill β-cells. Transgenic mice have been generated to express viral antigens on the β-cell surface using the insulin promoter to achieve cell specificity [207, 208]. When the mice are infected with virus, the generated CTL effectively kill the β-cells. These experiments are proof of principle of CTL-mediated β-cell killing to generate useful mice for diabetes studies. However, these studies do not provide answers as to what possible pathways a virus may use to enter β-cells, replicate, and express viral antigens on MHC class I molecules, thereby making the β-cell a target for virus-specific CTL. Regardless of the numerous reports of TD1 onset following viral diseases [209, 210], no conclusive pathogenic connection has been found between viral infection and human islet autoimmunity [33, 209, 211]. Virus diabetogenicity has been studied in rodents [209] and may be sustained by an aberrant immune response toward the β-cells. Interestingly, human pancreatic islets increase the expression of innate PRR when infected by virus or exposed to virus-related cytokines as IFN and IL-1-beta [212]. To be recognized by CTL virus antigen peptides need to be presented on MHC class I on the β-cell surface [213]. The critical question is to what extent a virus-infected β-cell is copresenting viral and β-cell antigens on MHC class I molecules. Some viral antigen sequences are similar to self-peptides and may mislead T-cell responses. This phenomenon of ‘molecular mimicry’ has been proposed between PC-2 antigen from Coxsackie B and GAD65 [96], between Rotavirus and IA-2 [214], for rubella [215], and cytomegalovirus [33]. However, it is possible that these events are more relevant to the amplification of the autoimmune process [216] and its maintenance after the resolution of the viral infection, than to the initial triggering of autoimmunity. As previously described, virus may activate β-cell intracellular signaling that induces altered expression of self-antigens on the β-cell surface (‘neoantigens’ or ‘cryptic antigens’) and participates to the cascade leading to β-cell apoptosis and insulitis [110]. Moreover, virus replication in the β-cell may result in its necrosis and in release of previously sequestered cellular constituents (‘hidden antigens’), lacking induced thymic tolerance [217]. The uptake and presentation

of these self-antigens by APC to CD4+ T cells may eventually lead to the formation of specific autoantibodies. Coxsackie B4 has been isolated from the β-cells of T1D new-onset patients [218]. Evidence for intracellular replication and induction of altered GAD65 islet expression [219] with production of specific autoantibodies has been reported in Coxsackie-infected mice [220]. In summary, there is wide evidence that virus infections may accelerate islet autoimmunity (step two) leading to a precipitation of clinical onset of T1D. The mechanism may be an increase in insulin resistance or a boost in β-cell killing induced by the virus infection. The major question to be answered is whether a virus infecting and replicating in human β-cells induces islet autoimmunity. The ongoing TEDDY (The Environmental Determinants of Diabetes in the Young) study may be able to answer this question [221, 222].

24.2.3.2 Cytotoxin-Induced β-Cell Killing

Alloxan [223], streptozotocin [223, 224], and the rodenticide Vacor [225] are β-cell cytotoxic agents. It is important to note that both alloxan and streptozotocin are more toxic to rodent than human β-cells. Other chemicals that may be potential human beta-cytotoxins are nitrosamine derivatives as well as dietary microbial toxins [226]. Epidemiological data suggest that an increase in nitrate-treated food items enhances the risk for children to develop T1D [227]. Experiments in both rats and mice have given insights into possible mechanisms by which beta-cytotoxic agents may induce islet autoimmunity apart from inducing diabetes by direct β-cell killing. First, low-dose streptozotocin treatment is inducing insulitis in a MHC and T-cell-dependent fashion [224, 228]. The use of streptozotocin in mice therefore offers a way to kill β-cells with a toxin that initiate β-cell autoimmunity. In rats treated with streptozotocin to induce β-cell destruction [229] it has been possible to detect circulating immunoreactive GAD65 following the β-cell killing, prior to hyperglycemia [230]. In previous rat studies with streptozotocin it was shown by electron microscopy that β-cell remnants including insulin granules could be detected in islet macrophages, possibly representing islet APC [13, 231]. Other compounds structurally related to streptozotocin or alloxan have been implicated as possible environmental agents, contributing to human T1D. Most prominently these compounds include the rodenticide pyriminil (Vacor) [225] that induces islet-cell surface antibodies and confirms that β-cell destruction in humans may cause islet autoimmunity [229].

In summary, several virus and chemical agents directly affecting islet cells may be causative in the initiation of an autoimmune β-cell destructive process. Alternatively these factors may potentiate a process initiated by other environmental factors, which are currently under scrutiny in the TEDDY study [221, 222]. In individuals prone to develop TD1, environmental chemicals may play a detrimental role by repeat injuries to the pancreatic β-cells over several years of life and in combination with a poor regenerative capacity of the β-cell and islet autoimmunity eventually induce diabetes.

24.2.4 Antigen Presentation in Pancreatic Lymph Nodes

Although specific mechanisms in humans remain unclear, APC loaded with β-cell antigens migrate from the islets to the pLN, where the processed antigens are presented to naïve CD4+ T cells (Th0) (Fig. 24.1). A recent study on human DC showed that antigen-specific DC/CD4+ T cells interaction allows DC migration through the dissolution of podosomes [232]. This led the authors to speculate that the same event may involve neighboring immature DC and induce their recruitment to the site of antigenic stimulation [232]. In the pLN, primed CD4+ T cells proliferate and differentiate into several subsets, as type 1 CD4+ T cells (Th1), IL-17-producing CD4+ T cells (Th17), and Treg cells, and activate naïve CD8+ T and B cells into CTL and plasma cells, respectively. The expansion of CD4+ T cells toward lineages of pro-inflammatory subtype (Th1 and Th17) is mainly promoted by the cytokine milieu, through IL-6, IL-12, and IL-23, whereas a balance toward IL-4 [233], IL-5, IL-13, and IL-25 would decrease the inflammation [234]. Th1 cells release IFN-γ, which activates macrophages, TNF-α, IL-12, and IL-18 [235]. The recent discovery of Th17 cells that are potent inducers of tissue inflammation and autoimmunity [236] is of interest, as they may have a role in islet destruction, as is reported for the NOD mouse [237]. Activation and differentiation of naïve CD8+ T cells to antigen-specific CTL is dependent on 'cross-priming,' namely the cognate recognition of the same antigen by the CD8+ and the CD4+ T cells on the same APC [238]. The interaction between CD40 on APC and CD154 on CD4+ T cells induces upregulation of costimulatory molecules for the activation of the CD8+ T cells [142] and increases the local production of pro-inflammatory cytokines such as TNF-α and IL-12 [239]. Alternatively, IFN-γ produced by CD8+ T cells could enhance CD4+ T-cell action [3]. The relative contribution of CD4+ and CD8+ T cells on diabetogenesis has been addressed by transfer experiments in mice [240, 241]. When cognate interaction occurs between B cells and activated CD4 + T cells, the B cells differentiate into plasma cells and start to secrete immunoglobulins with the same specificity of the previous membrane-bound immunoglobulin [242], upon stimulation of T-cell-released 'Th2' cytokines IL-4 and IL-5.

24.2.5 Homing of T Cells to Islets

Primed β-cell-specific effector T cells gain access to peripheral non-lymphoid tissues, migrate to the pancreas, and reach the β-cells [243] (Fig. 24.1). The molecular basis for this directed migration (homing) of autoreactive T cells to the islets and for endothelial transmigration is not completely clarified. The antigen specificity of infiltrating T cells has been amply demonstrated in mice [244, 245] and is reasonably postulated in humans, but the processes guiding islet autoantigen-specific T cells into islets are not known. In pancreas transplantations between monozygotic twins without immunosuppression, islets in the donor pancreas were infiltrated by CD8+ T cells in association with the loss of β-cell function [246]. These experiments

demonstrate the immunologic memory of the recipient, as well as β-cell killing by CTL, indicating that autoreactive CTL are reactivated. The mechanism of reactivation is unclear. It has been proposed that T cells can be programmed to a specific tissue tropism through a distinct 'homing receptor pattern' acquired at the site of priming [247]. In contrast to naïve cells, primed or memory T cells are significantly less dependent on a costimulatory signal and can proliferate with TCR engagement alone [248]. Upon second contact with cognate antigen in the islet, CTL are retained inside the islet tissue and initiate insulitis [197, 249] (Fig. 24.1). In the islets, β-cell-specific CTL may recognize antigens expressed on β-cells in association with MHC class I molecules. MHC class I overexpression on islet cells, previously described in pancreas with insulitis [12, 250, 251], is not likely to be involved in these early phases. It is noted in mouse studies that abrogation of MHC class I on β-cell does not blunt T-cell activation in pLN [249], but it may contribute to the local retention of self-reactive clonotypes. In the NOD mouse, the early infiltrate consists of activated macrophages and CTL that lead the initial accumulation at the vascular entrance (peri-insulitis), probably under the effect of chemotactic mediators as IL-1 [252] and chemokines as CXCl10 and CCl2 [253] that direct leukocyte migration and activation during the transition to adaptive immunity [110]. Of notice increased islet levels of CXCl10, CCl2, CCl20, and IL-15 are detectable in the NOD mice during the pre-diabetic stage [254, 255]. Although the routine investigation of the early phase (step 1) is not feasible in humans, immunocytochemistry on pancreas biopsy specimens from new-onset T1D patients in Japan indicates the presence of CD8+ T cells and activated macrophages secreting inflammatory cytokines [256]. The ongoing inflammatory islet milieu expands the recruitment of autoreactive CTL through the expression of chemokines and homing ligands from the β-cells. These may in turn secrete the chemokines as CXCL10, which specifically attracts autoreactive CD8+ T cells. Mouse β-cells may also express the CXC chemokine receptor 3 [257] to further promote the recruitment of T cells and macrophages to the islets [258]. A study on the homing of human diabetogenic T cells reported that IFN-γ is crucial for diapedesis and penetration into islets [176]. As physiological response to the inflammation, islet endothelium upregulates the expression of surface adhesion molecules that increase vascular permeability and facilitate the recruitment of effector cells. Adhesion and diapedesis of T cells are feasible through the interactions of T-cell surface molecules (integrins) such as leukocyte function-associated antigen-1 (LFA-1) and very late activation antigen-4 (VLA-4) with their counter ligands on VEC, such as intercellular adhesion molecules (ICAM) and junctional adhesion molecules (JAM-1) [259] that play a major role in the homing of diabetogenic T cells to the pancreas in the NOD mouse [260, 261]. This hyperexpression of adhesion molecules is documented in new-onset diabetes pancreas [11, 262] but may not fully account for the observed enrichment of infiltrating autoantigen-specific T cells. It is now proposed that after migration from pLN, activated T cells require an additional upregulation of LFA-1 functional activity for the successful adhesion to VEC [263]. Data support that the triggering of TCR, achieved through peptide antigen presentation by iVEC, is an important component of integrin functional activation [264] and may provide an additional grade of antigen specificity in T-cell recruitment. The

hypothesis that iVEC may participate in T-cell selective recruitment and adhesion in an antigen-specific fashion is intriguing. A recent study reported that GAD65 presentation by iVEC markedly promotes the in vitro transmigration of GAD65-autoreactive T cells across iVEC monolayers in an LFA-1-dependent fashion [174]. In this process, CD4+ T cells may also intervene by secreting various lymphokines that attract and activate other cell types such as monocytes, eosinophils, and natural killer lymphocytes (NK) [173]. Whether islet-specific autoantibodies secreted by plasma cells take part in the islets destruction, or are merely recruited upon the ongoing discharge of autoantigens, it is still a matter of debate, since a defined pathogenetic effect has not been proven. A pathogenetic involvement of the autoantibodies may be suggested from NOD mouse experiments in which B-cell-deficient animals are protected from diabetes [265], but clinical evidences in humans do not support this hypothesis [266]. Nonetheless, the observation of a cytotoxic effect of autoantibodies on human β-cell in vitro [267] may indicate possible harmful effects. Autoantibodies might exert either complement-mediated or antibody-dependent cellular cytotoxicity, but there is no clear evidence of these effects in vivo. Moreover, immunoglobulin deposits may [12] or may not be found in islets [3]. From previous experimental observations in mouse, the presence of islet autoantibodies does not seem to be sufficient [3] nor necessary [253] to β-cell destruction, and do not clearly correlate with the T-cell responses [268]. However, recent insights acquired from NOD mice [88] suggested that B cells may be more important players than formerly considered, and their relevance in physiopathology of human β-cell destruction is currently under investigation [269]. It is still matter of debate whether antibodies reacting to antigen-binding areas of autoantibodies (anti-idiotype) may be of relevance within the autoimmune process [270], through the blockade of circulating self-autoantibodies.

24.2.6 Insulitis and β-Cell Destruction

The progression from the initiating phase to an adaptive immune response is thought to take place very early during the insulitis and determine the final outcome toward the generation of a prolonged devastating autoimmune reaction, or the resolution of inflammation and preservation of islet integrity. Target-cell death further activates PRR that in turn promote the progression of insulitis [271] through IFN-α-mediated upregulation of MHC class I molecules on pancreatic islet cells [110, 272]. Among TLR ligands, HSPs are reported to promote antigen presentation [273, 274] and shift DC toward immunogenic phenotype in vivo [273]. IFN and other macrophage-derived cytokines prompt NK activation. These cells exert nonantigen-specific cytotoxicity through the release of perforin, after the activation of surface receptors, as NKG2D that recognizes viral products and other specific ligands [275]. NKT cells on the other hand may be considered as innate-like lymphocytes, as they may co-express NK cell surface markers including NK1.1 (human CD161) and TCR [276]. Most NKT cells recognize glycolipid antigens presented on the MHC class

I-like molecule CD1d [276]. The majority of human NKT cells display an invariant TCR-α chain (Vα24-Jα18) and limited number of beta chains [277], and it is often referred to as invariant NKT cells (iNKT) or 'classical' or 'type 1' NKT [276]. iNKT cells are potent producers of Th2-like reactivity in vivo [277] and are involved in autoimmune diseases in humans and mice [278]. The possible role of NK in β-cell damage has not yet been clarified, since neither a protective nor a detrimental effect of these cells has been consistently reported in humans or in mice [279–282]. Similarly, an unequivocal role in islet autoimmunity for iNKT has not been established, though a predominant immunomodulatory function has been proposed in the NOD mouse [283]. The effect may be exerted by inducing DC tolerogenic differentiation [284, 285] and conditioning the cytokine environment of pLN or islets [286]. Conversely, the exacerbation of insulitis for an iNKT-mediated enhance of IFN-γ-producing CTL has also been reported [287].

As the islet invasion progresses, chemokines-attracted macrophages contribute to the recruitment of other immune cells that also release multiple chemokines and pro-inflammatory cytokines. These inflammatory signals create an overall immunoactivatory environment that modifies DC phenotype [107], shifts CD4+ T cells toward 'Th1-like' responses which promote the expansion of CTL, and shelters them from peripheral tolerance [154, 288]. If this vicious circle is not interrupted, the maintenance and amplification of insulitis evolve in accumulation of immune cells and their cytotoxic mediators that may act synergistically to destroy the islets [289] (Fig. 24.1). In the later stages, the destructive process may be worsened in the course of β-cell failure as hyperglycemic environment may locally enhance insulin epitope presentation [290].

Apoptosis is probably the main form of β-cell death in T1D and is regulated in parallel to the inflammation, through the activation of similar intracellular signaling pathways [110]. β-cell injury in the course of insulitis is caused by both exposure to soluble mediators as cytokines and reactive oxygen species, secreted by infiltrating cells, and direct cell–cell contact with activated macrophages and CTL [252]. The role of cytokines in diabetes development was confirmed by the demonstration that suppression of cytokine signaling within β-cells completely prevents mice from diabetes, despite the presence of insulitis [291]. Cytotoxic inflammatory cytokines IL-1-β, TNF-α, and IFN-γ released by CTL and macrophages affect β-cell gene regulatory networks, influencing primarily transcription factors NFk-B, STAT-1, and AP-1, and activate apoptosis [292]. IL-1 exerts in vitro cytotoxic effects on islets [293] that express specific surface receptors [294]. Cytokine-induced β-cell death seems to be preceded by a functional impairment, as IL-1 [295, 296] and TNF-α [297] inhibit insulin secretion from isolated cells. This effect may be mediated by the action of nitric oxide (NO) [297, 298]. TNF-α, IL-1, and IFN-γ activate β-cell inducible NO synthase activity and enhance the production of endogenous NO [299]. For the lack of radical scavenging activity, β-cells are highly susceptible to reactive oxygen species [300] that directly participate to cell death through DNA injury, activation of the DNA repair enzyme poly-ADP-ribose polymerase, and depletion of nicotinamide adenosine dinucleotide (NAD) (87). A central role in cytokine-mediated β-cell death is ascribed to IFN-γ as observed in mouse

studies on anti-IFN-γ antibodies and IFN-γ transgenic expression [252]. In the islets INF-γ enhances T-cell cytotoxicity and participate to direct β-cell damage, probably through the upregulation of receptor Fas on β-cells [291]. Fas is a 45-kDa surface receptor which directly transduces the signal for apoptosis through translocation of phosphatidylserine, upon binding of its specific ligand (Fas-L) [301]. Although the precise mechanism is not defined in vivo, the Fas/Fas-L complex may act through caspase, which is thought to be a major effector enzyme in the apoptotic pathway [199, 204]. Effector T cells can trigger β-cell death through direct contact between their surface Fas-L and membrane-bound TNF-α and apoptosis-inducing receptors on β-cells or through the secretion of perforin [252]. Perforin acts facilitating the passage of protease (granzymes) and may be involved in more advanced stages of the destruction [87].

Further studies of early insulitis in humans will be needed to fully appreciate the initiating mechanisms of infiltration of immune cells. Effective prevention of T1D may require a better understanding of the early events of building chronic insulitis. We speculate that a chronic insulitis which includes APC presenting islet autoantigens within the islets as opposed to the pLN represents a refractory state to immunosuppression. This may explain why immunosuppression at the time of clinical diagnosis of T1D is ineffective. It cannot be excluded that immunosuppression therapy may be efficacious, provided that the treatment is used prior to chronic insulitis.

24.2.7 Is β-Cell Destruction Reflected in the Blood?

Assaying the cells involved in β-cell damage may give insights about the induction and maintenance of islet autoimmune destruction. Several possible immunological alterations have been searched in the peripheral blood of T1D patients and at-risk subjects to differentiate them from healthy subjects.

24.2.7.1 APC

Most alterations described in mice are consistent with the hypothesis of an increased DC capacity to activate CD4+ and CD8+ T cells [302], such as upregulation of costimulatory molecules, enhanced secretion of cytokines IL-12p70 and TNF-α [303], and downregulation of IDO [304]. An abnormal cytokine response by DC from T1D patients upon antigenic [305] or nonantigenic stimulation was proposed [306] but has not been confirmed by other studies [307]. More robustly, phenotypic characterization suggests that DC from recent-onset T1D patients exhibit an immature phenotype and may have a decreased T-cell stimulatory capacity, compared to controls [308]. DC may therefore indirectly participate to T1D autoimmunity through a reduced efficacy in stimulating Treg, as is also reported in mice [309] and BB rats [310]. This immature phenotype of T1D human DC may result from abnormal activation of the NF-kB pathway [311], consistently with the strong involvement of this transcription factor in the induction of self-tolerance in mice [157, 303].

Studies investigating the peripheral DC count reported a reduction in absolute number of blood DC in T1D children [307] and, more recently, a modest but significant increase in the relative frequency of pDC subset, strictly time-related with disease onset [312]. Individual, genetically determined antigen processing (internalization and proteasomic cleavage) has been demonstrated in different APC [313] and may account for disease-relevant epitope presentation in genetic susceptible individuals [314]. However, the present observations about DC in human diabetes rely upon studies on in vitro monocyte-generated DC that may not reflect the true in vivo situation (315).

24.2.7.2 T Cells

Plenty of studies on peripheral blood mononuclear cells (PBMC) of T1D patients aimed at detecting the presence of islet-specific CD4+ and CD8+ T cells, upon stimulation with synthetic peptides from islet antigens. The immunogenic epitopes are selected among putative immunodominant regions within the multiple diabetes-related islet autoantigens. Many of these studies report a higher frequency of islet-specific self-reactive T cells in T1D patients than in control subjects, when T cells are detected by either functional tests of antigen-induced proliferative [96] and cytokine-secretive response [91, 102, 316, 317] or tetramer staining [101, 318, 319].

CD8+ and CD4+ T cells from T1D patients target a wide array of epitopes within GAD65 molecule [92, 316, 318–322], insulin and proinsulin [91, 323–325], IA-2 [316, 317, 326], IGRP [323, 327, 328], I-A2b [327, 328], islet amyloid polypeptide (IAPP) [327–329], and glial fibrillary acidic protein (GFAP) [328] as comprehensively summarized in a review updated to the end of 2006 [330]. More recently, a few more epitopes have been described, as GAD536-545 [316], other IGRP fragments, among which IGRP 211–219 and 222–230 [331], and several novel insulin- and proinsulin-derived peptides targeted by CD8+ [323, 332, 333] and CD4+ T cells [334]. In conclusion, these investigations, mostly oriented toward epitope identification, provide evidence of multiple immunodominant β-cell regions targeted by CTL in human T1D, but do not fully clarify the development of the T-cell-specific responses during the progression of the disease. In fact, no single epitope has proven to be highly discriminatory, though a hierarchy of T-cell responsiveness has been proposed among proinsulin peptides [102]. In some ways, the choice of the epitope may also be misleading. Candidate sequences are usually selected on the basis of predicted TCR–pMHC-binding motifs [335] or affinity algorithms [336], whereas the strength of the TCR–pMHC complex interaction may inversely correlate with immunogenicity [327, 337], in accordance with an insufficient negative thymic selection; this bias can be avoided through the analysis of multiepitope, multiantigen panels [317, 337]. Moreover, epitopes that have been proved of relevance in mice may guide the searching efforts in humans, as is recently happening with IGRP peptides [331]. As already mentioned, a precise, reproducible, and standardized method for detection and identification of β-cell-specific autoreactive T cells to reliably identify the pathologic response of T1D patients is not available. However,

some authors reported that the use of multiple epitopes achieved more diagnostic sensitivity and discriminates T1D from controls [102, 324]. It is therefore still unclear to what extent all the data provided can be translated into evaluation of diabetes risk or disease condition. Moreover, autoreactive T-cells-specific responses for T1D self-antigens have been widely described in healthy individuals in stimulation assays with peptides from GAD65 [338, 339] and insulin [100, 102, 327]. Several differences have been proposed between self-reactive T CD4+ T cells from T1D and controls. Only GAD65-reactive T cells from T1D subjects seem to be fully autoantigen-experienced in vivo and express the memory T-cell marker CD45RA [338, 339] and are capable of activation in the absence of CD28/B7 costimulatory signals [248]. It was also recently proposed that CD4+ T cells from T1D subjects may have a lower threshold of activation, as compared to healthy controls [340].

Interestingly GAD65-specific T-cell TCR repertoire does not differ between T1D and controls [338], implying that central tolerance to GAD65 is the same among healthy and T1D subjects. Probably, in healthy individuals, self-reactive T cells are present but quiescent for the immunosuppressive action of Treg, as confirmed by the experimental observation that Treg in vitro depletion is followed by amplification of autoreactive T cells only in samples from healthy individuals [338]. Treg pool in human T1D has also been extensively investigated, and a deficiency in Treg peripheral frequency has been reported in patients compared to controls [341], but subsequent investigations have failed to uniformly replicate these findings [342] and have suggested that T1D nTreg may rather display an impaired immune suppressor function [342, 343]. Globally, it seems that a simple deficiency in the peripheral Treg repertoire is not confirmed [341], but a local impairment of Treg activity at the site of inflammation cannot be excluded. Notably, most defects in number and function of Treg observed in NOD mice [186, 344, 345] may be ascribable to the flogistic environment [346] that may actively inhibit Treg suppressive function through both the reduction in IL-2 [347, 348], mTGF-β [349], and TNF-α [350] and the increase in IL-21 [351, 352]. Interestingly, the peripheral blood from T1D patients may be evidence for a misbalance toward inflammation. Autoantigen-driven cytokine secretion by CD4+ T cells from T1D patients may be polarized toward INF-γ, while HLA-matched healthy controls display IL-10+ cTreg-like responses [102]. This 'regulatory phenotype' skewed toward IL-10 has also been reported in association with T1D later onset [102] and better glycemic control [353]. Increased levels of 'Th1 cell'-derived chemokines CCl3, CCl4, and CXCl10 [354, 355] and of adhesion molecules ICAM and L-selectin (CD62L) [356] have been found in serum of T1D patients. Several reports have addressed NK population in the peripheral blood of T1D patients [357] and have described a decrease in the peripheral frequency, in most cases temporally related to disease onset [4, 281], or a functional deficit [358], but these findings have not been universally replicated [357]. More recently, a larger study confirmed a functional impairment of NK cells in T1D patients, i.e., reduced surface expression of activating receptors and low levels of IFN-γ and perforin, and suggested that these alterations may be a consequence of T1D, since they are evident exclusively in long-standing disease [359]. It has also been reported that activated NK cells in T1D patients display a reduced expression

of NKG2D receptor [357]. It is possible that a downregulation of NKG2D receptor mediates the increased risk for T1D associated with polymorphisms of MHC class I chain-related (MIC) proteins [360] that are NKG2D natural ligands. Studies addressing a correlation between NKT cell levels in peripheral blood and T1D in humans have yielded variable results, since the reports of altered frequency [361] or cytokine secretion [224, 362, 363] have not been confirmed [364].

It must be noticed that the reported assays on cell repertoire and flogistic mediators in humans have been performed on peripheral blood samples. More disease-relevant alterations may be detected assessing the islets or the pLN, which would give a more realistic picture of locally generated signals. Some recent reports have tried to overwhelm this limit. Two studies in the mouse have suggested that, regardless the provenience of the T cells (periphery, islets, or lymph nodes), β-cell antigen-specific CD8+ T-cell pool shares TCR chain usage [365] and show conserved patterns of epitope immunodominance [366]. Another study has performed micro array analysis of the cytokine pattern of PBMC from healthy subjects after the exposure to sera from new-onset T1D patients and has reported an enhanced secretion of pro-inflammatory factors as IL-1, CCl2, and CCl7 [367].

24.2.7.3 B Cells and Autoantibodies

The assessment of eventual disorders of humoral immunity in T1D relies on the monitoring of circulating islet-reactive autoantibodies (Table 24.2). Autoantibodies against at least one of islet-cell antigens GAD65, IA-2, insulin, and ZnT8t are present in more than 95% of T1D patients [34] and in only 1–2% of general population [368]. Radio-binding assay of these autoantibodies has replaced the ICA assay. GAD65 antibodies are found in 70–75% of T1D patients [369] and show a diagnostic sensitivity of 70–80% and a diagnostic specificity of 98–99% [40]. Of interest, the titer of GAD is usually low at time of diagnosis. The major antigenic epitopes of GAD65 are the middle- [370] and C-terminal region [371, 372] and are in close proximity to T-cell disease-relevant determinants [170]. Differential epitope specificities, as identified by monoclonal antibodies to GAD65 epitopes within the C-terminal region, align with distinct autoimmune disease phenotypes [170, 373], and the binding of N-terminal epitope is associated with slowly progressive β-cell failure [374]. Finally, it was recently suggested that the presence of GAD65 antibodies in T1D patients may be the result of an ‘unmasking’ due to the lack of anti-GAD65-anti-idiotypic antibodies [375]. These anti-idiotypic antibodies are reported to highly discriminate T1D from healthy subjects and may be of some relevance in the pathogenesis of islet autoimmunity. IAA are found in approximately 50–70% of T1D patients [40, 140] and are first islet autoantibody to appear [376], suggesting an involvement of insulin as primary autoimmune triggering antigen, also in humans [377]. Epitopes targeted by IAA are placed within A and B chains and are shared between insulin and proinsulin [378]. IA-2 antibodies are detected in 60–70% of patients with new-onset T1D and tend to appear closer to the clinical onset [2, 40]. Epitopes for IA-2 antibodies are found exclusively within the cytoplasmic region of the molecule and predominantly within the

tyrosine phosphatase-like domain [379, 380]. Antibodies to ZnT8t are detected in 60–80% of newly diagnosed T1D [2, 65]. Finally, antibodies anti-alfa-2-amylase have been recently described in a subgroup of patients affected by autoimmune pancreatitis presenting with fulminant diabetes [381], a form that is commonly considered 'non-autoimmune.' In these patients, the lymphocyte infiltrate affecting the exocrine component in autoimmune pancreatitis is extended to the islets [381] and may reveal shared immune-mediated mechanisms, as seem to be suggested in the NOD mouse [382].

24.3 Prediction of β-Cell Destruction

Standardized methods [83] have made islet autoantibodies the most useful marker for T1D prediction [2, 40] and for enrollment of subjects into clinical prevention trails. The most accurate single predictor is GAD autoantibodies [383] with a positive predictive value for T1D of about 60% [40, 384], followed by IAA (30%) that is a better predictor among children [59, 385]. To enhance the predictive power, more markers in combination are actually used [386, 387], and the prediction power for T1D reaches 100% in case of multiple positivity (Fig. 24.2). Similarly, in case of single autoantibody, the correlation between islet autoimmunity and histological evidence of insulitis is weak [251, 388]. Longitudinal studies investigating DC and T cells in at-risk subjects are lacking. Some reports have found poor in vitro maturation and pro-inflammatory cytokine response in DC from children at genetic risk for TID [389, 390] and a number of attested T-cell responses from PBMC of at-risk subjects toward islet-specific autoantigens GAD65 [318, 319] and insulin/proinsulin [318, 328]. Finally, increased chemokines, such as CXCl10 [354] and adhesion molecules [356], have been detected in the plasma of at-risk individuals; more

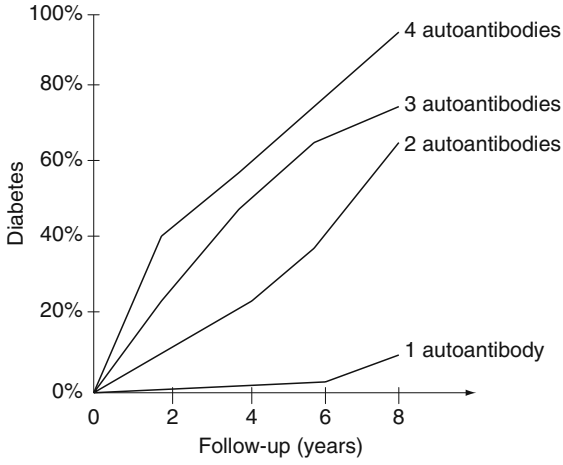

Fig. 24.2 Diagrammatic presentation of the effect of multiple islet autoantibodies on the risk of developing T1DM in the Diabetes Prevention Trial-Type 1 (DTP-1) (Courtesy of Jay Skyler)

recently, in three at-risk subjects followed until diagnosis these alterations were present years before the clinical onset [367]. However, this data cannot to date be translated into risk stratification.

24.4 Concluding Remarks

In conclusion, the β-cell in T1D is the major target for an autoimmune process that takes place in two steps. The first step is the development of an autoimmune reaction directed toward specific β-cell antigens. This step is reflected by circulating autoantibodies to β-cell autoantigens including GAD65, IA-2, ZnT8, and insulin. The number of autoantibodies predicts T1D risk. The second step is progression from islet autoimmunity to the clinical onset of T1D, which in humans is associated with a major loss of β-cells and insulitis. Insulitis appears late in the autoimmune process and can be recapitulated in pancreas and islets transplantation. The immunological memory of β-cell autoantigen is chronic. Efforts are needed both to detect intra-islet events that precede the development of autoantibodies and to disclose when islet autoantibody positivity is marking that the β-cell destructive process of insulitis is about to be established. A better understanding of step 1 and two events will be necessary for the ultimate prevention of β-cell destruction and of T1D.

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Chapter 25
Toll-Like Receptors and Type 1 Diabetes

Danny Zipris

Abstract Type 1 diabetes (T1D) is an autoimmune disease that results in the progressive loss of insulin producing cells. Studies performed in humans with T1D and animal models of the disease over the past two decades have suggested a key role for the adaptive immune system in disease mechanisms. The role of the innate immune system in triggering T1D was shown only recently. Research in this area was greatly facilitated by the discovery of toll-like receptors (TLRs) that were found to be a key component of the innate immune system that detect microbial infections and initiate antimicrobial host defense responses. New data indicate that in some situations, the innate immune system is associated with mechanisms triggering autoimmune diabetes. In fact, studies preformed in the BioBreeding Diabetes Resistant (BBDR) and LEW1.WR1 rat models of T1D demonstrate that virus infection leads to islet destruction via mechanisms that may involve TLR9-induced innate immune system activation. Data from these studies also show that TLR upregulation can synergize with virus infection to dramatically increase disease penetrance. Reports from murine models of T1D implicate both MyD88-dependent and MyD88-independent pathways in the course of disease. The new knowledge about the role of innate immune pathways in triggering islet destruction could lead to the discovery of new molecules that may be targeted for disease prevention.

Keywords Type 1 diabetes · Innate immunity · Toll-like receptors · Inflammation

25.1 Introduction

Type 1 diabetes is an autoimmune disease that results in the progressive loss of insulin producing cells [1]. How disease is triggered is still unclear; however, epidemiological data and evidence from animal models of T1D implicate viral infections in the course of disease [2]. The innate immune system is the first line

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of defense against microbial infections. It plays a critical role in detecting and eliminating microbial infections. Innate responses involve macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils, and NK cells [3]. In contrast to adaptive immunity that identifies antigens using highly specific antigen receptors expressed on T and B lymphocytes, the identification of microbes by the innate immune system is mediated via a limited number of receptors termed pattern recognition receptors (PRRs) that bind a broad range of molecular structures expressed by microbes [reviewed in Refs. 3–6]. Among the most studied PRRs are TLRs expressed by both innate and adaptive immune cells. Binding between TLRs expressed on DCs and their ligands leads to a complex response that involves upregulation of proinflammatory cytokines and chemokines and increased expression of MHC Class II and costimulatory molecules on dendritic cells (DCs). The latter response enhances the ability of DCs to prime antigen-reactive T cells to specifically attack invading pathogens [3–5, 7, 8].

Recent data have shown that TLR-induced activation of the innate immune system could result in the induction of proinflammatory pathways leading to autoimmunity. Indeed, there is new evidence that TLR activation by exogenous or host-derived ligands is linked with a number of autoimmune disorders such as arthritis, atherosclerosis, systemic lupus erythematosus (SLE), and autoimmune diabetes [reviewed in Ref. 9]. This review summarizes recent data from studies performed in humans and animal models of diabetes implicating the innate immune system and TLR pathways in the autoimmune process leading to islet inflammation and destruction.

25.2 Toll-Like Receptors

The innate immune system identifies pathogens via a limited number of germ line-encoded receptors termed pattern recognition receptors (PRRs). These receptors bind conserved structures expressed by microbes and termed pathogen-associated molecular patterns (PAMPs) [3–5, 9, 8]. The most characterized PRRs are toll-like receptors (TLRs). They were first described in the Drosophila. It was evident that the expression of TLR in this fly was crucial for the defense against fungal infection [10]. Thirteen mouse TLRs and 10 human TLRs have been discovered thus far, and as shown in the Drosophila, their expression in mammals is critical for host defense against microbial infections [11]. All TLRs have a similar structural organization, an extracellular or endosomal ligand-binding domain consisting of leucine-rich repeats (LRRs), a single transmembrane region, and an intracellular Toll/IL-1 receptor homology (TIR) signaling domain [for reviews, see Refs. 12, 13]. Following binding between TLRs and their ligands the cytoplasmic TIR domain associates with the TIR domain on the adaptor protein. There are four known TIR adaptors involved in TLR signaling, termed MyD88, TIRAP, TRIF, and TRAM, and different TLRs can sometimes be involved in recruiting more than one type of adaptors. The adaptor being recruited following receptor ligation governs the subsequent nature of the immune response. MyD88 is linked with TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, and TLR9 pathways. TIRAP is associated with TLR2

and MyD88-dependent TLR4 signaling, whereas TRIF is involved in MyD88-independent TLR3 and TLR4 pathways. TRAM is linked with MyD88-independent TRIF-dependent TLR4 signaling.

TLRs can bind a wide variety of chemical compounds expressed by microbes. For example, TLR1, TLR2, and TLR6 bind lipid and carbohydrate molecules expressed by Gram-positive bacterial cell walls. TLR2 recognizes peptidoglycan, lipoteichoic acid (LTA), and lipoproteins. In addition, TLR2 can form a heterodimer with TLR1 or TLR6 involved in the recognition of different lipoproteins with different lipid moieties. TLR4 identifies lipopolysaccharide (LPS) expressed by Gram-negative bacteria and this recognition requires the participation of accessory molecules such as MD-2-, CD14-, and LPS-binding protein (LBP). Some TLRs can be activated by proteins. For example, TLR5 recognizes bacterial flagellin and TLR11 senses the presence of profilin expressed by the parasite *Toxoplasma gondii*. In contrast to TLRs such as TLR4 that are located on the cell membrane surface, TLR7, TLR8, and TLR9 are found on cytoplasmic membrane compartments such as endosomes and lysosomes. TLR7, TLR8, and TLR9 recognize nucleic acids from bacteria and viruses. TLR3 binds viral double-stranded RNA (or a synthetic analogue called polyinosine polycytidylic acid (Poly(I:C)) [14]. TLR9 recognizes DNA containing hypomethylated CpG motifs expressed by bacteria and viruses [15–17], whereas TLR7 and TLR8 bind viral single-stranded RNA [18, 19]. It was recently shown that like viral and bacterial DNA, host-derived nucleic acids can under some circumstances activate endogenous TLR7 and TLR9 signaling pathways. It was hypothesized that this recognition could result in proinflammatory responses involved in triggering autoimmunity [20, 21].

TLRs are not the only molecules utilized by the innate immune system to sense microbial infections. Other intracellular cytosolic sensors such as retinoic acid-inducible gene I (RIG-I)-like helicases (RLH) and melanoma differentiation-associated gene-5 (MDA5) are also associated with innate immune responses against nucleic acids expressed by pathogens [5]. Hence, some pathogens may be recognized by multiple types of sensors. Like TLRs, RLHs were found to be evolutionarily conserved. Human and mouse RIG-I recognize RNAs from paramyxoviruses and orthomyxoviruses that harbor triphosphate on their 5′ termini.

Interactions between PAMPs and PRRs initiate a cascade of intracellular signaling leading to the induction of antimicrobial genes, proinflammatory cytokines, and chemokines, such as type I interferons, IL-6, IL-12, and CXCL-10. Interactions between TLR ligands and their receptors also induce a maturation program that results in upregulation of MHC Class II and costimulatory molecules on the surface of DCs, enhancing the capacity of these cells to present antigens to T cells [3–5, 7, 8].

25.3 The Natural History of T1D

Type 1 diabetes is thought to be a T cell-mediated organ-specific autoimmune disease associated with islet inflammation and in some cases a slow and progressive loss of insulin secretion [22]. The mechanism triggering islet destruction

is not yet fully understood, but published data suggest that islet β-cell death is linked with pancreatic infiltration of immune cells, including T and B lymphocytes, macrophages, and dendritic cells (DCs) [23]. Evidence from humans and animal models of T1D shows that T cells play a key role in the mechanism of islet cell death [24–29].

The appearance of autoantibodies against islet antigens in the peripheral blood is one of the early evidence for an ongoing autoimmune response against the pancreas prior to disease onset. This immune response plays a key role in disease prediction in genetically susceptible individuals. Prospective studies of the natural history of T1D such as the Diabetes Autoimmunity Study in the Young (DAISY), the Finnish DIPP study, and the German BabyDIAB study have characterized the autoantibody response [reviewed in Ref. 30]. On the basis of data from these studies, it is now possible to predict the development of T1D by measuring four anti-islet autoantibodies against insulin, GAD65, IA-2 (ICA512), and (Znt8)] [31]. These antibodies can in some cases be found in the peripheral blood of prediabetic individuals many years prior to disease onset [23]. Their role in the course of diabetes remains unknown; however, studies conducted in the NOD mouse suggest that B cells are important players in the mechanism leading to diabetes [32].

Islet-reactive autoantibodies are thought to develop sequentially. Insulin autoantibodies are typically the first to be expressed, particularly in young children [reviewed in Ref. 33]. It was documented that family members who express autoantibodies against insulin, GAD65, and IA-2 have a 75% 5-year risk of diabetes compared with a 25% 5-year risk in relatives who express only one of those autoantibodies. In addition, children who express anti-islet antibodies in their first 2 years of life have a fast progress to disease onset leading to hyperglycemia before the age of 10 years [34].

There is convincing evidence that genetic factors have a major impact on the risk for disease. Major susceptibility genes are located within the HLA region [35]. The HLA haplotypes associated with the highest risk for diabetes in Caucasian populations are HLA-DR3/4 DQ8 [34]. A major risk factor in diabetes is first-degree T1D family history, as about 10–13% of newly diagnosed children have a first-degree relative with T1D [36]. The Diabetes Prevention Trial 1 (DPT-1) study indicates that siblings of patients with T1D develop islet autoantibodies more frequently than offspring or parents of type 1 diabetic patients [37]. The risk for disease is increased if both parents or a parent and a sibling have diabetes compared with a single affected family member [34].

25.4 Viruses and T1D

Data from epidemiological studies support a role for environmental agents and viruses, in particular, in the mechanism triggering disease. It is, however, exceedingly difficult to demonstrate a causative link between microbial infections and T1D in humans [2, 38]. Perhaps one of the most compelling evidence associating

environmental agents with diabetes is the finding that the concordance rate for T1D in monozygotic twins is ~50% [39–41]. Consistent with the notion that the environment plays a role in diabetes is the observation that incidence rates of T1D have been rising over the past few decades worldwide by an average of 3% per year [42], an increase that cannot be a result of genetic drift [43]. It was hypothesized that the increase in the incidence of diabetes, particularly in industrialized countries such as Finland and Sweden, is linked with reduced infection rates during childhood as a consequence of improved hygiene and vaccine programs (The "hygiene hypothesis", see Refs. [44, 45]). This hypothesis is supported by data from retrospective studies indicating an inverse correlation between infections and diabetes [45–48]. The "hygiene hypothesis," however, remains controversial as other studies failed to observe a link between infections and the risk for disease development [49, 50].

Viruses [51, 52] and antivirus antibodies [53–56] were detected in patients with newly diagnosed disease, suggesting a role for microbial infections in diabetes. Cytomegalovirus [57], rubella [58], mumps [59], EBV [60], and coxsackie B [61] are among pathogens implicated in disease course. Coxsackie B virus, a single-stranded DNA enterovirus that belongs to the Picornaviridae family, has been most studied with respect to T1D, but its role in the course of disease remains unsettled [62]. This virus can be found more frequently in patients with T1D as compared with healthy individuals [63–69] and sometimes be detected in the pancreas of subjects with acute T1D but not nondiabetic individuals [51, 52]. In addition, a correlation may exist between coxsackie virus expression and the appearance of antibodies against islet antigens [70, 71]. Finally, injecting mice with coxsackie B virus isolated from the pancreas of patients with diabetes leads to islet destruction and diabetes in mouse recipients [2, 52, 72]. A number of studies addressing a connection between coxsackie B virus and diabetes failed to obtain evidence for such a link [73–75], and thus, further investigations are clearly required to define the role of this virus in disease mechanisms.

25.5 The Innate Immune System and Human T1D

Dendritic cells are involved in defending from microbial infections and were previously associated with mechanisms of autoimmunity [76]. For example, DCs were linked with cardiomyopathy [77, 78], systemic lupus erythematosus [79], and psoriasis [80]. In autoimmune diabetes, DCs can be detected in islets from patients with T1D and contribute to the process of islet destruction via the release of proinflammatory cytokines such as TNF-α and IL-1β [81]. DCs from the peripheral blood of diabetic patients express normal levels of proinflammatory cytokines and surface CD40 molecules upon TLR3 activation [82], but they are unable to upregulate normal levels of Class I and CD40 molecules following TLR4 ligation [83]. Summers and colleagues found that DCs from individuals with long-term diabetes express diminished amounts of IFN-α and IL-12 upon CD40 activation [84]. This

is in agreement with another report demonstrating reduced cytokine expression in DCs from children with T1D and individuals at risk for disease development [85]. Altered DC phenotype and functions such as diminished expression levels of costimulatory molecules and impaired ability to stimulate autologous T cells were seen in cytokine-induced DCs from prediabetic individuals [86], implying that innate alterations may be linked with early events leading to disease onset [86]. Work done by Allen and coworkers indicates that plasmacytoid DCs (pDCs) from patients with T1D have enhanced ability to process and present islet autoantigens to CD4 lymphocytes in the presence of sera containing autoantibodies of the relevant specificity [87]. The authors speculated that the mechanism of diabetes may involve a synergy between the proinflammatory capacity of pDCs and islet cell autoantibodies. There are reports linking IL-1 cytokine family members, including IL-1β, IL-1R1, and IL-1R2 with diabetes [88], and these data are consistent with previous evidence demonstrating the presence of elevated levels of various proinflammatory cytokines and chemokines, including IL-1α, IL-1β, IL-6, CXCL-10, and IFN-γ in sera from patients with newly diagnosed diabetes [89–93].

Noteworthy is the observation that a mutation in differentiation-associated gene-5 (MDA-5), an intracellular molecule involved in recognizing double-stranded RNA, was recently linked with human T1D [94]. It is presently unknown if this genetic alteration influences the ability of MDA-5 from patients or genetically susceptible individuals to sense viruses.

Overall, the data support the notion that innate immune alterations exist in T1D; however, whether and how these aberrations are linked with the mechanism initiating diabetes remain to be seen. Because hyperglycemia and insulin treatments, can by themselves, alter innate immune functions [95, 96], further studies will have to be conduced in subjects at risk for diabetes prior to hyperglycemia to elucidate the role of the innate immune system in T1D.

25.6 TLR Pathways and T1D in the Rat

25.6.1 The BB and LEW1.WR1 Rat Models

There are two inbred strains of BB rats, the diabetes-prone BB (BBDP) develops spontaneous diabetes and the diabetes-resistant BB (BBDR) in which diabetes is inducible [24, 97]. The BBDP is severely deficient of T lymphocytes due to a mutation in the gene encoding the mitochondrial membrane protein Ian4, a defect that results in a decrease in the peripheral T-cell life span [98]. BBDR rats were derived from BBDP forebears by selection for the absence of disease [99]. Like BBDP, BBDR rats express *RT1*^{u} MHC haplotype [24, 100]. In contrast to BBDP, BBDR rats have normal levels and function of peripheral $CD4^{+}$ and $CD8^{+}$ T cells [24, 100]. Spontaneous diabetes does not occur in these animals in viral antibody-free environment [101]; however, islet infiltration, diabetes, and severe ketosis develop

in ~100% of animals following a treatment with the TLR3 ligand Poly(I:C) plus depletion of regulatory ART2$^+$ T cells ([Table 25.1 and Ref. [101]), or upon infection with Kilham rat virus (KRV) [102]. The disease in the BBDR rat is thought to be immune mediated as evident by the ability of lymph node cells from diabetic animals to transfer disease to nondiabetic recipients [28].

Another rat model suitable for addressing the role of environmental factors in diabetes is the LEW1.WR1 rat. The MHC haplotype of this rat is *RT1.A^{u}B/D^{u}C^a*. Like the BBDR rat, the LEW1.WR1 rat has normal levels and function of CD4$^+$ and CD8$^+$ T lymphocytes [103]. There is evidence that ~2% of LEW1.WR1 rats develop spontaneous disease between the ages of 49 and 86 days in viral antibody-free facility [103]. As seen for the BBDR rat, infection of LEW1.WR1 rats with KRV leads to diabetes characterized by selective loss of islet β-cells, glycosuria, ketonuria, and polyuria (Table 25.1 and Refs. [101, 103]).

25.6.2 Kilham Rat Virus

Kilham Rat Virus is an environmentally ubiquitous rat virus that belongs to the family of *Parvoviridae*, a group of small single-stranded DNA viruses with an average genome size of 5 Kbp encapsidated by protein [104]. This virus family infects several species, including rodents [105] and humans [106]. KRV encodes three overlapping structural proteins, VP1, VP2, and VP3, and two overlapping nonstructural proteins, NS1 and NS2 [105]. H-1 parvovirus is a close homologue of KRV; the NS proteins of these viruses are 100% homologous, whereas the VP proteins are ~80% homologous. Infection of BBDR rats with H-1 virus with or without innate immune upregulation does not trigger diabetes (Table 25.1 and Ref. [107]). Infection with KRV leads to insulitis, islet destruction, and diabetes in BBDR and LEW1.WR1, but not other rat strains, and disease is virus specific, as sialodacryoadenitis [102], H-1, or vaccinia [108, 109] viruses are unable to induce diabetes (Table 25.1 and Refs. [101, 110]). KRV is lymphotropic, capable of infecting CD4$^+$ and CD8$^+$ T cells as well as B cells [111]. It is currently unclear whether and how this ability is linked with disease mechanisms.

The ability of KRV to induce diabetes is dependent on the expression of class I A^u and class II B/D^u [101, 110]. The RT1^u haplotype, however, is not sufficient for disease development, since Wistar Furth (WF) rats that express RT1^u are resistant to KRV-induced disease [101, 110]. Infection of PVG.R8 (A^{a}B/D^{u}C^u), PVG.R23 (A^{u}B/D^{a}C^a), and Lewis (A^{l}B/D^{l}C^l) rats with KRV does not lead to disease onset (Table 25.1 and Ref. [101]). Depletion of regulatory ART2.1$^+$ T cells combined with KRV infection increases the incidence of diabetes in BBDR and LEW1.WR1, but not PVG.RT1^u or other rat strains (Table 25.1 and Refs. [101, 110]). Disease triggered by KRV is T-cell-mediated, since diabetes can be blocked by a treatment with various antibodies against T cells [101, 110], and transfer of spleen cells from virus-infected BBDR rats to class II^u compatible rats adoptively transfers insulitis and diabetes [101, 110].

Table 25.1 TLR pathways and T1D in the rat

Strain	MHC (RT1 haplotype A, B/D, C)	Method of diabetes induction	TLR pathways involved in diabetes	Exogenously activated TLRs	Effect of exogenous TLR activation on diabetes	References
BBDR	$RT1^{u/u/u}$	KRV infection	TLR9	–	–	[109, 118]
		KRV infection plus innate immune upregulation	TLR9	TLR2, TLR3, TLR4, TLR7/8, and TLR9	Exacerbation	[109, 118]
		Innate immune upregulation	–	TLR3 (high dose)	Induction	[121]
		Innate immune upregulation plus ART2.1 T regulatory cell depletion	–	TLR3	Induction	[124]
BBDP	$RT1^{u/u/u}$	Spontaneous	?	–	–	[164]
		Innate immune upregulation	–	TLR3	Exacerbation	[120, 165]
		Innate immune upregulation	–	TLR3	Prevention (very low doses of Poly(I:C))	[119]
		RCMV	TLR9?	–	Disease exacerbation	[166]

Table 25.1 (continued)

Strain	MHC (RT1 haplotype A, B/D, C)	Method of diabetes induction	TLR pathways involved in diabetes	Exogenously activated TLRs	Effect of exogenous TLR activation on diabetes	References
LEW.1R1	$RT1^{u/u/a}$	Spontaneous in a small portion of animals in virus-free antibody environment	Unknown	–	–	[110, 125]
		KRV infection	TLR9?	–	Induction	[101]
		KRV infection plus TLR upregulation	TLR9?	TLR3	Exacerbation	[103]
PVG.$RT1^{u}$	$RT1^{u/u/u}$	Innate upregulation	–	TLR3	Induction	[101, 110]
WAG	$RT1^{u/u/u}$	Innate upregulation	–	TLR3	Induction	[110]

There are currently two members of the *Parvoviridae* family known to be pathogenic in humans [112], B19 and bocavirus [113] and there is no evidence linking them with human T1D. There are, however, reports that human parvovirus B19 is associated with other inflammatory autoimmune diseases, such as acute myocarditis [114, 115], rheumatoid arthritis [116], systemic lupus erythematosus [117], Sjögren's syndrome, and a series of other autoimmune disorders [116]. Infection with B19 was linked with the appearance of elevated levels of autoantibodies against nuclear antigens and double-stranded DNA [116].

25.6.3 TLRs and T1D

As discussed earlier, infection of BBDR and LEW1.WR1 rats with KRV leads to diabetes in a portion of infected animals (Table 25.1, unpublished observations, and Refs. [103, 109]). Several lines of evidence implicate TLR signaling pathways in the mechanism whereby KRV induces diabetes [109]). KRV acts as a TLR9 agonist and induces innate immune responses in vitro in plasmacytoid DCs (pDCs) but not myeloid DCs (mDCs) and B lymphocytes, and in vivo in the spleen and pancreatic lymph nodes [109, 118]. In addition, injecting BBDR and LEW1.WR1 rats with purified agonists of TLRs or heat-killed *Escherichia coli* and *Staphylococcus aureus* synergizes with KRV to increase diabetes incidence rates from 25 to 40% in animals treated with virus only to ~90–100% in animals pretreated with TLR agonists such as Poly(I:C) and CpG DNA (Table 25.1, unpublished observations, and Refs. [103, 109]). Injecting TLR agonists only at doses that induce diabetes when combined with KRV does not result in autoimmune diabetes, underlining the importance of virus infection in disease induction. Virus infection and TLR-induced innate upregulation induces diabetes in rat strains other than BBDR and LEW1.WR1 rats and susceptibility to diabetes is dependent on the expression of MHC Class II^u (Table 25.1 and Ref. 110]). Finally, injecting BBDR rats with Poly(I:C) induces diabetes by virus titers that by themselves are nondiabetogenic [109], providing further support to the notion that upregulation of the innate immune system, under some circumstances, can increase disease penetrance.

TLR upregulation can also block diabetes, as administration of Poly(I:C) at very low doses to BBDP rats prevents disease via mechanisms that may be linked with upregulation of regulatory T cells (Table 25.1 and Ref. [119]). High Poly(I:C) doses, however, exacerbate disease in BBDP rats (Table 25.1 and Refs. [120, 121]). Of note is the finding that infection of BBDP but not BBDR rats with rat cytomegalovirus (RCMV) accelerates the spontaneous disease, suggesting that RCMV enhances but does not initiate proinflammatory responses in pancreatic islets (Table 25.1 and Ref. [102]).

ART2 is an extracellular mono-ADP-ribosyltransferases expressed on mature peripheral T cells, but not by thymocytes or recent thymic migrants in the rat [122]. Injecting ART2+ T cells into rats susceptible to diabetes can block disease development [123], whereas depletion of ART2+ T cells with a specific antibody leads

to diabetes onset [124]. These findings led to the notion that ART2 is a marker of regulatory T cells. Indeed, TLR upregulation combined with ART2.1 depletion dramatically increases the incidence of diabetes in BBDR and LEW1.WR1 rats, but not PVG.RT1^{u} or other rats. It is hypothesized that the latter rats do not develop diabetes due to the presence of low frequency of autoreactive T cells (Table 25.1 and Ref. [101]).

TLR upregulation leads to diabetes in rat strains that are not susceptible to spontaneous or virus-induced diabetes [110, 125]). For example, injecting high doses of Poly(I:C) induces diabetes in WF, WAG, and PVG rats (Table 25.1 and Refs. [110, 125]), rat strains that express Class II^{u} molecules, but not in animals that express Class II^{a} or Class II^{c} [110], findings that are consistent with the key role that Class II antigens play in conferring susceptibility to human diabetes [23, 126]. Among mechanisms hypothesized to be involved in diabetes induced by Poly(I:C) are upregulation of macrophages and CD8^{+} T cells and increased expression levels of Class I molecules in pancreatic islets [110], as reported for spontaneous disease in BBDP and virus-induced disease in BBDR rats [24, 97, 99, 127]. The disease may also be linked to elevated expression of endothelial ICAM-1 and VCAM-1 and infiltration of macrophages and T cells into the pancreatic interstitium [110].

25.6.4 Inflammation and T1D

We recently proposed that proinflammatory responses induced by KRV in BBDR rats are linked with diabetes mechanisms [109]. This is compatible with the finding that blocking inflammation with chloroquine, an immunosuppressant agent, correlates with reduced diabetes incidence in the BBDR rat [118]. Using a DNA microarray approach, we found that KRV, but not the homologous H-1 parvovirus, induces profound alterations in the global gene expression profile in pancreatic lymph nodes, leading to an increase in the expression of 569 genes (our unpublished observations). It was evident that KRV induces transcripts for a vast array of proinflammatory chemokines and cytokines, including CXCL-9, CXCL-10, CXCL-11, IL-1α, IL-1β, IL-6, the p40 subunit of IL-12 and IL-23, and IL-18, and genes associated with interferon production and signaling, such as IRF7, STAT-1, and STAT-2 (our unpublished observations). Ex vivo and in vitro studies indicate that KRV upregulates proinflammatory cytokines and chemokines in B lymphocytes and Flt-3L-induced plasmacytoid DCs (pDCs). The exact role of the proinflammatory response in the mechanism of diabetes is not yet clear; however, it could be involved in the upregulation of autoreactive T lymphocytes [109].

We hypothesize that the mechanism of virus-induced diabetes is associated with upregulation of proinflammatory cytokines and chemokines in the microenvironment of pancreatic lymph nodes. This innate response in conjunction with Treg downmodulation induced by KRV early following infection [108] may lead to the recruitment and upregulation of islet-specific autoreactive T cells, resulting in islet β-cell destruction and diabetes [118].

25.7 TLR Pathways and T1D in the Mouse

25.7.1 Transgenic Mouse Models

The hypothesis that TLR pathways are involved in triggering autoimmune diabetes is supported by data from transgenic mouse models of T1D (Table 25.2). It was documented that upregulation of TLR3 and RLH pathways with Poly (I:C) results in diabetes in mice expressing B7.1 molecules in their pancreatic islets (B6/RIP-B7.1) via mechanisms linked with the upregulation of APCs and autoreactive T cells [128].

Activation of TLR pathways induces autoimmune diabetes in a transgenic mouse expressing an LCMV peptide in pancreatic islets (RIP-LCMV), an animal model of molecular mimicry and autoimmunity [129]. Immunizing these mice with the LCMV peptide does not lead to autoimmune diabetes; however, upregulation of TLR3 and TLR7, but not TLR9, leads to disease via mechanisms that appear to involve type I interferon and MHC Class I upregulation in pancreatic islets (Table 25.2 and Ref. [130]).

TLR3, TLR4, and TLR9 pathways are implicated in diabetes in transgenic mice expressing OVA in their islet β-cells (RIP-OVA). When these mice are injected with supraphysiological numbers of OVA-specific CD8 T cells, diabetes develops in a relatively small portion of animals [131]. However, the incidence of diabetes is increased and the number of CD8 T cells required for disease induction is reduced when mice are treated with agonists of TLR2, TLR3, TLR4, and TLR9. When a physiological number of OVA-specific CD8 cells are injected to RIP-OVA mice, TLR activation is insufficient for disease development unless OVA-specific CD4 T cells are provided [131], suggesting that specific CD4 help is a key component in the mechanism leading to TLR-induced diabetes in this mouse model [131].

25.7.2 The NOD Mouse

Microbial infections and upregulation of TLR signaling pathways in the NOD mouse are linked with diabetes prevention [132–141]. Administering NOD mice with viruses [134, 135, 142], or purified agonists of TLRs [136, 138, 140] leads to disease prevention via TLR pathways induced in dendritic cells (Table 25.2 and Ref. [143]). Only infection with coxsackie B viruses [144], a family of single-stranded DNA viruses, induces diabetes in NOD mice with already established insulitis [2, 52, 72] via mechanisms that may involve TLR4 [145] and/or TLR 7/8 pathways [144].

Interactions of intestinal microbes with the innate immune system were recently hypothesized to play a beneficial role in the susceptibility of NOD mice to T1D [146]. Work done by Wen and coworkers showed that in contrast to wild-type NOD mice, MyD88-deficient NOD mice do not develop diabetes when housed in specific pathogen-free conditions, and this effect is not seen in mice lacking TLR2, TLR3, and TLR4 (Table 25.2 and Ref. [146]). The authors also observed that in the

Table 25.2 TLR pathways and T1D in the mouse

Strain	Transgene and site of expression	Method of disease induction	Exogenously activated TLRs	Effect of TLR activation or disruption on diabetes	References
NOD	–	Spontaneous	–	–	[167]
	–	–	TLR3, TLR4, TLR9	Prevention	[136, 138, 140, 168]
	–	Coxcackie B Virus infection	TLR4, 7/8	Acceleration when administered to mice with insulitis	[144, 145]
NOD *TLR2–/–*	–	–	–	Prevention	[147]
NOD *TLR2–/–*	–	–	–	No effect	[146]
NOD *TLR3–/–*; NOD TLR4–/–	–	–	–	No effect	[146]
NOD *MyD88 –/–*(in SPF environment)	–	–	–	Prevention	[146]
Germ-free NOD *MyD88 –/–*	–	–	–	Robust diabetes	[146]
Germ-free NOD *MyD88–/–* colonized with commensal microbes	–	–	–	Prevention	[146]

Table 25.2 (continued)

Strain	Transgene and site of expression	Method of disease induction	Exogenously activated TLRs	Effect of TLR activation or disruption on diabetes	References
C57BL/6	–	Streptozotocin-induced islet injury	–	–	[147]
C57BL/6 *MyD88 –/–*	–	Streptozotocin-induced islet injury	–	Prevention	[147]
C57BL/6 *TLR2 –/–*	–	Streptozotocin-induced islet injury	–	Prevention	[147]
C57BL/6 *TLR4 –/–*		Streptozotocin-induced islet injury	–	No effect	[147]
B6/RIP-B7.1	B7.1 molecules in pancreatic islets	Insulin immunization	TLR3	Induction	[128]
LCMV RIP-GP	LCMV peptide in pancreatic islets	LCMV peptide immunization	TLR3, TLR7	Induction	[130]
RIP-mOVA	OVA in pancreatic islets	Transfer of OVA-specific CD4 and CD8 T cells	TLR2, TLR3, TLR4, TLR9	Induction	[131]

MyD88 knockout mice, the pancreatic lymph nodes have fewer numbers of diabetogenic T cells as compared with MyD88-sufficient NOD mice, and these T cells show diminished proliferation and interferon-γ production following activation with islet-derived peptides [146]. The protective effect observed in MyD88 knockout mice could be a consequence of alterations in the immune response to intestinal bacteria, since MyD88-deficient NOD mice treated with antibiotics have increased incidence of diabetes. In addition, MyD88-deficient NOD mice housed in germ-free conditions and therefore lack gut bacteria have increased levels of insulitis and a higher incidence of diabetes as compared with MyD88-deficient NOD mice with normal gut bacteria. Colonization of the germ-free mice with normal gut bacteria reduces the incidence of diabetes, implying that normal commensal flora protects from diabetes onset. It was also evident that the flora from the gut of MyD88-deficient mice was different than that seen in MyD88-sufficient NOD mice, implying that MyD88 deficiency changes the composition of the gut microbiota. Exposure of the germ-free MyD88-deficient NOD mice to the "protective" microbiota from the gut of specific pathogen-free MyD88-deficient NOD mice decreased pancreatic islet infiltration by lymphocytes. These data indicate that commensal gut bacteria have a protective role against type 1 diabetes development that is not MyD88 dependent [146].

25.7.3 Streptozotocin-Induced Diabetes

Host-derived TLR ligands were implicated in the mechanism by which streptozotocin induces diabetes in disease susceptible mice. It was demonstrated that apoptotic insulinoma cells undergoing secondary necrosis, but not intact apoptotic cells, upregulate proinflammatory responses in macrophages and DCs in vitro via the TLR2 signaling pathway (Table 25.2 and Ref. [147]). Diabetes induction in mice treated with streptozotocin appears to be also associated with TLR2 pathways, since disease is blocked in TLR2 and Myd88-deficient mice [147]. Support for the potential role of TLR2 pathways in diabetes is provided by the observation that NOD mice deficient of TLR2 are protected from diabetes [147], a finding that is not consistent with a recent report [146]. It could be argued that TLR2 pathways recognize endogenous ligands released by necrotic islet β-cells leading to the activation of islet-specific T cells and diabetes [147]. The hypothesis that host-derived ligands are involved in autoimmune diabetes is consistent with previous data implicating endogenous TLR4 and TLR9 ligands in arthritis [148] and EAE [149], respectively.

25.8 Type I IFNs and T1D

Type I IFNs are produced following microbial infection via mechanisms involving upregulation of a number of TLR pathways such as TLR3, TLR4, TLR7/8, and TLR9, as well as other intracellular TLR-independent sensors of viral infections [150]. Data obtained from humans implicate IFN-α in mediating diabetes. There are reports that IFN-α treatment for chronic hepatitis C virus infection results in the

Table 25.3 Type I IFNs and T1D in animal models

Animal model	Method of type I IFN induction or neutralization	Effect on diabetes	Mechanisms involved in diabetes induction or prevention	References
BBDP	IFN-α administration	Downmodulation	Inhibition of inflammation	[152]
NOD	IFN-α ingestion (in mice with established insulitis)	Downmodulation	Inhibition of inflammation, upregulation of regulatory cells	[153]
	IFNα/β receptor blockade	Prevention	Induction of IL-4 and IL-10; increase in immature DCs in pancreatic lymph nodes	[154]
	IRF1–/–	Prevention	Upregulation of Th2 type responses	[156]
NOD/RIP-IFN-β	IFN-β expressed in pancreatic islets	Acceleration	MHC Class I hyper-expression in pancreatic islets	[155]
CD1/RIP-IFN-α and C57BL/6/RIP-IFN-α	IFN-α expressed in pancreatic islets	Induction	Recruitment of leukocytes to pancreatic islets	[157, 158]
C57BL/6/RIP-B7.1	IFN-α administration	Induction	?	[159]

development of various autoimmune disorders including diabetes [151]. Evidence from rat and mouse models of T1D demonstrates that under some circumstances, type I IFN pathways can mediate and, conversely, prevent diabetes onset. In the BBDP rat, treatment with IFN-α or very low doses of Poly(I:C), a potent inducer of type I IFNs, blocks diabetes by interfering with pancreatic inflammation (Table 25.3 and Ref. [152]). In NOD mice with established insulitis, ingestion of IFN-α down-modulates autoimmunity by upregulating regulatory T cells [153]. Injecting BBDR rats as well as other rat strains with Poly(I:C), a known inducer of type I IFNs, induces or exacerbates diabetes (Table 25.1 and Ref. [109]). In the NOD mouse, blocking IFN-α pathways in young animals protects from disease by upregulating IL-4 and IL-10 and increasing the frequency of immature DCs in pancreatic lymph nodes (Table 25.3 and Ref. [154]). The expression of IFN-β in pancreatic islets of NOD mice accelerates disease course [155], and disrupting IRF-1 pathways in this mouse prevents autoimmunity [156]. In addition, transgene expression of type I IFNs in pancreatic islets in mouse strains that do not develop spontaneous disease induces autoimmune diabetes [157, 158]. Finally, injecting Poly (I:C) or IFN-α to transgenic B6/RIP-B7.1 leads to diabetes, and neutralizing type I IFNs in these mice following Poly(I:C) treatment prevents diabetes (Table 25.3 and Ref. [159]). Type I IFNs could promote autoimmunity through mechanisms that involve upregulation of both the innate and the adaptive immune systems [150]. Type I IFNs stimulate cytotoxic activity on a variety of cells of the immune system, including T cells and NK cells [160]. They also upregulate DCs, leading to increased expression of MHC class I and II, chemokines, and chemokine receptors, and costimulatory molecules [150]. In addition, they induce upregulation of IFN-γ production by DCs and T-cells, thus facilitating the induction of a Th1 type response [161–163].

25.9 Summary

Rat and mouse models of T1D have proven to be a promising experimental tool in studying the role of the environment in diabetes mechanisms. Data obtained thus far support the notion that interactions between microbes and innate immune genes play a key role in the upregulation of islet-reactive T cells. Future studies will be required to identify innate immune pathways directly involved in the course of diabetes and mechanisms whereby they lead to islet cell destruction. Proinflammatory molecules identified to be linked with initiating autoimmunity against islet β-cells may be targeted to prevent disease in genetically susceptible individuals.

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Chapter 26
Prevention of β-Cell Destruction in Autoimmune Diabetes: Current Approaches and Future Prospects

Saikiran K. Sedimbi and Carani B. Sanjeevi

Abstract Type 1 diabetes (T1D) is an autoimmune disease resulting from the destruction of pancreatic β-cells. The main aim of treatment should be to prevent β-cell destruction and preserve existing β-cells in individuals with progressive autoimmunity. This can be achieved in several ways and in this chapter the authors have reviewed recent approaches that are currently being tested in animal models and human T1D patients under the following categories: i) antigen based therapy, ii) antibody-based therapy iii) other forms of therapy and iv) failed therapies.

Keywords Type 1 Diabetes · β-cell · Antibody-based therapy · Antigen-based therapy

Type 1 diabetes mellitus (T1D) results from autoimmune destruction of pancreatic β-cells. Autoimmunity is thought to occur in genetically predisposed individuals after exposure to one or more environmental triggers such as dietary factors, viruses, etc. Infiltrating T cells, B cells, and NK cells initiate the autoimmune response and progressively destroy the insulin-producing β-cells. The entire process of β-cell destruction can take anywhere from a few months to a few years, finally resulting in hyperglycemia. HLA-DQ8 and HLA-DQ2 have been associated with high risk to T1D and 89% of newly diagnosed children from Sweden are positive for these HLA alleles [50]. Association of these HLA alleles with T1D has been shown to be inversely proportional to age [61].

Latent autoimmune diabetes in adults (LADA) is a slowly progressive form of autoimmune diabetes. Patients initially diagnosed as classical type 2 diabetics are identified as LADA according to the following criteria of Immunology of Diabetes Society: (i) adult age (>30 years) at onset of diabetes, (ii) the presence of circulating islet autoantibodies, and (iii) lack of a requirement for insulin for at least 6 months after diagnosis.

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Considering this sequence of events, preventing β-cell destruction is vital to preserving the residual β-cells in individuals with progressive β-cell loss and those at risk of developing T1D and LADA (referred to as autoimmune diabetes). Antigen-specific and nonspecific immune therapies that aim to reduce islet cell autoimmunity are in different phases of clinical development. Recent insights into the autoimmune process are elucidating the etiology of autoimmune diabetes, conceivably identifying therapeutic targets. Stand alone and/or combinational therapies that reduce autoimmunity in islets, regenerate β-cells, and restore insulin secretion appear to be the future of autoimmune diabetes intervention. Aggressive autoimmunity appears significantly earlier than overt disease and therefore pursuing therapeutic strategies before disease presentation should be beneficial for susceptible patients. Early intervention before the autoimmunity is initiated is the best. Second best is intervention after autoimmunity is initiated but before the disease becomes insulin requiring.

Preservation of β-cells is advantageous in autoimmune diabetes as it may significantly reduce both short- and long-term complications (hypoglycemia, retinopathies, etc.) while at the same time stabilize blood glucose levels and improve quality of life. To this end, pharmaceuticals are being developed using the available knowledge to generate target antigen-specific immune response. Ideally, tolerance induction would be a short time course, leading to a long-lasting tolerant stage, without debilitating the capability of the immune system to mount effective immune response against invading pathogens. In the following sections, the authors have discussed recent strategies employed to prevent β-cell destruction and preserve residual β-cells in autoimmune diabetic patients in the following categories: (i) antigen-based therapy, (ii) antibody-based therapy, (iii) other forms of therapy, and (iv) failed therapies in the past (summarized in Table 26.1).

Table 26.1 List of therapeutics used in prevention of β-cell death in autoimmune diabetes

S. No	Therapy	References
1.	Antigen-based therapies	
	(i) Alum-formulated GAD65	[29, 30]
	(ii) Insulin	[33–36]
	(iii) Insulin–Cholera toxin B conjugates	[31]
	(iv) DiaPeP277	[24–26]
2.	Antibody-based therapies	
	(i) Anti-CD3 monoclonal antibodies	[7]
	(ii) Anti-CD20 monoclonal antibodies	[21]
3.	DNA vaccination	
	(i) GAD	[49, 50]
	(ii) Microsphere-based vaccine	[39]
4.	Cyclosporin	[41]
5.	Vitamin D	[51]
6.	Nicotinamide	[43, 44]
7.	BCG	[45]
8.	Anti-inflammatory agents	[54–56]

26.1 Antigen-Based Therapy

26.1.1 GAD65

Glutamic acid decarboxylase isoform 65 (GAD65) is a major autoantigen in T1D. Studies in NOD mouse have shown that destruction of islet β-cells was associated with T cells recognizing GAD65. Kaufman et al. showed that GAD65 effectively prevents autoimmune β-cell destruction and reduce and delay the development of spontaneous diabetes [28].

Diamyd evaluated this by using alum-formulated human recombinant GAD65 in LADA patients. They selected diabetic patients of both sexes aged 30–70 years, diagnosed with T2DM and positive for GAD65 antibodies. These patients were treated with either diet or oral tablets. A total of 34 patients and 13 controls were tested with 4, 20, 100, and 500 μg dose. This was injected subcutaneously twice but 4 weeks apart. No serious adverse effects were reported. In the follow-up, the C-peptide level (both fasting and stimulated) was significantly elevated in the group receiving 20 μg dose compared to placebo. Likewise the HBA1C and mean glucose levels were significantly lowered in the 20 μg dose compared to placebo. The $CD4^+CD25^+$ T cells which reflect the increase in regulatory T cells associated with nondestructive response to β-cell were elevated in the 20 μg dose but not in other doses. All these findings were relevant even after a follow-up period of 24 months (www.diamyd.com, 29). It is thought that the prevention of β-cell destruction and β-cell recovery is due to shifting of immune response from destructive to nondestructive which is mediated by the Diamyd GAD65 vaccine.

Subsequent phase IIb trials in T1D patients with alum-formulated GAD showed significant preservation of β-cell function 30 months after the first 20 μg dose administrations. It also induced antigen-specific T-cell population, cytokines involved in regulation of immune system, and a long-lasting B cell memory, suggesting that modulation of general immune responses to GAD can be helpful in preserving residual β-cells [30]. Large-scale phase III clinical trials are being conducted in Europe and the United States to confirm these initial findings.

Alum-formulated GAD is the only antigen-based vaccine candidate which has been shown to be effective in both T1D and LADA. LADA is often misdiagnosed as type 2 diabetes and treated accordingly. This may lead to additional stress on an already declining β-cell mass (due to autoimmune destruction). Hence diagnosis and treatment of LADA are vital.

26.1.2 Oral Tolerance

Oral tolerance is a term used to describe the tolerance, which can be induced by the exogenous administration of antigen to the peripheral immune system via the gut. It is a form of antigen-driven peripheral tolerance and appears to involve two main mechanisms, which are in part dependent on antigen dose. The tolerance induced by

lower doses of orally administered antigen appears to be mediated predominantly by active suppression whereas higher doses tend to induce clonal deletion. The active suppression of low doses of oral antigen appears to be mediated by the oral antigen-generating regulatory T cells that migrate to lymphoid organs and to organs expressing the antigen administered orally and confer suppression via the secretion of down-regulatory cytokines including IL-4, IL-10, and TGF-β. Th2-type immune responses are preferentially generated by antigen presentation via the gut, and consequentially oral tolerance and resultant protection against autoimmune disease are in some ways analogous to the Th1/Th2 paradigm.

26.1.3 Insulin and Cholera Toxin

A mechanism of tolerance induction that is currently showing promise is oral insulin conjugated to β-subunit of the cholera toxin (CTB) [31]. It has been shown recently that oral administration of microgram amounts of antigen coupled to the CTB subunit can effectively suppress systemic T-cell reactivity in animal models. Bergerot et al. report that feeding small amounts (2–20 μg) of human insulin conjugated to CTB can effectively suppress β-cell destruction and clinical diabetes in adult nonobese diabetic (NOD) mice [31]. The protective effect could be transferred by T cells from CTB-insulin-treated animals and was associated with reduced lesions of insulitis. Furthermore, adoptive cotransfer experiments show concomitant reduction in islet cell infiltration. These results suggest that protection against autoimmune diabetes can be achieved by feeding minute amounts of a pancreas islet cell autoantigen linked to CTB and appears to involve the selective migration and retention of protective T cells into lymphoid tissues draining the site of organ injury.

CTB subunit carries the insulin to the intestine and helps in the transfer of the insulin molecule across the intestinal barrier. The CTB conjugation also helps in the reduction of the dosage of insulin that can be administered orally without causing hypoglycemia. Further, this approach has also been tried successfully by intranasal administration. Both approaches have prevented the development of diabetes in the NOD mouse model of the autoimmune disease. CTB-insulin β-chain fusion protein produced in silk worms has been shown to suppress insulitis in NOD mice [32].

26.1.4 Insulin

The Diabetes prevention trial 1 (DPT-1) was performed to access the capability of insulin administered as injections to prevent T1D. The study however failed to demonstrate any beneficial preventive outcome [33]. Another approach in the DPT-1 was to administer insulin orally in first-degree relatives of T1D patients; however, the treatment failed to delay or prevent T1D [34]. Administration of nasal insulin in children carrying high-risk HLA (for T1D) soon after detection of autoantibodies failed to prevent or delay the disease [35]. The Pre-POINT (Primary Oral/intranasal

Insulin Trial) is a dose-finding safety and immune efficacy pilot study aiming primary prevention in children genetically at risk to T1D, using oral or intranasal insulin [36].

26.1.5 DiaPeP277

Heat shock protein 60 (hsp60) is a 60 kDa protein which is one of the self-antigens in T1D. p277 (DiaPeP277) is a 24 amino acid peptide analog which comprises 24 residues, 437–460 (www.develogen. com). Administration of DiaPep277 in NOD mice arrested the disease [24]. A randomized double-blind phase II trial using DiaPeP277 in human subjects with newly onset disease (<6 months) resulted in preservation of the endogenous insulin production compared to the placebo group [25]. In a follow-up study [26] the findings were reiterated. In both studies, immunomodulation was observed and associated with down-regulation of Th1 cells and upregulation of IL-10-producing T cells. The immune responses were antigen specific as T-cell responses to bacterial antigens remained unaffected. However the trials had a maximum follow-up up to 13 months in adults. However studies performed in children did not show any improvement in the preservation of β-cell function or metabolic control [27].

26.2 Antibody-Based Therapy

26.2.1 CD3 Antibodies

OKT3, the first monoclonal antibody specific for the CD3 T-cell epitope (Orthoclone OKT3, Ortho Biotech), was used to reverse ongoing renal allograft rejection in humans [1]. Experiments in the early 90s in NOD mice demonstrated that hamster-derived anti-CD3 monoclonal antibodies reversed diabetes in hyperglycemic mice [2, 3]. These series of experiments demonstrated several unique features of the antibody therapy. First, continuous immune suppression was not required, second the ability of the antibody to reverse disease after hyperglycemia has occurred was demonstrated. Treated NOD mice were resistant to transfer of diabetes by diabetogenic spleen cells, implying the involvement of active immune regulation preventing diabetes [3]. When this approach was translated to the humans, adverse events were induced due to excessive release of cytokines. Hence modification of the antihuman CD3 OKT3 monoclonal antibodies was thought to be the next alternative. Fc-mutated (Fc-nonbinding) monoclonal CD3 antibodies were engineered and these were found to be less mitogenic, but were equally tolerogenic compared to functional Fc CD3 antibodies [2, 3]. Two humanized CD3 antibodies are in different stages of clinical trials. They are the ChAglyCD3 antibody (www.tolerx.com), having a single mutation (Asn→Ala) at residue 297 in the Fc region that prevents glycosylation, derived from rat YTH 12.5 antibody [14],

and the hOKT3Ala-Ala antibody (www.macrogenics.com), having two mutations at residues 234 (Lue→Ala) and 235 (Lue→Ala) in the Fc region. This antibody is derived from OKT3 [15].

Promising Therapies:

- *Alum formulated GAD65:*Specific Modulation of long lasting immune response to β-cells GAD
- *Anti-CD3 antibodies:* Prevention of β-cell destruction by depletion of T cells
- *Anti-CD20 antibodies:* Prevention of β-cell destruction by depletion of B cells.
- *DiaPep277:* Immunomodulation and shift from Th1 response to a Th2 response.

The FcR nonbinding CD3 antibody (CD3 antibody with a mutated Fc portion) therapy was effective only if the immune response was primed and ongoing. Locally, they target autoreactive T cells and the strength of the T cell receptor (TCR)/CD3 is important in determining the efficacy. Thus it can be hypothesized that though CD3 is expressed on all T cells, CD3 antibodies mediate signaling depending on the functional stage of the target T cell whether it is naive or effector or memory. Administration of CD3 antibodies induces depletion of effector T cells in the target tissue and lymphoid organs. In pancreas draining lymph nodes, apoptosis is induced in effector T cells compared to regulatory T cells and resting T cells [4–6]. Apoptotic effector T cells are engulfed and digested by phagocytes (macrophages and immature dendritic cells (DC)). These phagocytes secrete large amounts of transforming growth factor (TGF-β) which creates a noninflammatory environment and also plays a major role in maturation of DCs. TGF-β production has been suggested and experimental data demonstrate that TGF-β is central to the tolerance induced by FcR nonbinding CD3 antibodies. TGF-β-neutralizing antibodies are shown to completely neutralize the tolerogenicity induced by anti-CD3 therapy [7]. Local production of TGF-β has been shown to have the capability to convert a proinflammatory environment to a noninflammatory and tolerogenic one [8]. A high concentration of TGF-β also promotes upregulation of inhibitory receptor ligands (programmed cell death ligand 1, ICOS ligand) and down-regulation of MHC and costimulatory molecules on antigen-presenting cells [8, 9]. This in turn induces the induction or expansion of $CD4^+CD25^+FOXP3^+$ T regulatory cells [9]. From the available experimental data it has been proposed that the FcR nonbinding CD3 antibody treatment triggers a massive local production of TGF-β, by phagocytes engulfing activated effector T cells [10].

26.2.2 Improving the Existing CD3 Antibody Therapy

CD3 monoclonal antibody therapy induces tolerance and effector T-cell deactivation. However this is not complete, as some degree of T-cell activation is still observed in patients treated [11, 12]. These can be avoided by developing CD3 antibodies devoid of the Fc portion, which can prevent cytokine release completely.

Administration of drugs which promote β-cell survival and growth (such as exendin-4, glucagon-like peptide-1 (GLP-1), etc.) may increase the β-cell growth and replication in the 'tolerant' environment. In NOD mice combination of exendin-4 and CD3 monoclonal antibodies let to effective reversal or the disease with increased insulin content of the β-cell as compared with individual exendin-4 or CD3 monoclonal antibody treatment [13]. Frequent side effects because of interferences with the T-cell population in proximity with treatment periods and recurrent autoimmunity might be a problem in CD3 antibody-treated individuals. Repetitive treatment can be a possible way out in such a situation but formation of anti-idiotypic antibodies should be taken into consideration.

26.2.3 CD20 Antibodies

B cells constitute ~60–70% of the immune cells infiltrating the pancreatic islets [16]. Until recently B cells were thought to play an important role in priming T cells [17]. However a recent study showed for the first time that B cells promote the survival of $CD8^+$ T cells in the islets and thereby promote the disease [18]. CD20 is a cell surface marker expressed on all mature B cells. Rituximab (Roche/Genentech), a humanized anti-CD20 monoclonal antibody (CD20 mAb), has been shown to successfully deplete human B cells from peripheral circulation via mechanisms involving Fc and complement-mediated cytotoxicity and probably via proapoptotic signals [19, 20]. Given the important role of B cells in maintaining the T1D, depleting B cells is a very interesting therapeutic option. Transgenic NOD, mice engineered to express human CD20 on B cells, when treated with a single dose of CD20 mAb gave interesting results [21, 22]. First, treatment of mice in early stage of the disease (insulitis) prevented or delayed the progression to disease. Second, clinical hyperglycemia could be reversed in over one-third of the experimental animals. Third, B cell levels were restored to predepletion levels within 3 months of treatment, but the progression to T1D was delayed almost indefinitely. TrailNet is currently testing the efficacy of rituximab (CD20 mAb) in a new-onset trial involving 4-week course treatment with the antibody [23].

26.3 Other Forms of Therapy

26.3.1 DNA Vaccination

DNA vaccination involves administration of a gene that encodes the target antigen, instead of the antigen as in classical vaccination. Variety of vectors can be used to transfer the target gene as DNA or RNA, along with genes encoding immunomodulatory molecules. Several studies have been performed using administration of plasmids encoding antigens such as insulin B chain, GAD, immunoglobulin G–Fc fusion constructs in animal models. However plasmids carry unmethylated CpG

motifs (ISS: immunostimulatory sequences) which activate the innate immune system. Therefore DNA vaccination against T1D should block or overcome the effect of such stimulatory elements. DNA vaccine hold good promise in treatment of autoimmune diseases as they have been used, in experimental models, to direct the immune response toward a Th1 or a Th2 response [48].

26.3.1.1 DNA Vaccination with GAD65

Intramuscular injections of plasmid containing GAD65 fused with IgG–Fc and IL-4 were reported to generate a GAD65-specific Th2 response, protecting NOD mice from developing T1D [49]. A study performed to evaluate two different modes of delivery of a plasmid coding for GAD65 reported the elicitation of IL-4 secreting T-cell response. Two methods of plasmid delivery, intramuscular and a novel gene gun method, were tested in this study. Intramuscular injections fail to stop the ongoing β-cell autoimmunity, whereas the gene gun method was successful in eliciting immunomodulation, significantly delaying the disease onset in NOD mice [50].

26.3.1.2 Microsphere-Based Vaccine

Microparticulate carriers have the capability to shape the functional phenotype of dendritic cells (DC) [37, 38]. A nucleic acid-based vaccine using antisense oligonucleotides coated on microspheres, directed against CD40, CD80, and CD86 (costimulatory molecules important in DC maturation), has been shown to prevent T1D in NOD mice as well as reverse new-onset disease [39]. Microspheres administered are taken up by DCs by phagocytosis. Inflammation in pancreatic islets associated with β-cell apoptosis is suggested to drive the antisense oligonucleotide loaded DCs to acquire the β-cell antigen(s). This is followed by accumulation of these DCs in the pancreatic lymph nodes, where they are hypothesized to interact with regulatory T cells inducing a β-cell-specific immune hyporesponsiveness or functional tolerance to β-cell antigens [40]. The detailed mode of action of the microsphere-based vaccine is yet to be established and clinical trials in human subjects will decide the efficacy of this approach in prevention of T1D.

26.3.2 Use of Anti-inflammatory Agents

Use of anti-inflammatory drugs such as aspirin [54], statins [55] and glitazone [56] has been shown to be beneficial in type 2 diabetes. These drugs have been shown to have anti-inflammatory effect either by affecting the signaling pathways (such as NFkB signaling) or cytokines involved in inflammation. Such drugs can be vital in bringing down the overall islet inflammation and thereby creating a better islet environment which can respond to other forms of treatment.

26.3.3 Vitamin D

Vitamin D has been shown to suppress proinflammatory responses by suppressing enhanced activity of immune cells taking part in autoimmune processes. In NOD mice vitamin D has been shown to prevent autoimmune diabetes [51]. Supplementation of vitamin D has been shown to be protective in children against T1D. High dosage and the timing of the dose have also been shown to play a role. A randomized open label, pilot trial is currently under way (NCT00141986), where increased dose of vitamin D (2,000 IU/day instead of the current practice of 400 IU/day) is administered to children genetically at risk of developing T1D.

26.4 Past Trials

26.4.1 Cyclosporin

Cyclosporin was one of the first immunosuppressive drugs used in treatment of T1D, which could delay the onset of the disease [41]. However cyclosporin achieved immunosuppression by targeting intracellular processes, which is nonspecific and unrelated to autoantigens involved in the disease. Withdrawal of the treatment resulted in invariable recurrence of the pathogenic immune response. Considering the nephrotoxic potential of the drug, it was not a choice of long-term treatment and therefore it was not considered for therapy [42].

26.4.2 Nicotinamide

Nicotinamide Diabetes Intervention Trial (ENDIT) tested the efficacy of nicotinamide in preventing diabetes in human subjects. Previous studies in animal models demonstrated that the administration of nicotinamide can prevent T1D [43]. Nicotinamide is speculated to confer protection by inhibiting DNA repair enzyme poly-ADP-ribose polymerase and prevent the depletion of β-cell NAD depletion. However in the ENDIT, nicotinamide treatment did not result in successful prevention of T1D [44].

26.4.3 BCG

Bacille Calmette-Guerin (BCG) vaccination has been proposed as an adjuvant therapy to prevent T1D. A study reported that administration of BCG vaccination soon after T1D onset preserves β-cell function [45]. However this was not the case in the trials that followed. BCG vaccination could not prevent the development of T1D in children genetically at risk [46].

26.5 Ongoing Prediction Studies

Several international collaborative efforts are under way. These studies will identify potential population/risk groups who would benefit from various therapies for prevention of β-cell death.

Potential therapies aiming at prevention of β-cell death would directly benefit patients suffering from autoimmune diabetes (T1D/LADA). Successful therapies can also benefit prediabetics, first-degree relatives of T1D patients, and individuals at risk of developing autoimmune diabetes.

TEDDY: The environmental determinants of diabetes in the young (TEDDY) study are an effort to screen more than 360,000 children around the world to the environmental factors that might play a role in T1D pathogenesis [47]. Several genome-wide association scans have been completed and are under way, with an aim to identify the T1D risk loci across the human genome. Identification of environmental and genetic factors involved in the etiology of T1D can broaden the scope of therapeutic interventions.

Trail Net: It is an international consortium of clinical research centers working toward achieving prevention of T1D [23].

TRIGR: Trial to reduce IDDM in the genetically at risk (TRIGR) study is another collaborative effort, which aims at testing the hypothesis that weaning to an extensively hydrolyzed infant formula will decrease the incidence of T1D in children who carry high-risk HLA and in those who have a first-degree relative with T1D [52]. Initial findings from TRIGR suggest that introduction of cow's milk at an early age in children with dysfunctional gut immune system might result in aberrant immune response, leading to T1D [53].

DAISY: The DAISY study (The Diabetes Autoimmunity Study in the Young) aims at elucidating the interaction between genes and the environment that can trigger T1D. Children who are genetically at risk or those who have a first-degree T1D relative are being studied and followed up.

BABY-DIAB: BABY-DIAB is [62] a prospective study conducted from birth among children of mothers with IDDM or gestational diabetes or fathers with IDDM to investigate the temporal sequence of antibody responses to islet cells (ICA), insulin (IAA), GAD (GADA), and the protein tyrosine phosphatase IA-2/ICA512 (IA-2A). A total of 78.6% of children (17,055 out of 21,700) born in the southeast of Sweden were entered in the *ABIS* (All babies in southeast Sweden) study with an aim to study environmental factors affecting the development of immune-mediated diseases in children, with special focus on T1D [63]. The *DIPP* (Diabetes Prediction and Prevention Project) was launched in 1994 in Finland. In the study, general population newborns are screened for increased genetic risk for type 1 diabetes in the University Hospitals of Turku, Tampere, and Oulu.

26.6 Future Directions

Intervention/prevention of β-cell destruction in T1D is the final goal resulting in good metabolic control of blood glucose. Balancing the risks and benefits in

intervention/prevention of T1D is very complicated. Individual response to a particular therapy might differ. Biomarkers which can identify individuals who would or would not respond to a particular therapy are the need of the hour. T1D is associated with end-organ complications. The number of adverse events in an individual undergoing a particular therapy might differ from another, depending upon the time and intensity of progress to end-organ complications. Therefore identification of those at risk becomes important while considering therapy.

Disease diagnosis is another important factor. T1D is usually diagnosed when the existing β-cells fail to meet the insulin needs of the body and thereby insufficient metabolic control. Earlier identification of existing autoimmunity is very crucial.

Research in the past few decades has highlighted many ways in which this can be achieved. Several promising candidates (such as alum-formulated GAD65 and anti-CD-3 antibodies) have also reached different stages of clinical trials. Alum-formulated GAD is being tested in several phase III clinical trials, including a trial in nine countries in Europe apart from Norway and a 4-year follow-up in Sweden (www.diamyd.com). It is interesting to note that although GAD alum and anti-CD3 seem to show similar efficacy the GAD-alum product has not been associated with any relevant side effects and moreover it is easy to administer. Considering the complex etiology of the disease, involving several susceptibility factors and immune cells, it is possible that multi-therapy, involving more than one therapeutic agent, may be of advantage. With the increasing insights into the etiology of the disease more and more targets are being identified for prevention/intervention.

26.6.1 Strategies on Islet Expansion

Nutrient ingestion stimulates the gastrointestinal tract to secrete incretin hormones to enhance glucose-dependent insulin secretion, thereby maintaining glucose homeostasis. The success of several therapies in reversing islet cell autoimmunity has led to the search of agents that enhance β-cell preservation or restoration. Incretins such as glucagon-like peptide-1 (GLP-1) or its mimetics can be used for treatment. One such analog is exendin-4. Exendin-4 is thought to act on β-cells by stimulating cellular proliferation, inhibiting apoptosis and aiding in recovery of residual β-cell function in NOD mice [64–66]. Dipeptidyl peptidase (DPP)-IV inactivates GLP-1 (and its analogs) rapidly. Therefore to aid the action of GLP-1 analogs DPP-IV inhibitors such as sitagliptin [67] and vildagliptin [68] were used and found effective in NOD mice as well as type 2 diabetic patients [69]. Glucose-dependent insulinotropic polypeptide (GIP), another incretin hormone, cannot be used for treatment because the diabetic β-cell is resistant to GIP action [70]. A phase II clinical trial using combination therapy of alum-formulated GAD (Diamyd®) and DPP-IV inhibitor sitagliptin has received FDA approval and is currently under way (www.diamyd.com).

26.6.2 Probiotic Approach

Identification of the role of environmental agents (viruses and more recently bacteria) and their potential use as therapeutics throws open a vast range of possibilities.

Use of food supplements or even a probiotic yoghurt containing 'friendly bacteria' in prevention of autoimmune diabetes has been speculated. The idea seems far-fetched but considering the influx of information on the disease etiology, it is not completely impractical; however such concepts should be approached with extreme caution. In conclusion, therapies aiming at preserving/preventing β-cell function should aim at providing safe, long-term, and clinically relevant improvements over standard insulin therapy.

Key Points

- Therapeutic interventions can be benificial to individuals identified at risk and to individuals with existing autoimmunity to prevent the damage to residual β-cells.
- Modern therapies aimed at reducing β-cell autoimmunity should ideally be short-term treatment which can induce long-lasting 'tolerance,' but does not debilitate the capacity of immune system to fight pathogens.
- Successful intervention using autoantigen-specific therapies like alum GAD is the need of the hour.
- Combinatorial therapies can be very helpful in β-cell regeneration and arresting aggressive β-cell autoimmunity.
- New approaches such as DNA vaccines can be beneficial, but should be approached with caution.

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Chapter 27
In Vivo Regeneration of Insulin-Producing β-Cells

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Abstract Type 1 and type 2 diabetes mellitus are considered to be caused by defective control of blood glucose resulting from a reduced β-cell mass. Thus, the restoration of a functional β-cell mass by replacing the damaged β-cells or stimulating β-cell regeneration is a logical approach for the treatment of diabetes. Strategies for increasing the β-cell mass include stimulating β-cell replication and differentiation and inhibiting β-cell death. Treatment with various growth factors such as GLP-1, BTC, HGF, and EGF and forced expression of β-cell transcription factors such as Pdx-1, NeuroD, and MafA resulted in the regeneration of β-cells in vivo. Another approach is the administration of stem/progenitor cells, which can differentiate into insulin-producing cells. However, there are no satisfactory methods yet for clinical application. Understanding the mechanisms of the regenerative process of pancreatic β-cells will pave the way for the development of regenerative medicine for treatment of diabetes.

Keywords β-cell · Regeneration · Differentiation · Growth factor · Transcription factor

27.1 Introduction

Diabetes mellitus is a metabolic disorder that is characterized by hyperglycemia. Both type 1 and type 2 diabetes result from an inadequate mass of insulin-producing pancreatic β-cells. Type 1 diabetes is caused by autoimmune-mediated destruction of pancreatic β-cells, resulting in an absolute deficiency of insulin [1, 24, 94]. Type 2 diabetes is caused by insulin secretion that is insufficient to compensate for insulin resistance [7, 34, 45, 95]. The β-cell mass is reduced in later stages of type 2 diabetes, and exogenous insulin therapy is eventually required for control of blood

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glucose levels. The discovery of insulin and the development of insulin analogs are essential for the treatment of hyperglycemia in diabetic patients at the present time. However, insulin therapy cannot restore the perfect control of insulin production in response to physiological changes of blood glucose levels as accomplished by pancreatic β-cells. Approaches to express insulin under the regulation of glucose-responsive transcription in non-β-cells have been tried, but do not equal pancreatic β-cells for the rapid increase in insulin secretion in response to glucose and the strict regulation of insulin release in response to minute-to-minute fluctuations of plasma glucose.

Restoration of a functional β-cell mass is a logical approach for the treatment of diabetes. Allogeneic islet transplantation into diabetic patients is a promising method for restoring the β-cell mass, but the limited supply of islets cannot meet patient demand and post-transplant immunosuppression can produce serious side-effects [29, 65, 66]. To overcome these limitations, various methods are being investigated to provide an alternative source of insulin-producing cells, such as differentiation of insulin-producing cells from embryonic and adult stem cells, differentiation of non-β-cells, and proliferation of β-cells in vitro and in vivo.

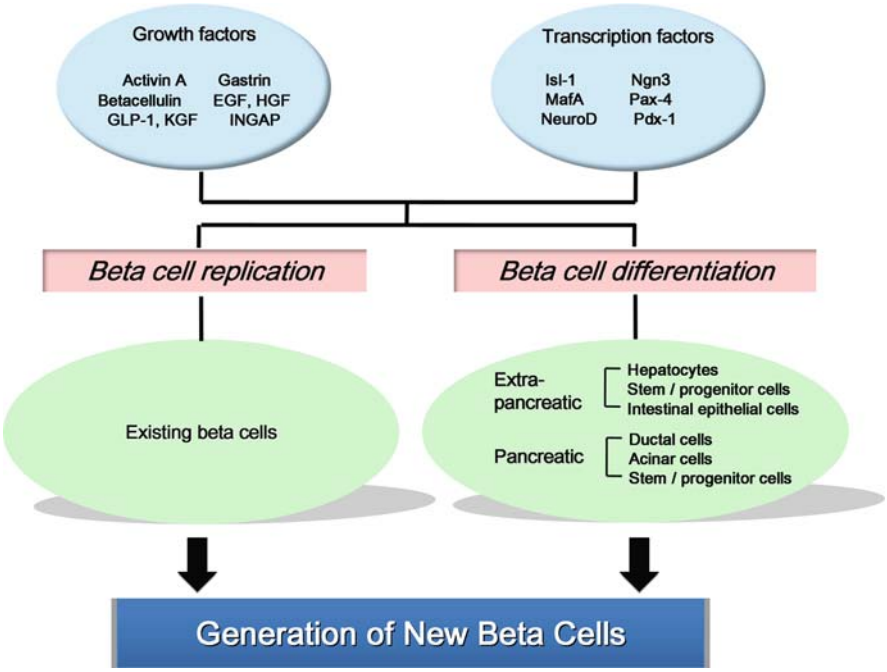

Fig. 27.1 Generation of new β-cells in vivo. Treatment with growth factors and expression of β-cell transcription factors can stimulate the replication of existing β-cells and differentiation of stem/progenitor cells and other cells inside or outside the pancreas into insulin-producing cells, thus remitting diabetes. GLP-1, glucagon-like peptide-1; EGF, epidermal growth factor; HGF, hepatocyte growth factor; INGAP, islet neogenesis gene associated protein; MafA, v-maf musculoaponeurotic fibrosarcoma oncogene homolog A; Ngn3, neurogenin3; Pdx-1, pancreatic and duodenal homeobox-1

Of considerable interest are methods designed to regenerate β-cells in vivo, thus avoiding complications of tissue matching and surgical procedures.

Mature β-cells can replicate throughout life [19, 84], and considerable evidence has shown that division of pre-existing β-cells is a major mechanism by which neonatal growth of the β-cell mass and regeneration after trauma are achieved [12, 15, 23, 53, 85]. However, it has been argued that neogenesis from pancreatic progenitor cells also contributes to the increase in β-cell mass during normal growth and after injury [5]. In accord with this, a recent study reported that neurogenin-3 (Ngn-3)-positive progenitor cells in the injured adult mouse pancreas differentiated into functional β-cells [91]. However, in type 1 diabetes, β-cell replenishment, by whatever mechanism, is outpaced by β-cell destruction; therefore much research is focused on methods to augment β-cell regeneration while suppressing the autoimmune response. In vivo strategies for increasing the number of insulin-producing cells include the expansion of remaining β-cells, differentiation of β-cells progenitors, and transdifferentiation of non-β-cells into insulin-producing cells, both inside and outside the pancreas. Two major avenues of research have been the use of growth factors and β-cell transcription factors (Fig. 27.1). This review will explore current developments in these areas.

27.2 Regeneration by Growth Factor Treatment

27.2.1 Background

The β-cell mass is dynamic and increases in response to environmental and physiological changes and insulin resistance [15, 62]. In fact, β-cell replication can be stimulated by pregnancy [72], diabetogenic stimuli such as glucose and free fatty acids [2, 74], and growth factors such as hepatocyte growth factor (HGF) [22], a combination of epidermal growth factor (EGF) and gastrin [6], betacellulin (BTC) [32], and glucagon-like peptide-1 (GLP-1) or its long-lasting homolog, exendin-4 [49]. In addition, members of the regenerating protein family such as Reg protein [88] and islet neogenesis gene associated protein (INGAP) [64] can stimulate proliferation of β-cells. Therefore, growth factors have been investigated as potential therapies for diabetes.

27.2.2 Betacellulin

BTC, a member of the epidermal growth factor family, is known to induce proliferation and differentiation of insulin-producing insulinoma cells [32] and convert an exocrine pancreatic cell line (AR42J) into insulin-expressing cells when combined with activin A [51]. When administered systemically, BTC improved glucose tolerance in alloxan-induced diabetic mice [92], streptozotocin (STZ)-induced diabetic mice [47], and rats made diabetic by 90% pancreatectomy [46] by promoting the formation of new β-cells. As well, the combined treatment of activin A and BTC resulted in the regeneration of pancreatic β-cells in neonatal STZ-treated rats [48].

However, multiple systemic injections of recombinant BTC into diabetic animals failed to completely restore normoglycemia, probably owing to the short half-life of the BTC protein [92].

To overcome the short half-life of BTC, a recombinant adenoviral vector was constructed that would produce high levels of endogenous BTC when injected into diabetic animals. This adenoviral vector contained the cytomegalovirus promoter/enhancer for strong constitutive expression, beta-globin chimeric intron for high levels of transgene expression, and the albumin leader sequence to facilitate secretion, followed by cDNA encoding mature BTC (rAd-BTC) [69]. When rAd-BTC was injected intravenously into chemically induced diabetic mice, high serum levels of BTC were seen for about 1 week, and serum BTC levels declined thereafter, presumably due to clearance of the virus. This transient expression of BTC was sufficient to increase the β-cell mass, evidenced by increased positive staining for insulin in the pancreas and increased pancreatic and serum insulin levels [69]. The increase in the β-cell mass and resulting increase in insulin levels were sufficient to remit diabetes, shown by normal glucose clearance and normal blood glucose levels in rAd-BTC-treated diabetic mice. Furthermore, blood glucose levels in chemically induced diabetic mice remained normal up to 100 days post-treatment, long after serum levels of BTC had dissipated. This suggests that once β-cells have regenerated, further expression of BTC is unnecessary. When rAd-BTC was injected into autoimmune diabetic NOD mice, blood glucose levels were also lowered, but only transiently, probably a result of autoimmune attack against the newly generated β-cells. Treatment with complete Freund's adjuvant to suppress immune responses prior to rAd-BTC injection resulted in long-term remission of diabetes for up to 100 days, when the experiment was terminated [69].

27.2.3 Glucagon-Like Peptide-1

GLP-1 is produced from gut endocrine cells and has been shown to stimulate β-cell proliferation and neogenesis [16, 17, 75, 90, 96] and have anti-apoptotic effects on β-cells [16]. Injections of GLP-1 or exendin-4, a stable analog of GLP-1, can increase the β-cell mass and improve hyperglycemia. However, the potential for using GLP-1 as a possible method to regenerate pancreatic β-cells is limited by its short biological half-life, due to rapid degradation by dipeptidyl peptidase IV (DPPIV) [13, 54]. For example, continuous infusion of GLP-1 was required to induce normal glucose tolerance and increased endocrine cell mass in old, glucose-intolerant rats [61]. To overcome this problem, GLP-1 analogs that are long-acting receptor agonists or resistant to DPPIV degradation and inhibitors of DPPIV have been investigated for the treatment of diabetes [31]. Clinical trials are under way for β-cell regeneration in type 1 diabetes using synthetic exendin-4 (AC2993) along with immune suppressors (National Institute of Diabetes and Digestive and Kidney Diseases, protocol number 03-DK-0245.)

Continuous release of GLP-1 via treatment with a recombinant adenovirus constitutively expressing GLP-1 (rAd-GLP-1) remitted diabetes and increased the β-cell

mass in STZ-induced diabetic mice [50]. However, similar to the case with rAd-BTC, autoimmune diabetic mice injected with rAd-GLP-1 showed only a transient remission of hyperglycemia [50]. In the case of rAd-GLP-1-treated autoimmune diabetic mice, normoglycemia could be prolonged by pretreatment of animals with complete Freund's adjuvant, in this case, up to 1 year [50]. Another strategy is the use of anti-lymphocyte serum to abrogate autoimmunity in exendin-4-treated NOD mice, which resulted in complete remission in 88% of mice treated within 75 days [60].

In addition to generating insulin-producing cells in the pancreas [50], rAd-GLP-1 treatment also produced insulin-positive cells in the intestine (Jun et al., unpublished results), suggesting that induction of high intestinal levels of GLP-1 might induce insulin-producing cells capable of remitting diabetes.

27.2.4 Growth Factors in Combination with Gastrin

Both GLP-1 and EGF have been combined with gastrin, a hormone produced by the parietal cells of the stomach, as a therapy to restore the β-cell mass. Therapy with GLP-1 and gastrin, but not with GLP-1 or gastrin alone, restored normoglycemia in autoimmune diabetic NOD mice both by restoring the β-cell mass and by downregulating the immune response [79]. A study using diabetic immunodeficient NOD.scid mice implanted with human islets and treated with this combination therapy showed that the expanded β-cells arose largely from cytokeratin 19-positive pancreatic duct cells associated with the islets [78].

EGF itself appears to be ineffective as a β-cell regenerative agent; however, a combination of EGF and gastrin has been successfully used. Treatment of chemically induced diabetic rodents with EGF and gastrin by either systemic injections [6] or continuous infusion using osmotic minipumps [63] lowered blood glucose, improved glucose tolerance, and increased the β-cell mass. In autoimmune diabetic NOD mice, treatment with EGF and gastrin for 2 weeks restored normoglycemia and increased the β-cell mass three-fold as compared with diabetic mice. In addition, the autoimmune response was downregulated, evidenced by delayed diabetes onset by adoptive transfer of diabetogenic cells into immunodeficient NOD mice [77]. In a similar study, combination therapy with EGF and gastrin induced neogenesis of human β-cells from pancreatic duct cells in vitro as well as from human pancreatic cells implanted into immunodeficient NOD.scid mice [76].

Clinical trials are under way using injectable EGF and gastrin analogs (E1-INF^{TM}) for type 1 and type 2 diabetes [87].

27.2.5 Other Factors

Although most research on the use of growth factors for β-cell regeneration has focused on BTC and GLP-1, there is evidence for a stimulatory effect of other growth factors, Reg family proteins, and growth factor-like plant extracts

on β-cell regeneration in vivo. Intravenous injection of a plasmid encoding HGF promoted β-cell proliferation and inhibited β-cell death, resulting in the mitigation of hyperglycemia in STZ-induced diabetic mice [11]. Administration of keratinocyte growth factor into neonatal diabetic rats for 4 days increased proliferation of presumed precursor cells in the pancreatic ducts, thus increasing the β-cell mass [55].

The Reg gene family belongs to the calcium-dependent lectin (C-type lectin) gene superfamily and is divided into subclasses based on the primary structures of the encoded proteins. When rat Reg protein, a member of the Reg I family, was intraperitoneally administered for 2 months to rats made diabetic by subtotal pancreatectomy, blood glucose was decreased and the β-cell mass was increased in the residual pancreas [88]. Injection of human Reg Ia gene product into diabetic NOD mice increased the β-cell mass and ameliorated diabetes when combined with an immunoregulator [26]. INGAP was first characterized from a soluble tissue fraction from partially obstructed hamster pancreas and was subsequently found to be a member of the Reg III family [20]. When STZ-induced diabetic mice were given INGAP peptide (a 15-amino acid fragment of INGAP) for 39 days, diabetes was reversed and islet cell neogenesis was increased [64]. INGAP peptide has been used in phase 2 clinical trials with type 1 and type 2 diabetic patients. Although the effects on stimulated C-peptide and HbA1c were promising, no consistent treatment effects on fasting glucose, insulin, and C-peptide were seen [20].

With regard to growth factor-like effects of plant extracts, an acetone extract of the fruit of *Momordica charantia* (also known as bitter gourd, bitter melon, or karela) lowered blood glucose in alloxan-induced diabetic rats. As well, some treated animals showed small islets among the acinar tissue, which was interpreted as neoformation of islets from pre-existing islet cells [71]. A chloroform extract of the water lily, *Nymphaea stellata*, lowered blood glucose levels in diabetic rats and significantly increased the β-cell mass and insulin content [80]. For both of these plant extracts, over 30 days of treatment was required for the observed effects. In addition, orally administered plant extracts containing conophylline, a vinca alkaloid that can induce differentiation of insulin-producing cells from an acinar cell line [39], reduced blood glucose levels in Goto–Kakizaki rats, a model of type 2 diabetes [21].

27.3 Regeneration by Transcription Factor Expression

27.3.1 Background

Understanding the molecular mechanisms for pancreatic development has been pivotal for designing methods by which non-β-cells can be driven into the β-cell lineage. With the transgenic mouse technologies, great advances have been made in unveiling the key transcription factors which play a role in the differentiation of pancreatic β-cells.

During development, the endoderm germ layer forms the foregut, which depends on the proper anterior–posterior patterning of the endoderm [89]. Transforming growth factor, fibroblast growth factor (FGF), and wingless-type MMTV integration site family (Wnt) signaling pathways are important for posterior endoderm development [14, 52]. The foregut gives rise to the thyroid, lungs, liver, stomach, and pancreas. FGF, retinoic acid, and hedgehog signaling pathways are required for establishing the pancreatic organ within the developing gut tube, and transcription factors such as SRY (sex determining region Y)-box (Sox)17, homeobox gene HB9 (Hlxb9), hepatocyte nuclear factor (HNF)-6, HNF-3beta (also known as foxhead box A2, Foxa2), pancreatic and duodenal homeobox-1 (Pdx-1), and pancreas-specific transcription factor-1a (Ptf-1a) are required for proper pancreatic development [33, 35, 37, 38, 57]. All pancreatic progenitor cells express Pdx-1, and inactivation of Pdx-1 after bud formation prevents both islet (endocrine) and acinar (exocrine) cell differentiation [30]. Neurogenin-3 (Ngn-3) is a key regulator of endocrine development and is expressed exclusively in endocrine precursor cells [27, 68], whereas Ptf-1a is involved in exocrine acinar cell development [44]. The expansion and differentiation of pancreatic progenitor cells appear to be regulated by Notch signaling [28, 56]. Inhibition of Notch signaling upregulates Ngn-3 and increases endocrine formation [3, 33], and activation of Notch1 prevents endocrine differentiation [28, 56].

Many transcription factors such as Pdx-1, ISL LIM homeobox 1 (Isl-1), Ngn-3, NK2 homeobox 2 (Nkx2.2), NK6 homeobox 1 (Nkx6.1), NeuroD, Hlxb9, Pax-4, and paired box gene (Pax)-6 have been identified as islet differentiation factors. Ngn-3 is a key transcription factor for endocrine development and is absolutely required for islet cell development [25]. Nkx6.1 and Pax-4 are downstream of Ngn-3 and appear to act as β-cell-determining factors [67, 73]. Nkx2.2 is required for the final differentiation of β-cells and production of insulin [10, 82]. Pax-6 is required for islet cell proliferation, morphology, and β-cell function [4].

Among these transcription factors, Pdx-1 has been most extensively utilized to drive both neogenesis of β-cells in the pancreas and transdifferentiation of pancreatic and extra-pancreatic cells into insulin-producing cells.

27.3.2 Pancreatic Transduction

Delivery of the Pdx-1 gene or protein to the pancreas has been shown to be a successful method for generating new insulin-producing cells. Delivery of an adenoviral vector containing the *Pdx-1* gene into the mouse pancreas via the common bile duct induced β-cell neogenesis and ductal proliferation [83]. Delivery of the *Pdx-1* gene along with the *BTC* gene into the pancreas of STZ-induced diabetic rats via ultrasound-targeted microbubble destruction normalized blood insulin and C-peptide and maintained blood glucose below 200 mg/dl. However, regeneration of insulin-producing islets was not seen, rather insulin was produced ectopically by acinar (exocrine) cells [8]. An alternative strategy for delivery of Pdx-1 which avoids the potential side-effects associated with the use of viral vectors is injection of

recombinant Pdx-1. Recombinant Pdx-1 has a protein transduction domain, which facilitates its entry to cells. Intraperitoneal injection of recombinant Pdx-1 into STZ-induced diabetic mice increased islet cell number and proliferation in pancreata and upregulated the expression of insulin and other genes related to pancreas regeneration in the pancreas, contributing to the restoration of normoglycemia [43].

Recently it was reported that reexpressing a specific combination of three transcription factors, Pdx-1, Ngn3, and MafA, induced differentiation of pancreatic exocrine cells into β-cells, which were indistinguishable from endogenous islet β-cells in size, shape, and ultrastructure, resulting in amelioration of hyperglycemia [97].

27.3.3 Extra-Pancreatic Transduction

In addition to stimulating new insulin-producing cells in the pancreas, transcription factors such as Pdx-1 and NeuroD have been used to confer β-cell-like characteristics to non-islet tissues, such as the liver and the intestine. The aforementioned study using systemic injection of recombinant Pdx-1 found expression of insulin and other genes related to pancreatic function not only in the pancreas but also in the liver [43]. Adenoviral-mediated delivery of the *Pdx-1* gene to the liver of normal mice resulted in a substantial increase in both hepatic immunoreactive insulin content and plasma immunoreactive insulin levels, compared with that in mice treated with control adenovirus. The hepatic immunoreactive insulin was processed to mature insulin and ameliorated hyperglycemia in STZ-induced diabetic mice [18]. A similar strategy of ectopic Pdx-1 expression in the liver also improved blood glucose levels in cyclophosphamide-accelerated autoimmune diabetic NOD mice [70]. Adenoviral-mediated systemic delivery of Pdx-1 carrying the VP16 transcriptional activation domain (Pdx-1/VP16) increased the expression of insulin biosynthesis in liver, particularly in the presence of NeuroD or Ngn-3, resulting in the improvement of glucose tolerance [36]. Delivery of the *NeuroD* gene by a helper-dependent adenoviral vector partially reversed STZ-induced diabetes and completely reversed it when combined with the *BTC* gene. Insulin transcripts and other islet-specific transcripts, including proinsulin-processing enzymes, β-cell-specific glucokinase, and the sulfonylurea receptor, were detected in liver of the treated mice. Insulin-, glucagon-, pancreatic polypeptide-, and somatostatin-producing cells were found to be organized into islet clusters within the liver [42]. Intravenous delivery of the *Ngn-3* and *BTC* gene by helper-dependent adenovirus to STZ-induced diabetic mice resulted in two waves of insulin production from the liver. The first wave was from terminally differentiated hepatocytes, which only lasted 1–6 weeks. The second wave was from the transdetermination of oval cells (multipotent hepatic progenitors) into islet-like cell clusters, which lasted 6 weeks to 6 months [93].

With respect to the intestine, forced expression of Pdx-1 [41] or v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) [59] in intestinal epithelia by adenovirals-mediated gene transfer induced the expression of insulin and lowered blood glucose levels in STZ-induced diabetic rodents.

27.4 Regeneration by Injection of Stem/Progenitor Cells

A large body of research has been directed to the differentiation of isolated stem/progenitor cells into insulin-producing cells that can be used therapeutically. Whereas much of this work has focused on in vitro differentiation and subsequent transplantation, some have used the approach of injecting undifferentiated stem/progenitor cells directly. However, whether or not these injected stem/progenitor cells differentiate into insulin-producing cells in vivo or exert other effects that preserve or increase the β-cell mass is unclear. Injection of allogeneic splenocytes in combination with complete Freund's adjuvant (to prevent anti-islet autoimmunity) corrected diabetes in diabetic NOD mice [40]. This study suggested that the injected splenocytes are the source of new insulin-producing cells, but later studies failed to show evidence of donor splenocyte-derived differentiation of insulin-producing cells [9, 58, 81]. A successful clinical trial involving autologous hematopoietic stem cell transplantation and immune suppressor in diabetic patients was reported [86]. Although β-cell function was shown to be increased, the mechanism of action was not clear.

27.5 Conclusions

Several experimental models including pancreatectomy, STZ treatment, and ductal ligation suggest that pancreatic β-cells regenerate in vivo. Therefore, one promising strategy for a permanent cure for type 1 and type 2 diabetes is to stimulate the regeneration process in the pancreas in vivo, along with treatments to suppress the autoimmune response in type 1 diabetes. A related strategy is to transdifferentiate extra-pancreatic cells into insulin-producing cells; whether these cells avoid subsequent autoimmune attack has yet to be determined. Regeneration of β-cells and transdifferentiation into β-cells have been tried using β-cell growth and differentiation factors, expression of β-cell transcription factors, and injection of stem/progenitor cells. So far, most of the results have been obtained from studies with animal models and very little has been tried clinically. Identifying the source of regenerated new β-cells will be useful for the development of regenerative medicine to treat diabetes; that is, determining which cells give rise to new β-cells (existing β-cells or stem/progenitor cells in the pancreas or other tissues) and identifying biomarkers for potential stem/progenitor cells that can differentiate into β-cells. In addition, understanding how β-cells develop and are maintained postnatally and the underlying mechanisms for the normal renewal process in adults is important, and considerable effort will be required to translate this knowledge into clinical applications.

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Chapter 28
Customized Cell-Based Treatment Options to Combat Autoimmunity and Restore β-Cell Function in Type 1 Diabetes Mellitus: Current Protocols and Future Perspectives

Fred Fändrich and Hendrik Ungefroren

Abstract Type 1 diabetes mellitus (T1D) is considered a classical autoimmune disease which commonly starts during childhood but may appear later in adulthood in a proportion of 30–40% of affected individuals. Its development is based on a combination of a genetic predisposition and autoimmune processes that result in gradual destruction of the β-cells of the pancreas and cause absolute insulin deficiency. Evidence for an autoimmune origin of T1D results from measurable islet β-cell autoantibody directed against various autoantigens such as proinsulin or insulin itself, glutamic acid decarboxylase 65, the islet tyrosine phosphatase IA-2, and the islet-specific glucose-6-phosphatase catalytic subunit-related protein. In addition, T-cell lines with specificity for insulin or glutamic acid decarboxylase have been identified within peripheral blood lymphocytes. Importantly, in most instances the pathogenesis of T1D comprises a slowly progressive destruction of β-cell tissue in the pancreas preceded by several years of a prediabetic phase where autoimmunity has already developed but with no clinically apparent insulin dependency. Unless immunological tolerance to pancreatic autoantigens is re-established, diabetes treated by islet cell transplantation or stimulation/regeneration of endogenous β-cells would remain a chronic disease secondary to immune suppression related morbidity. Hence, if islet cell tolerance could be re-induced, a major clinical hurdle to curing diabetes by islet cell neogenesis may be overcome. Targeted immunotherapies are currently explored in a variety of clinical studies and hold great promise for causative treatment to readjust the underlying immunologic imbalance with the goal to cure the disease. This chapter will outline possible treatment options to stop or reverse the β-cell–specific autoimmune and inflammatory process within pancreatic islets. Special emphasis is given to stem cells of embryonic, mesenchymal, and haematopoietic origin, which, besides their use for regenerative purposes, possess potent immunomodulatory functions and thus have the potential to suppress the

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autoimmune response. At the end of this chapter we will introduce a novel type of in vitro modified monocytes with immunosuppressive and anti-inflammatory properties. These tolerogenic monocytes provide a feasible option to be used as autologous cellular transplants to halt autoimmunity and to protect still viable β-cells within Langerhans islets.

Keywords Autoimmune disease · β-cell · Stem cell · T cell · Tolerance

Abbreviations

AHST	adult haematopoietic stem cell transplantation
AICD	activation-induced cell death
AIRE	autoimmune regulator
APC	antigen-presenting cell
AUC	mean total area under the curve
BM	bone marrow
CTL	cytotoxic T lymphocyte
DA	Dark-Agouti rat strain
DC	dendritic cell
DM	diabetes mellitus
DSS	dextran sodium sulphate
EBV	Epstein–Barr virus
ESC	embryonic stem cell
GAD	glutamic acid decarboxylase
GVA	graft-versus-host autoimmunity
GVHD	graft-versus-host disease
γIFN	gamma-interferon
HSC	haematopoietic stem cell
IAA	insulin autoantigen
IA-2	insulin antigen 2 specific antibody
IDDM	insulin-dependent diabetes mellitus
IDO	indoleamine 2, 3-dioxygenase
IGRP	islet-specific glucose-6-phosphatase catalytic subunit-related protein
IL-10	interleukin-10
iNOS	inducible nitric oxide synthase
LEW	Lewis rat strain
mAb	monoclonal antibody
M-CSF	macrophage colony-stimulating factor
MSC	mesenchymal stem cell/multipotent stromal cell
mTOR	mammalian target of rapamycin
NIDDM	non-insulin-dependent diabetes mellitus
NK	natural killer
NOD	nonobese diabetes (an inbred mouse strain)
PC	phosphatidylcholine

PCMO	programmable cell of monocytic origin
RESC	rat embryonic stem cell
scid	severe combined immunodeficiency disease
STIC	self-tolerance inducing cell
TAIC	transplant acceptance inducing cell
T1D	type 1 diabetes mellitus
Treg	regulatory T cells

28.1 Introduction

Diabetes mellitus (DM) is still thought to be an incurable metabolic disease characterised by hyperglycaemia which can either be caused by an autoimmune response-mediated destruction of the insulin-producing β-cells within the endocrine tissue of Langerhans cells, or, alternatively, by increased resistance of peripheral tissues to the hypoglycaemic action of insulin. In the early 1950s a clear distinction between insulin-dependent diabetes mellitus (IDDM), now designated type 1 diabetes mellitus (T1D), and non-insulin-dependent diabetes mellitus (NIDDM), now called type 2 DM, was made. Both forms of clinically apparent DM usually are associated with severe clinical consequences, loss of life expectancy, and quality of life in affected patients.

T1D can be envisaged as an autoimmune triggered disease with both genetic and environmental factors implicated in its aetiology. Based on a considerable number of immunologic, genetic, and clinical studies, a comprehensive description of the disease pathogenesis of T1D is now possible. It is this knowledge which is necessary to understand current and future strategies to prevent and restore insulin function of the pancreas without the need of presently used recombinant insulin substitutes as a palliative treatment option to achieve normoglycaemia in T1D patients. This chapter will outline possible treatment options to sustain viable β-cell mass and to restore insulin function in patients suffering from T1D. As such, the pathogenesis underlying the autoimmune origin of the disease will be described first.

Putative autoantigens, their trigger mechanisms, and related effector mechanisms of the immune system will be addressed to get a broader picture of potential treatment options to relieve the tissue of Langerhans from the underlying autodestructive and inflammatory process in the pancreas.

As mentioned above, T1D does not occur overnight. Usually, a long prediabetic phase of several years precedes the onset of T1D [31] during which autoimmune responses against β-cell-specific antigens have already developed but are insufficient to destroy enough tissue to cause clinically overt insulin dependency. It is still not possible to accurately quantify the number of persistent β-cells at the time of clinical onset. Since β-cell-specific autoantibodies to proinsulin, insulin, glutamic acid decarboxylase 65 (GAD65), or IA-2 are probably not directly pathogenic, their blood levels are not useful markers to estimate the extent of viable, insulin-releasing cells in the pancreas. These islet-specific antibodies are rather generated secondary

to T-cell-mediated activation but have a remarkable predictive value [73]. Clinical observations led us to believe that there is a small window of a few months preceding insulin dependency, where infusion of glucose will not stimulate an adequate rise in C-peptide levels, an important indicator of metabolic dysfunction in these patients. Consequently, if it were possible to identify prediabetic patients early enough with reliable biomarkers at hand, dampening overshooting autoimmune responses during this critical period could possibly protect the mass of still functional β-cells in the pancreas, restore full β-cell function, and avoid major life-threatening complications related to T1D.

28.2 Autoimmune Diseases: Role of T Cells

A current concept addressing the initiation of autoimmune diseases in humans considers a breakdown of the balance between autoregulatory immune pathways and pathogenic autoreactivity as a major causative mechanism [70]. Subsequently, autoaggressive T-cell-mediated reactions and autoaggressive antibodies directed

Fig. 28.1 Potential mechanisms of autoimmunity. Peripheral tolerance is the mechanism by which mature T cells that recognize self-antigens in peripheral tissues become incapable of subsequently responding to these antigens. Peripheral tolerance comprises varies mechanisms including *anergy* (if T cells use the inhibitory receptor for B7 molecules, CTLA-4, to recognize costimulators on APCs at the time the cells are recognizing antigen), *deletion* (repeated stimulation of T lymphocytes by persistent antigens results in death of the activated cells by a process of apoptosis, also called activation-induced cell death (AICD), see (**b**), or *suppression* (mediated by regulatory T cells able to block or inhibit functions of effector T lymphocytes). (**a)** Weak interactions between APCs and self-peptide-MHC complex on T cells can lead to homeostatic maintenance of T cells which over time undergo death by neglect. Instead, strong interactions between APCs and T cells disturb T-cell survival and trigger T-cell expansion and activation. Activated T cells (red nucleus) trigger B-cell (grey nucleus) maturation (b) and antibody formation (c) which can cause systemic autoimmunity if the interaction between T and B cells is sustained. Normal interactions between T cells and DCs can also lead to peripheral tolerance as outlined in (b). (**b**) Following interaction of T cells with APCs, expansion and activation of stimulated T cells depend on the avidity and affinity of bound antigen to the T-cell receptor. Subsequent excessive proliferation of T cells normally is counterbalanced via AICD, for instance by Fas–FasL interaction. Subsequently, activated T cells are either deleted or become anergic (*upper panel*). Absence of AICD leads to overshooting proliferation and clonal expansion of T lymphocytes with the risk to cause defective peripheral tolerance and autoimmunity to islet antigens can ensue. An alternative mechanism includes hyperactivated mature APCs which promote sustained activation of autoantigen-specific T-cell clones triggering autoimmunity (*lower panel*). (**c**) Pathogens taken up by mature DCs and presented indirectly within the MHC class II complex can activate CD4+ T cells which in turn cause clonal T-cell activation necessary to eliminate pathogen-infected cells. Strong cross-reactivity of these activated T cells with self-peptides expressed, for instance, on islet cells may cause vigorous T-cell responses (a, brown round antigen complex). Alternatively, weak cross-reactivity (b, green round antigen complex) may result in minimal autoimmunity with organ infiltration but no destruction. If DC-primed pathogen-specific T-cell clones do not cross-react (c, green rectangular antigen complex) no autoimmunity will ensue

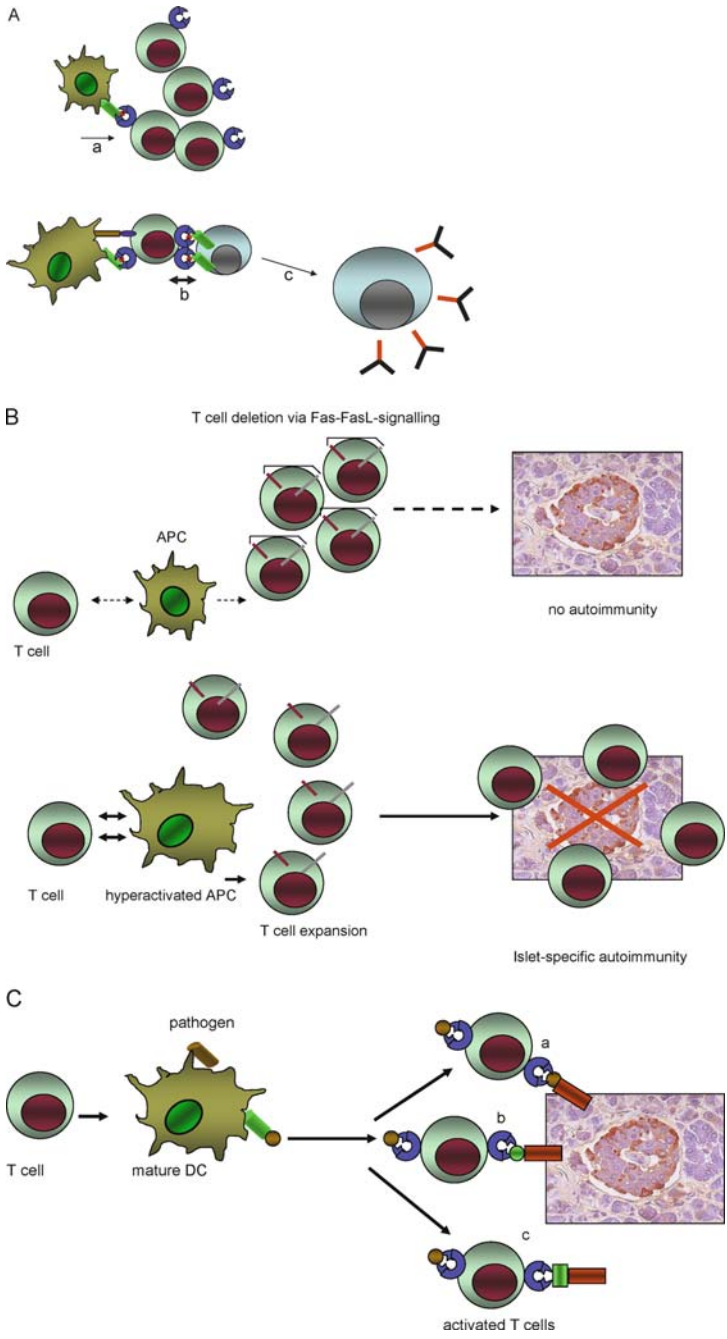

Fig. 28.1 (continued)

against the host's own antigens ensue. In general, specific self-molecules of a defined tissue entity cause an immune response and, if not stopped early enough, can manifest clinically as autoimmune disease. The human immune system surveys overwhelming immune responses under normal circumstances and utilises a variety of self-limiting regulatory mechanisms. Two inherent mechanisms which control self-reactive T cells comprise defined immune tolerance processes which are either located centrally in the thymus [45] or peripherally in secondary lymphoid tissue compartments [81]. Despite the existence of potent inherent tolerance mechanisms, these occasionally fail to prevent autoaggressive immune responses. An overwhelming T-cell response caused by a specific self-antigen may not be sufficient to cause autoimmunity. However, a variety of genes and environmental factors modulate disease susceptibility and contribute to the onset of autoimmunity in different ways (Fig. 28.1). One form includes (i) *altered homeostasis*, where homeostatic maintenance of T cells following weak interactions with self-peptide MHC is disturbed. Subsequently, survival of partially activated T cells leads to splenomegaly and lymphadenopathy and triggers secondary B-cell responses which in turn can stimulate autoantibody production and onset of clinically apparent autoimmune disease. Another form (ii) comprises *peripheral T-cell tolerance*. Here, upon encounter of T cells with high-affinity/-avidity antigen on resting antigen-presenting cells (APCs), typical expansion and subsequent deletion or anergy of activated T-cell clones is defective due to blocked apoptotic signaling. Disturbed peripheral tolerance ensues either from an inability to directly delete the self-reactive cell or from a lack of cross-presentation of self-antigen, eventually leading to autoimmunity. A third form, (iii) *molecular mimicry*, describes the existence of potentially autoreactive T lymphocytes in the peripheral T-cell repertoire which can be activated under specific circumstances such as infections with pathogens. After elimination of the pathogen weak cross-reactivity of these T-cell clones with self-peptides on parenchymal cells can result in either weak or transient autoimmunity or, alternatively, if T cells vigorously react against self-antigen may cause organ-specific autoimmunity. It is still a matter of controversy at which stage regulatory T cells (Tregs), which are able to attenuate autoaggressive T cells, act to prevent autoimmunity. Early studies in autoimmunity suggested that Tregs are activated by antigens derived from host tissues, and the view that Tregs are specific for self-antigens became widely accepted. Research on Tregs has identified specific markers which allow these cells to be distinguished from other T cells; among those, CD25 (a high-affinity interleukin 2 receptor) and Foxp3, a transcription factor, are most important, even not exclusive to Tregs. In addition, other markers such as CD39, CD62L, CTLA-4, GITR, and CD127 are used to define Tregs. Intrathymically generated CD4+/CD25+/Foxp3+ Tregs, also designated "naturally occurring" Tregs, are able to prevent autoimmune disease by suppressing effector T cells [51]. This process involves a number of "transcriptional signatures" independent of Foxp3 and relies on signaling via TGF-β, IL-2, and possibly other cytokines [37]. Our current knowledge on the putative suppressor mechanism exhibited by naturally occurring Tregs comprises (i) cell–cell contact, possibly involving coinhibitory receptor–ligand interactions (see below); (ii) cytokine deprivation by CD25 binding of IL-2; (iii) bystander

suppression via secretion of anti-inflammatory cytokines such as TGF-β, IL-10, and IL-35; and (iv) cytolytic activity due to granzyme B and perforin release [84]. In addition, Foxp3-expressing T cells can be induced in peripheral lymphoid tissue [6] and requires antigen presentation in the absence of costimulation, for instance antigen presentation under subimmunogenic conditions. In addition to positive costimulatory receptors, T-cell reactivity is controlled by negative regulatory receptors (so-called coinhibitory molecules), such as CTLA-4 and PD-L1. CTLA-4 attenuates T-cell activation by competing for CD28 ligation. The importance of this regulatory control mechanism becomes evident in CTLA-4-deficient mice, which develop massive lymphoproliferation [89]. Accordingly, reduced expression of a soluble form of CTLA-4 (sCTLA-4) is associated with limited costimulatory molecule blockade and enhances the capacity of APCs to stimulate autoreactive T cells. Moreover, conversion of naïve T cells into Tregs depends on intact TGF-β signaling, underscoring the important role of TGF-β in this process [58].

28.3 Type 1 Diabetes Mellitus: Evidence for an Autoimmune-Based Origin

T1D is considered a multifactorial disease evolving from complex interactions between genetic and environmental risk factors. Based on family and twin studies genetic factors strongly determine susceptibility to develop T1D. The concordance rate for monozygotic twins ranges between 21 and 70%, while for dizygotic twins it is only 0–13% [76]. However, the absence of a 100% concordance between monozygotic twins points to other confounding factors, such as environmental triggers [75]. Various loci were described as part of the human leukocyte antigen complex which are linked with an inherited risk for disease susceptibility whereas others confer protection [74, 55]. Among those susceptibility loci, IDDM1, IDDM2, and IDDM12 play an important role in the pathogenesis of T1D. The IDDM1 locus confers about 40–50% of the inherited risk. Located on chromosome 6p21 within the major histocompatibility complex various defined HLA genes have an important role in the modulation of the immune system. The risk of T1D is mostly linked with class II genes, particularly DR3/DQ2 and DR4/DQ8 which are known to be carried by more than 90% of white Caucasian individuals and fewer than 40% of normal controls. DR3/DR4 heterozygosity is associated with approximately 50% of children who develop diabetes at an age younger than 5 years, in 20–30% of adults suffering from T1D and in only 2.4% of the general population [76]. In addition, there is a notable association between the pattern of autoantibodies for insulin autoantigen (IAA) and IA-2 with the DR4-DQ8 haplotype whereas the GAD65 antibody is more commonly found in patients with DR3-DQ2 haplotype [59]. Recently, genome-wide analyses have identified several additional T1D-linked genes or genetic regions [90]. Environmental factors which contribute as confounding parameters to the pathogenesis of T1D include exposure to various pathogens, and complex foreign antigens, such as cow's milk, gluten, or other cereal-derived proteins [9, 52, 93].

A couple of hallmarks defining an autoimmune disease exist and can be reliably documented for T1D, often referred to as the prototypic organ-specific autoimmune disease. Healthy human islets of Langerhans comprise a core of about 80% β-cells surrounded by other endocrine cell types, α, δ, and PP cells, producing glucagon, somatostatin, and pancreatic polypeptide, respectively. Evidence that T1D is triggered by an autoimmune mechanism is based on the following observations: (i) The presence of circulating activated T cells directed against β-cell targets [7, 72], (ii) the presence of circulating autoantibodies directed against antigens on the β-cell [55], (iii) the coexistence of other autoimmune diseases, likewise thyroiditis or Addison disease [5], (iv) the clinical responses to immunosuppressive treatment protocols [13, 36, 48], (v) the existence of relevant spontaneous animal models (NOD mouse, BB rats) [8], and (vi) the passive transfer of disease via CD4+ and CD8+ T lymphocytes generated from nonobese diabetic (NOD) mice into naïve animals [22].

28.4 Diabetes-Specific Antigens and Associated Effector Cells

28.4.1 T Cells

Our current view on the pathogenesis of T1D disease development consider T lymphocytes which recognize and bind to islet autoantigens to be central for the inflicted autodestructive process. Several laboratories have identified antigens which are presented by diabetes susceptible MHC class II molecules and subsequently stimulate CD4+ T-cell proliferation. As outlined below, this view is supported by the fact that therapeutic agents who inhibit or block T-cell-driven effector mechanisms are able to preserve β-cell function and mass at least for the period of the time medication is applied [87]. Other evidence for a T-cell-driven disease process in T1D results from observations following BM transplantation (for haematological malignancy) from T1D-positive donor siblings where nondiabetic recipients have developed T1D a few months to years after reconstitution of the ablated BM which included depletion of their own immune system as part of the conditioning regimen [60, 61]. These findings are in line with observations from NOD mice (a mouse strain that spontaneously develops T1D, as described in detail elsewhere in this compendium, chapters 24 and 26), where isolation of autoaggressive T cells from infiltrated islets and draining lymph nodes is possible. These lymphocytes have the capacity to transfer the autoimmune disease upon transfer to NOD recipients [33]. Testing the diabetogenicity of T cells constitutes a real challenge. Since some T-cell clones transfer a mild insulitis or none at all, this poses the question of whether antigen specificity or an inherent property of a particular T cell is responsible for the weak immune response. One hypothesis is that autoantigens trigger the initial stage of disease and when inflammation sets in, concomitant “epitope spreading” ensues. As potentially potent autoantigens, those which are heavily expressed and/or those with

extraordinarily high avidity for specific autoaggressive T-cell receptors are envisaged [34]. In this context, proinsulin and insulin itself appear to represent strong autoantigens for T cells as outlined cogently by Jaeckel et al. [43]. After successful engineering of class II gene constructs which express the IAA they found that mice presenting this antigen by thymic APCs presented with a reduced or complete absence of disease. Noteworthy, control experiments which used other nondiabetes-related autoantigens (third-party controls) yielded negative results. These and other studies point to central thymic presentation and related negative selection, Tregs, and antigen presentation problems as a possible cellular basis in the pathogenesis of T1D in NOD mice [88, see Ref. 83 for comprehensive review]. In addition, cytotoxic T lymphocytes (CTLs, CD8+ T cells) significantly contribute to the diabetic process as shown upon transfer of spleen-derived CD8+ T lymphocytes from diabetic NOD mice into nondiabetic mice. In addition, CTLs are generally found in insulitis upon fine-needle biopsy of the Langerhans cell clusters and may exert a direct cytotoxic function as they are known to secrete toxic enzymes such as perforin and granzyme B. CTLs with high-affinity TCRs (high-avidity T cells) cause most of the harm, while those with low-affinity TCRs (low-avidity T cells), according to recent experimental evidence, accumulate as memory T cells during the disease and may be protective in NOD mice. It has been hypothesized that such low-avidity T cells afford disease protection either by crowding the islets of Langerhans or by killing APCs [for review see Ref. 49]. In contrast to CD8+ T cells, autoantibodies on their own are unable to passively transfer the disease into animals or unborn foetus despite their presence in cord blood which strongly argues against autoantibodies being per se responsible for initiating β-cell damage [91].

28.4.2 Antigen-Presenting Cells

So far, at least 15 distinct peptides derived from β-cells and their corresponding CD4+ T cells have been identified [86, 96]. The presentation of β-cell antigens is a complex issue as β-cells themselves do not express MHC class II molecules. It can be surmised that presentation of β-cell-specific antigens is mediated by APCs within islets of Langerhans. These professional dendritic cells (DCs) are able to load the peptide groove of their MHC class II complexes with peptides derived from β-cell granules [18]. In this context, local lymph nodes draining the pancreas are crucial to the selection and activation of diabetogenic T cells [29]. Here, the question arises, how the β-cell antigen presentation takes place. It is not clear yet, whether this occurs via migration of islet DCs to the lymph nodes or, instead, by drainage of β-cell products directly to the nodes and subsequent uptake by DCs in the draining lymph nodes. Based on our knowledge gathered from the NOD mouse, β-cell autoimmunity progresses in relatively well-defined “checkpoints”. A first checkpoint is marked by DC infiltration of islets in 2- to 3-week-old NOD mice. Early detection of DCs and macrophages is followed by CD8+ and CD4+ T

cells, NK cells, and B cells. During islet cell infiltration these cells encounter β-cell autoantigens such as GAD65 and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP). The β-cell destruction resulting from inflammatory damage leads to release of cell contents including GAD65 and other autoantigens. Subsequently, these can be taken up by activated endothelial cells able to process and present disease-related epitopes of the GAD65 autoantigen. This in turn accelerates T-cell priming and aggravates the detrimental β-cell-specific immune response [32]. On the cytokine level it could be shown that DC-driven CD4+ helper T cells present in the blood of T1D patients release proinflammatory cytokines, such as γ-interferon (γIFN) and tumor necrosis factor upon stimulation with islet autoantigens, e.g. proinsulin, GAD65, and IA-2 [7, 72]. As such, the detection of autoantibodies in patients with T1D is after β-cell damage has occurred and autoantibodies are considered markers of disease progression.

It remains to be clarified what exactly initiates the disease process in T1D and what mechanisms operate in the host to allow T cells to become specifically activated. As outlined above, thymic deletion of potentially autoreactive T cells might fail but for those lymphocytes that escape thymic selection peripheral mechanisms are – under normal circumstances – in place. Thymic insulin mRNA levels are decreased for defined polymorphisms in the *INS* gene and in the absence of the *AIRE* (autoimmune regulator) gene and in both situations are associated with increased diabetes susceptibility. Alternatively, peripheral mechanisms related to a lack of Tregs are defective in T1D patients [63]. In this context, a polymorphism in the gene encoding CTLA-4 (a negative regulator of immune responses) has been described in Tregs of patients suffering from T1D [92].

28.5 Surrogate Markers Preceding Autoimmunity in T1D

Based on these findings, biological markers which would predict islet autoimmunity in candidates at risk for T1D are urgently needed for preemptive treatment. The risk determinants of T1D and the exact mechanisms which regulate progress towards β-cell failure as well as factors which determine time of presentation of clinical overt diabetes are still poorly understood. There is preliminary evidence that changes in the serum metabolome are correlated with preautoimmune alterations which may open a time window for novel T1D prevention strategies. Work from Orescic et al. [71] compared serum metabolomes from 56 children progressing to T1D with 73 controls who remained nondiabetic and permanently autoantibody negative. They report that individuals who developed T1D had reduced serum levels of succinic acid and phosphatidylcholine (PC) at birth, decreased triglycerides and antioxidant ether phospholipid levels throughout the follow-up, and increased levels of proinflammatory lyso-PCs several months before the onset of seroconversion to autoantibody positivity. Lipid changes were independent from known HLA-associated genetic risk factors. These findings indicate that autoimmunity is a relative late response to ongoing early metabolic disturbances.

28.6 Treatment Options to Prevent and Cure T1D

28.6.1 Targeted Immunotherapies

By use of diabetes associated risk factors, such as HLA genes and autoantibodies which are now at hand, algorithms could be designed and calculated which predict future diabetes manifestation in first-degree relatives of diabetic patients. Estimates are as such that 40–50% of positively tested candidates will contract the disease within 5 years. This possibility allows interfering with disease progression at a relatively early stage as demonstrated by two pioneering multicentre studies which tested the feasibility of such an approach. The US-based Diabetes Prevention Trials either administered low-dose parenteral [24] or daily oral insulin [79] to high-risk individuals. This approach was based on observations from mice demonstrating that preemptive insulin treatment by a number of different routes could prevent diabetes progression, either by restoration of tolerance to the causative autoantigens or by allowing β-cell recovery [78]. However, none of these studies showed significant clinical benefit except for a subgroup of patients with high titre IAA that was treated with oral insulin. An alternative option to treat patients with autoimmune diseases focuses on immune tolerance protocols by taking advantage of various immune pathways regulating peripheral tolerance in order to avoid autoreactivity. As for T1D, resetting β-cell function within the endocrine pancreas will necessitate a short inductive therapy which abrogates pathogenic reactivity to autoantigens without hampering the patient's immune system to mount normal immune responses against foreign pathogens. The availability of defined disease-specific autoantigens, for instance insulin in T1D, myelin basic protein in multiple sclerosis, acetylcholine receptor in myasthenia gravis, or desmoglein-3 in pemphigus vulgaris allows the generation of therapeutic vaccines that selectively inhibit antigen-specific immune responses, as demonstrated successfully in a variety of different experimental animal models [4, 46, 47, 50, 66]. However, so far it was not possible to transfer these results into man with appropriate success rates [19, 26, 80].

At current stage, targeted immunotherapies which either take advantage of biological compounds, soluble ligands, monoclonal antibodies, and/or in vitro modified cellular products are tested clinically in different approaches to promote immune tolerance. It is assumed that interventions that lead to retention of insulin secretory capacity would be expected to improve glucose control. The development of Fc-receptor non-binding CD3-specific monoclonal antibody (mAb) represents one example of an innovative biological to preserve endogenous insulin-secreting β-cell mass. Humanized anti-CD3 mAbs have been developed over the past years for clinical application in man in order to alter autoimmune processes and to sustain and/or restore β-cell function. In the mid-1990s, a first demonstration that anti-CD3 mAb can reverse TID in NOD mice was published. A brief 5-day course at the time of clinical manifestation of diabetes in these mice was able to restore insulin function and prevent recurrent immune responses, even towards second-set transplanted syngeneic pancreatic islets [21]. Consistent with these findings, clinical studies showed that a short intervention of the autoimmune process with

these humanized Fc-mutated anti-CD3 mAbs (e.g. hOKT3γ1[Teplizumab®] or ChAglyCD3) led to maintained or increased C-peptide responses and β-cell function for at least 24 and 18 months, respectively [35, 48]. Interestingly, the effect of ChAglyCD3 mAb was most pronounced among patients with initial residual β-cell function at or above the 50th percentile of a cohort of 80 patients tested [48]. According to findings from the Diabetes Control and Complications Trial, a stimulated C-peptide release of $\geq$ 0.2 pmol/ml is associated with improved metabolic control in patients with T1D. It is generally assumed that by the time someone is diagnosed with T1D, a substantial proportion – likely 60–85% – of a patient's β-cell mass is already non-functional with respect to production and release of insulin. Patients treated with Teplizumab showed continued benefits in terms of C-peptide production and use of insulin over a period of 3 years when compared to control groups [35]. However, a significant proportion of probands experienced a decline in insulin production by two years follow-up. A retreatment trial with Teplizumab is currently underway through support of the Immune Tolerance Network (Abate Trial/www.abatetrial.org/whatis.html). The European multicentre phase II placebo-controlled clinical trial [48] which tested ChAglyCD3 preserved β-cell function very efficiently, as evidenced by significantly higher endogenous insulin secretion and substantially lower insulin requirement in treated patients when compared with the control cohort. Especially patients with a high β-cell mass at study entry (higher than the median value of the whole population) required insulin doses of less than 0.25 U/kg/day at 18 months, a dose requirement which can be clinically judged as insulin independency [20]. A caveat in both studies was the finding of transient reactivation of Epstein Barr virus (EBV) as assessed by an increase in numbers of EBV copies in peripheral blood mononuclear cells. It is important to note that those patients were able to mount an effective anti-EBV response suggesting that the antibody treatment did not impair immune responses to unrelated exogenous antigens. This observation hints to a state of operational immune tolerance following anti-CD3 mAb treatment.

28.6.2 Alternative Treatment Strategies

In order to prevent recurrence of the autoimmune process and to improve sustained insulin production, combinations of CD3 mAbs with other immunotherapies are currently the focus of intensive research. Based on our experience from cancer therapies which have highlighted the importance of combination regimens, the most effective therapy to reverse autoimmunity and stabilize immune tolerance might involve synergistic effects between different therapies. In order to successfully translate such combination trials into clinical application, it will be crucial to use a panel of efficacy biomarkers and indices of prognoses to define the optimal therapeutic windows for administration of various drugs and compounds.

Possible options to synergize the large array of newly available drugs are outlined here. The combination of FcR-non-binding CD3-specific mAb with autoantigens to induce antigen-specific tolerance might enhance a more selective induction

of antigen-driven Tregs to combat pathogenic autoaggressive T lymphocyte subpopulations. When administered together with intranasal proinsulin peptide in a mouse model, anti-CD3 mAbs showed a higher efficacy in terms of sustained β-cell function than antigen or antibody given alone [16]. In addition, this treatment accomplished to induce insulin-specific Foxp3+CD4+CD25+ Tregs and transfer of these regulatory cells achieved dominant tolerance in immune competent NOD recipient mice [16]. These results are currently tested in a clinical trial sponsored by the Juvenile Diabetes Research Foundation. An alternative approach uses B-cell-specific antibodies (CD20 mAb, Retuximab®) which have emerged as a potential tool to defeat overt autoimmunity, for instance in rheumatoid arthritis. The hypothesis is based on the assumption that Rituximab would eliminate pathological autoantibodies and limit the biological role of potentially potent APCs involved in the autoimmune process. Based on the complementary role of T- and B-cell activity in the pathogenesis of autoimmune diseases, a combination therapy which takes advantage of T- and B-cell-specific mAbs or other drugs that target B-cell survival (B-cell-activating factor BAFF/BLYS; Belimumab®) are possible candidates for this type of Ab-linked treatment protocols. Other conceivable drugs are molecules or Abs which inhibit the costimulatory signaling cascade [11], such as Abatacept®, a CTLA-4–immunoglobulin fusion protein already proven efficaciously in psoriasis [2, 3] and refractory rheumatoid arthritis [30, 56, 57]. Further targets to be regulated in the process of T-cell activation include the CD40-CD154 and ICOS-ligand pathways.

Special interest is dedicated to rapamycin, an mTOR (mammalian target of rapamycin) inhibitor which regulates IL-2 signaling associated with T-cell proliferation [97]. Whereas apoptosis and subsequent deletion of autoaggressive T cells is considered to be mediated by rapamycin, this compound is also believed to spare Tregs, which are thought to resist rapamycin-induced apoptosis. Hence, a synergistic mode of action should be accomplished by combination of rapamycin and CD3-specific antibodies. Another phase I clinical trial has been launched to investigate the combination effect of Proleukin® (IL-2) and Rapamune® (sirolimus) as this regimen has been found to prevent T1D in NOD mouse models [20].

28.6.3 Stem Cell-Based Strategies for the Induction of Tolerance to Autoantigens

In pursuit of maintaining or restoring a critical mass of viable and functional β-cells in T1D patients various possibilities are currently explored. These include (i) stimulation of β-cell regeneration/proliferation in vivo through hormone and growth factor treatment, (ii) direct conversion by in vivo reprogramming of adult pancreatic exocrine cells to transform into β-cells [98], and (iii) isolation and in vitro differentiation of pancreatic progenitors, embryonic stem cells (ESCs), or extrapancreatic adult stem cells. Alternatively, pluri- or multipotency may be reestablished in adult-differentiated cells by induced pluripotent stem (iPS) cell

technology, or through targeted reprogramming with soluble factors or small molecule compounds, followed by tissue-specific differentiation [85]. The growth factor-mediated reprogramming of extrapancreatic adult stem cells will be discussed elsewhere in this compendium (chapter 29). Besides their potential use in islet cell regeneration [77], one may also exploit the immunosuppressive and anti-inflammatory properties of stem cells that are shared among ESCs [27, 53] and several types of non-haematopoietic stem cells (HSCs), including multipotent BM stromal cells/mesenchymal stem cells (both designated MSCs) [42]. The induction of haematopoietic chimerism and subsequent donor-specific immune tolerance via BM transplantation has long been known as an ideal approach for islet transplantation to treat T1D and has stimulated trials which seek to counteract autoimmunity by applying stem cell-related products to regain immune balance in overt T1D.

ESCs: Due to their pluripotent nature the use of ESCs has historically focussed on their regenerative capabilities. However, available data indicate that ESCs, or their in vivo derivatives, also have immunomodulatory capacities and can even induce transplantation tolerance. We have previously shown that injection of a rat ESC line (RESC) into the portal vein of non-immunosuppressed allogeneic recipient animals led to a state of tolerance as recipient animals indefinitely accepted heterotopic heart allografts from donors syngeneic to RESC, but acutely rejected third-party allografts in a way similar to untreated rats [27]. Transfusion of RESC gave rise to a limited state of chimerism which basically consisted of RESC-derived monocytes and B cells [28]. Murine ESCs reversibly and in a dose-dependent manner inhibited T-cell proliferation to various stimuli and the maturation of APCs induced by lipopolysaccharide. Inhibition of both was owed at least in part to production of transforming growth factor (TGF)-β by the ESCs. Thus, murine ESCs exert "immunosuppression" locally, enabling engraftment across allogeneic barriers [53]. Interestingly, ESC-derived HSCs have been shown to poorly express MHC antigens allowing for their long-term engraftment in sublethally irradiated recipients across MHC barriers without the need for immunosuppression. Low level chimerism was maintained in the BM of these mice over 100 days and chimeric animals were protected from rejection of donor-type cardiac allografts [12]. Verda et al. [94] demonstrated that ESC-derived HSC could produce islet cell tolerance, a phenomenon termed graft versus autoimmunity (GVA), and may thus be used to prevent autoimmune DM in NOD mice without causing graft-versus-host disease (GVHD) or other adverse side effects. This study represents the first instance of ESC-derived HSCs treating disease in an animal model [94].

HSCs: HSCs have long been used for BM reconstitution following myeloablation. Recent studies suggest that adult HSCs can reintroduce tolerance to autoantigens. HSC chimerism achieved through BM transplantation may affect T1D in two ways: (i) inducing tolerance to pancreas and islet cell transplants and (ii) reversing the autoimmune process prior to the development of terminal complications. Transplantation of BM from normal donors into patients with haematologic malignancy and coexistent T1D has reversed the systemic diabetic autoimmune process [25]. A more recent clinical study by Voltarelli et al. [95] tested safety and metabolic effects of high-dose immunosuppression followed by non-myeloablative adult HSC

transplantation (AHST) in 15 newly diagnosed T1D patients treated within 6 weeks before diagnosis. As reported, over a mean follow-up period of 18.8 months AHST led to independence of exogenous insulin between 1 and 35 months in 14 enrolled patients, aged between 14 and 35 years. An increase in C-peptide AUC (mean total area under the curve) and lower levels of haemoglobin A_{1c} and GAD65 antibodies were reported in 13/14 patients. However, severe adverse effects including pneumonia and late endocrine dysfunction in a total of three patients currently hamper a wider application of this approach. Although being still a matter of debate and controversy among stem cell scientists, there are numerous reports which state that HSCs may also be able to switch lineage and, therefore, be a convenient source of stem cells for islet cell regeneration and repopulation [17].

MSCs: The use of MSCs as a potential therapeutic strategy for T1D with emphasis on immunomodulation has recently been reviewed extensively [1]. Possible mechanisms by which MSCs regulate immune responses include (i) reducing the generation and differentiation of DCs; (ii) increasing the number of Tregs through production of TGF-β, or promoting the generation of regulatory DCs producing IL-10; and (iii) suppressing effector T cells through various growth factors, inducible nitric oxide synthase (iNOS), heme oxygenase (HO)-1, prostaglandin, or indoleamine 2,3-dioxygenase (IDO). MSCs may also act through downregulation of immunoglobulin production by B cells and by inhibiting NK cell cytotoxicity and proliferation [1].

Until now there are only few reports on the use of MSCs in animals models of diabetes. Lee et al. [62] employed streptozotocin-diabetic NOD/scid mice to study the effects of human MSCs (hMSCs) in DM development. They observed lower glucose and higher mouse insulin levels in the blood of hMSC-treated as compared with untreated diabetic mice. In the former, there was an increase in pancreatic islets and β-cells producing mouse insulin, but only few islets contained human cells that colabelled for human insulin or Pdx-1. Most of the β-cells in the islets were mouse cells that expressed mouse insulin. In kidneys of hMSC-treated diabetic mice, mesangial thickening and macrophage infiltration were decreased, and a few of the human cells appeared to have differentiated into glomerular endothelial cells. The results raised the possibility that hMSCs may be useful in enhancing insulin secretion and perhaps improving the renal lesions that develop in patients with DM. Itakura et al. [41], who examined the potential of MSCs in the induction of chimerism and islet allograft tolerance, noted that engraftment of the primary islet allografts with stable chimerism was achieved by the addition of a 2-week peritransplant administration of 15-deoxyspergualin. GVHD was not observed in any of the recipients infused with MSCs. These results indicate a potential use of MSCs for induction of haematopoietic chimerism and subsequent immune tolerance in clinical islet transplantation [41]. In the most recent publication, Boumaza and co-workers [14] induced sustained normoglycaemia by administering, following or prior to diabetes induction, in vitro-expanded syngeneic BM-derived rat MSCs into a rat model of streptozotocin-induced β-cell injury, suggesting that the bioactive factors secreted by MSCs support β-cell activation/survival in the pancreas. These authors observed that MSCs homed to the pancreas and enhanced insulin secretion consistent with

higher levels of Pdx-1 in the islets. Notably, peripheral T cells exhibited a shift towards the Th2 phenotype and higher frequencies of Foxp3-positive cells, suggesting anti-inflammatory and immunoregulatory effects on T cells. While at the time of this article's submission, ~60 clinical trials involving application of MSCs, were listed at http://www.ClinicalTrials.gov, results from clinical trials for T1D with MSCs have not yet been reported. However, a study sponsored by the Juvenile Diabetes Research Foundation and Osiris Therapeutics is currently in the phase of recruiting participants to evaluate the immunomodulatory effects of Prochymal, a formulation of immunomodulatory adult BM-derived MSCs, for the treatment of recently diagnosed T1D.

Splenocytes: One of the most provocative findings in recent years postulated transdifferentiation of splenocytes to form new β-cells as a future strategy to reverse T1D [54]. Unfortunately, three independent studies were unable to confirm these results and ruled out donor splenocytes to contribute to islet recovery [23, 68, 82]. However, the presence of surviving islets and functional β-cell mass in female NOD mice that had been diabetic for 2–3 weeks is more likely explained by immunomodulation during early stages of diabetes which allows for some preservation of β-cell function, the extent of which depends on the degree of injury before treatment is initiated. It now appears that with the treatment protocol of Kodama et al. [54] allogeneic splenocytes eliminated a significant fraction of preexisting diabetogenic T cells. More recent studies using the same protocol report on the presence of diabetogenic T cells which form a peri-islet lymphocyte infiltration, but appear to be in a "quiescent" state, unable to further damage adjacent islet tissue [82].

28.7 Own Work

In order to circumvent the necessity of non-myeloablative conditioning regimens with subsequent reconstitution of the recipient's immune system with donor BM or blood borne HSCs, we have focussed on peripheral tolerance induction by use of a subpopulation of macrophage-like cells with tolerogenic properties. It has been known for about 30 years that macrophages can suppress T lymphocyte activation [69]. A hallmark of myeloid cells is their ability to adopt different states of activation with both the potential to activate or to inhibit T-cell responses. It is generally agreed that mature DCs are the most potent APCs for T lymphocyte activation, while immature DCs can induce antigen-specific T-cell anergy and tolerance [67]. Monocyte/macrophages can be phenotypically polarized by the microenvironment to undergo specific functional activation programs. Polarized macrophages can be broadly classified into classically activated macrophages (or M1), whose prototypical activating stimuli are γIFN and lipopolysaccharide, and alternatively activated macrophages (or M2). M1 exhibit potent microbicidal properties and promote strong IL-12-mediated Th1 responses, while M2 support Th2-associated effector functions. Beyond infection M2 polarized macrophages play a role in resolution of inflammation through high endocytic clearance capacities and trophic

factor synthesis, accompanied by reduced proinflammatory cytokine secretion [64]. A large body of work has been devoted to the characterization of the phenotype and function of suppressive macrophages. Possible modes of action include direct suppression of T-cell proliferation through the production of IDO and the subsequent catabolism of the essential amino acid tryptophan [65]. An alternative mechanism is the generation of Tregs in order to promote peripheral tolerance.

As mentioned above, following injection of RESC into the portal vein of non-immunosuppressed allogeneic recipient animals, we identified a particular subset of monocytes with tolerogenic properties derived from the inoculated RESC [27]. Comparative marker analysis identified a similar subpopulation of macrophages in the mononuclear fraction of spleen, blood, and BM of rats. These cells, designated TAICs (for transplant acceptance inducing cells), were equally capable of promoting allograft acceptance in a rat model of heart transplantation (Fig. 28.2).

Generation of these monocyte-derived cells is achieved after initial adherence to cell culture plastics and subsequent culture in the presence of the macrophage growth factor M-CSF (macrophage colony-stimulating factor) for 5 days and stimulation with γIFN on day 4 for 24 hours. A recently published series of manuscripts describe this type of immunoregulatory macrophages in the setting of living-related and unrelated kidney transplantations [38, 39, 40]. Preoperative injection of TAICs, defined as CD14low/+CD13+CD33+HLA-DR+CD80lowCD83-CD206+ cells, was able to induce a semi-stable state of alloantigen-specific graft tolerance which allowed for stable minimization of immunosuppressive drugs in transplant

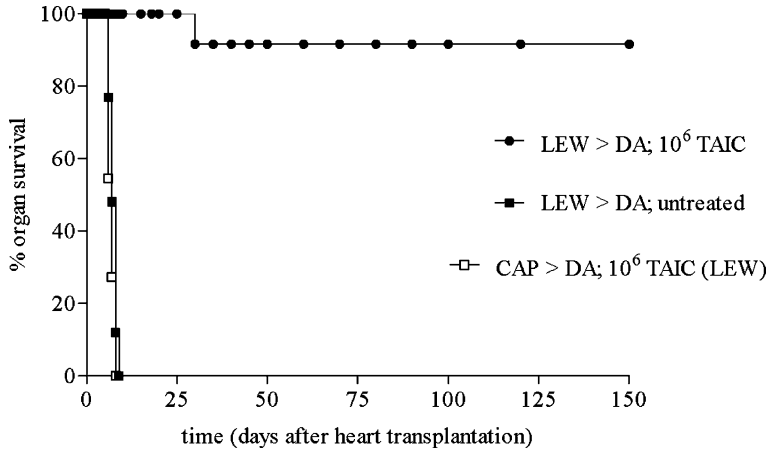

Fig. 28.2 Allogeneic TAICs induce donor-specific tolerance in rats. Kaplan–Meier survival plots of Dark–Agouti (DA) recipient rats heterotopically transplanted intraabdominally with Lewis (LEW)-donor hearts. Intravenous injection of 5×10^6 donor TAICs of donor origin 7 days prior to transplantation induced long-term (>150 days) tolerance, whereas untreated DA rats acutely rejected donor heart grafts within 7 days following transplantation. Graft acceptance was donor specific as transplantation with third-party heart grafts of CAP inbred rats into LEW-donor TAIC-primed DA recipient rats did not prolong allograft survival

recipients. These experimental observations in animals and man inspired the notion that autologous γIFN-induced macrophage-like cells could be equally useful as a therapeutic tool for causative treatment of autoimmune diseases.

Regarding their mechanism of action, TAICs have direct effects on alloreactive T cells which are depleted in an antigen-specific manner which – for the time being – appears to be related to induction of apoptosis. In addition, TAICs appear to expand the pool of Tregs in culture. The suppressive potential of TAICs to kill allogeneic cytotoxic T cells as tested in vivo and in vitro (manuscript in preparation) was additionally investigated in two different models of experimentally induced colitis [15]. As demonstrated in a dextran sodium sulphate (DSS)-driven colitis model, a single injection of 5×10^6 M – CSF/γIFN-stimulated monocytes generated from either BM, spleen, or blood into mice with overt colitis (after 5 rounds of DSS application) was sufficient to reverse the disease process and restore gut function and morphology in the majority of mice [15]. In a T-cell-triggered (adoptive transfer of CD62L+ T cells) scid mouse model of colitis, a single injection of these tolerogenic monocytes reliably reversed the autoimmune process and more than 80% of treated animals fully recovered within 21 days after intravenous injection. Since during culture of these tolerogenic monocytes, designated here as self-tolerance inducing cells (STICs), the number of contaminating lymphocytes (derived from the mesenteric lymph follicles of DSS-induced colitis animals) dramatically declined over time, it is assumed that the underlying mechanism involves deletion of alloreactive T cells. Time course experiments demonstrated a near-linear reduction in lymphocyte numbers over time reaching completion after 48 hours. A comparison of lymphocyte killing showed no difference between stimulator lymphocytes derived from colitis mice and those from healthy animals. However, non-activated lymphocytes, as judged by physical characteristics and lack of CD25 expression, prevailed in culture and were not phagocytosed within the observation period of 48 hours [15]. Consistent with this idea is the finding that the T-cell inhibitor cyclosporine was able to block lymphocyte killing in coculture experiments with autologous STICs. In addition, concanavalin A-stimulated lymphocytes cocultured with STICs were more rapidly deleted. We conclude from these observations that STICs actively delete activated T cells independently of their antigenic specificity. Furthermore, it was shown that lymphocyte killing was mediated via a caspase and cell contact-dependent mechanism as demonstrated by use of the general caspase inhibitor zVAD-FMK and coculture experiments, respectively. In these experiments, no detectable killing of colitis lymphocytes ensued. The use of PD-L1 blocking Abs or preparing STICs from PD-1 knock-out mice was unable to block deletion of activated T cells. Likewise, STICs generated from IDO-deficient mice, or blocking iNOS by use of N6-(iminoethyl)-L-lysine, had no effect on T-cell elimination in coculture experiments [15].

Another set of experiments addressed the fate of lymphocytes that survived coculture with γIFN-stimulated monocytes. Coculture with STICs expanded the pool of CD4+/CD25+(high) lymphocytes which were also positive for cytoplasmic CTLA-4, CD103, and Foxp3 expression, whereas control lymphocyte populations grown in the absence of STICs or the presence of control monocytes did not show

enrichment of Tregs. At a functional level, these CD4+/CD25+ T cells isolated from STIC cocultures were able to block polyclonally activated T cells (stimulated with CD3/CD28 mAbs), an observation which was unique to this CD4+/CD25+ double-positive population as CD4+/CD25- single-positive T cells failed to inhibit T-cell proliferation in comparable experiments. Hence, we assume that STICs promote the expansion of regulatory CD4+T cells. Based on results from similar in vitro experiments with STICs of human origin, human STICs might share this ability with their murine counterparts.

In preliminary experiments M-CSF-expanded and γIFN-pulsed monocytes were used in a model of rheumatoid arthritis (Edward K. Geissler, unpublished observation) and, in order to prevent overt diabetes, in NOD mice (our own work). In both models the clinical outcome underlines the feasibility of the concept to balance immune dysfunction with regulatory macrophages as an efficacious treatment [44]. Intravenous injection of 5×10^6 mouse-derived autologous STICs substantially improved the clinical disease score in both models. Interestingly, STIC injection administered intravenously at week 8 (before the onset of DM) was able to prevent clinical manifestation of the disease in 60% of treated recipients, whereas no treatment or treatment with autologous control monocytes (not exposed to M-CSF and γIFN) caused DM in 100 and 70% of animals, respectively (Fig. 28.3).

TAICs and STICs are generated from peripheral blood monocytes by a protocol very similar to that for generation of the stem cell-like PCMOs (see chapter 29). Not surprisingly, TAICs and PCMOs share many features such as adherent growth,

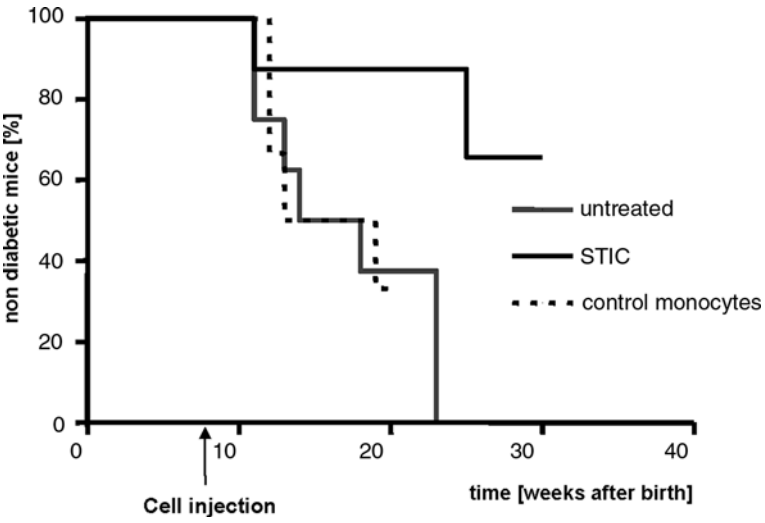

Fig. 28.3 Treatment with autologous STICs can prevent DM-related autoimmunity. Intravenous injection of 5×10^6 autologous STICs into NOD mice at week 8 after birth prevents overt diabetes and hyperglycaemic blood glucose levels in approximately 60% of female mice. In contrast, 100% of untreated animals and 70% of animals injected with 5×10^6autologous control monocytes (not exposed to M-CSF and γ-IFN) suffer from severe DM

flat morphology, and a (partially) dedifferentiated phenotype [39, and unpublished observations]. Interestingly, while PCMOs share with MSCs some non-haematopoietic markers, adherent growth, the capacity for (limited) self-renewal, and differentiation into tissues of mesodermal origin (see chapter 29), TAICs and STICs share with MSCs the ability to induce (antigen-specific) Tregs and to suppress effector T cells. Since monocyte-derived cells can be alternatively endowed with either stem cell-like or tolerogenic properties, it may even be possible to combine both within the same cell, providing a convenient cellular source for both tolerance induction and islet cell regeneration.

Taken together, future strategies are being developed to provide tools to stop autoimmune processes causing β-cell destruction and DM. The clinical goal to restore tolerance to autoantigens and to reorchestrate autoaggressive T and B cells within the lymphocyte compartment will be achieved by use of innovative, individualized treatment strategies. These take advantage of newly designed monoclonal antibodies and autologous (stem) cell types with regulatory properties to retune peripheral tolerance. Efficient multidisciplinary translation of new groundbreaking results in the fields of immune tolerance and stem cell biology may thus pave a new avenue for patient-customized protocols which circumvent the long-term need for insulin replacement. Consequently, severe late complications as caused by DM will be spared for the benefit of our patients.

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Chapter 29
The Programmable Cell of Monocytic Origin (PCMO): A Potential Adult Stem/Progenitor Cell Source for the Generation of Islet Cells

Hendrik Ungefroren and Fred Fändrich

Abstract Adult stem or programmable cells hold great promise in diseases in which damaged or non-functional cells need to be replaced, such as in type 1 diabetes. We have recently demonstrated that peripheral blood monocytes can be differentiated in vitro into pancreatic β-cell-like cells capable of synthesizing insulin. The two-step phenotypic conversion commences with growth factor-induced partial reprogramming during which the cells acquire a state of plasticity along with expression of various markers of pluripotency. These cells, termed "programmable cells of monocytic origin" (PCMOs), can then be induced with appropriate differentiation media to become insulin-producing cells (NeoIslet cells). Expression profiling of transcription factors known to determine endocrine and β-cell development in vivo indicated that NeoIslet cells resemble cells with an immature β-cell phenotype. Current efforts focus on establishing culture conditions that (i) increase the plasticity and proliferation potential of PCMOs by enhancing the reprogramming process and (ii) improve insulin production by mimicking in vivo lineage specification and normal pancreatic endocrine development. Combining these two strategies has great potential in generating large amounts of blood-derived cells suitable for both autologous and allogeneic therapy of type 1 diabetes.

Keywords β-cell · Differentiation · NeoIslet cell · PCMO · Stem cell

Abbreviations

BMP	bone morphogenetic protein
EGF	epidermal growth factor
FGF	fibroblast growth factor
ESC	embryonic stem cell
IL-3	interleukin-3
HGF	hepatocyte growth factor

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M-CSF	macrophage colony-stimulating factor
MSC	mesenchymal stem cell
PCMO	programmable cell of monocytic origin
RA	retinoic acid
RT-PCR	reverse transcription polymerase chain reaction
Shh	sonic hedgehog
TGF-beta	transforming growth factor-beta

29.1 Introduction

In both type 1 and type 2 diabetes mellitus, insufficient numbers of insulin-producing β-cells are a major cause of defective control of blood glucose, ultimately resulting in a variety of severe complications and an overall shortened life expectancy. Replacement of insulin-producing cells represents an almost ideal treatment for patients with type 1 diabetes. Reversal of diabetes can be achieved through (i) transplantation of pancreas and islet which, although being successful in experienced centres, cannot be applied widely because of the overall shortage of donor organs and (ii) stimulation of endogenous regeneration of the β-cell mass or the proliferation of β-cells in vivo or in vitro, which requires an understanding how β-cells maintain themselves in the adult pancreas. There has been much debate over whether β-cell proliferation, as a means of self-renewal, predominates over the existence and differentiation of a pancreatic stem cell or progenitor cell population [22]. These drawbacks prompted an intensive search for alternative sources of β-cells/insulin-producing cells for transplantation therapy in treating diabetes, such as human β-cell lines, or through the guided differentiation of stem or precursor cell populations. Stem cells are progenitor cells which are pluri- or multipotent and possess the capacity of self-renewing (and hence represent a potentially inexhaustible source) and differentiation in fully mature cells depending on the culture conditions. Stem cells with the potential to differentiate into insulin-producing cells include both embryonic and adult stem cells. The use of human embryonic stem cells (ESCs) is hampered by ethical concerns, but research with these types of cells may help us to decipher important steps in the differentiation process in vitro since almost all information available on pancreas development is based on animal studies [56]. The possibility of generating insulin-secreting cells with adult pancreatic stem or progenitor cells has been investigated extensively (reviewed by Bonner-Weir and Weir 2005 [2]). Alternatively, adult stem cells from other tissues including the liver, intestine, bone marrow, adipose tissue, and brain may be used. Yet another option is the transdifferentiation of more plentiful adult somatic differentiated cell populations, like the conversion of hepatocytes, or exocrine pancreatic duct, or acinar cells into β-cells [52, 53].

Several studies have reported the generation of insulin-secreting cells from ES and adult stem cells that normalized blood glucose values when transplanted into diabetic animal models. Due to β-cell complexity, insulin-producing cells generated

from stem cells do not possess all β-cell attributes. This indicates the need for further development of strategies and methods for differentiation and selection of completely functional β-cells. Recent progress in generating insulin-producing cells from ESCs has shown promise, highlighting the potential for trying to mimic normal developmental pathways which, however, requires a thorough understanding of pancreas development and β-cell formation [1]. Pancreas development is coordinated by a complex interplay of signaling pathways and transcription factors that determine early pancreatic specification and the later differentiation of exocrine and endocrine lineages as well as factors that relate specifically to the emergence of endocrine β-cells from pancreatic endoderm [29]. Current therapeutic efforts to generate insulin-producing β-cell-like cells from ESCs have already capitalized on recent advances in our understanding of the embryonic signals and transcription factors that dictate lineage specification and will most certainly be further enhanced by a continuing emphasis on the identification of novel factors and regulatory relationships. Although fully functional islets have not yet been derived from any stem cells, the use of stem cells is still the most promising approach on the way to establish a treatment protocol for the cure of type 1 diabetes in the future [37].

In patients with type 1 diabetes, autoreactive T cells are programmed to recognize the insulin-producing β-cells, and current therapeutic strategies for type 1 diabetes therefore also focus on an arrest of autoimmunity. Hence, for therapeutic replacement tissues, it may be more sensible to derive cells from non-β-cell origin that behave like β-cells but avoid the autoimmune response [8]. However, diabetic patients may benefit also from therapeutic strategies based on autologous stem cell therapies addressing late diabetic complications [40]. Autologous cell material for transplantation may be derived from human ESCs generated by somatic cell nuclear transfer, induced pluripotency (see below), or from adult stem cell populations such as bone marrow-derived stem cells. There is mounting evidence that candidate stem cells residing in the haematopoietic compartments, such as autologous self-renewing rat mesenchymal stem cells (MSCs), participate in regeneration of pancreatic islets following chemical and autoimmune β-cell injury in vivo [5]. The apparent major mechanisms include immunomodulation, revascularization, support of endogenous β-cell regeneration and (trans)differentiation into units capable of sensing, producing, and secreting insulin [5**,** 8]. Transdifferentiation or *de*differentiation and subsequent *re*differentiation of adult somatic cells into insulin-producing cells represent another interesting option. In its most extreme variation this process requires dedifferentiation of an adult differentiated somatic cell type towards a pluripotent intermediate, equivalent to an ESC or a pluripotent cell generated by somatic cell nuclear transfer. This has in fact been achieved through a novel technique called “induced pluripotency” simply by virus-mediated ectopic expression of only four transcription factors, namely Oct4 (also known as Pou5f1), Sox2, Klf4, and c-Myc [42]. Once the protocols have been improved to generate induced pluripotency without Myc [19] and genome-integrating viruses [41] to circumvent potential risk of carcinogenesis, it may find wide application for engineering β-cells of autologous origin. Zhou and colleagues recently employed a different strategy for directing cell reprogramming without reversion to a pluripotent stem cell state,

e.g. direct conversion into other mature cells or progenitors by re-expressing key developmental regulators in vivo. A specific combination of the three transcription factors Neurogenin3 (Ngn3), Pdx1, and MafA was identified that reprogrammes differentiated pancreatic exocrine cells in adult mice in vivo into cells that closely resemble β-cells with respect to phenotype, gene expression, and function [55]. As outlined in the next section, we have generated from human peripheral blood monocytes (which originally come from the bone marrow) in vitro and by nongenetic means a stem cell-like cell that can be used for the generation of insulin-expressing cells.

29.2 The "Programmable Cell of Monocytic Origin" (PCMO): A Partially Dedifferentiated Monocyte with Stem Cell Characteristics

The peripheral blood monocyte is an extraordinarily versatile progenitor cell that gives rise to very diverse cell types. It ultimately derives from the haematopoietic stem cell, which is the precursor of the common myeloid progenitor (CMP). From the CMP arises the granulocyte/monocyte progenitor which represents the precursor population for monoblasts. Monoblasts are the earliest form committed to becoming monocytes and having differentiated by stages to monocytes, their progeny emigrate from the bone marrow into the peripheral blood. Peripheral blood monocytes, when appropriately stimulated, will migrate to sites of inflammation and extravasate into the tissues, acquiring the characteristics of an activated macrophage. Alternatively, when not recruited to inflammatory lesions, monocytes are able to undergo a time-dependent maturation into several classes of tissue-resident macrophages [14].

Several cultured human cell populations that originate from circulating monocytes and have the capacity to differentiate into non-phagocytes have been described [21, 32, 54]. Recently, we have developed a protocol to induce from human monocytes by in vitro culture an apparently more plastic derivative, which we named "*p*rogrammable *c*ell of *m*onocytic *o*rigin" (PCMO). These cells following a 6-day treatment with macrophage colony-stimulating factor (M-CSF) and interleukin-3 (IL-3) can be induced upon exposure to appropriate differentiation media to convert into cells resembling endothelial cells [17], chondrocytes [30], and osteoblasts [26]. Our particular interest has been in PCMO-derived gastrointestinal cells like insulin-expressing cells (NeoIslets) and hepatocyte-like cells (NeoHeps). NeoHeps express a variety of hepatocyte markers which closely correlate with induction of hepatocyte-specific functions [34, 35, 12] making these cells an attractive alternative to primary human hepatocytes for studying drug metabolism in vitro [12]. Recently, it was claimed that NeoHeps improve survival in a rat model of acute liver failure [13], and monocyte-derived cells show promise in the treatment of decompensated liver disease [49, 17]. NeoIslet cells upregulate not only the insulin and glucagon genes but also transcription factors involved in pancreatic β-cell differentiation ([34] and see below).

Various mechanisms have been implicated in the acquisition of plasticity by adult somatic cells, such as *trans*differentiation, *de*differentiation to a more stem cell-like progenitor and subsequent redifferentiation along a new lineage pathway, or cell fusion [46]. During dedifferentiation, cells silence tissue/cell type-specific genes and eventually reacquire more primitive features, such as expression of markers of self-renewal and pluripotency. This is consistent with previous observations that M-CSF/IL-3-conditioned monocytes silence various genes encoding monopoietic transcription factors such as *PRDMI* (the human homologue of murine *BLIMP-1*), *ICSBP/IRF8* [34], and *Klf4*. Furthermore, they downregulate markers associated with specialized (immune) functions of monocytes or mediators of monocyte → macrophage differentiation, such as CD14 surface expression, toll-like receptors 2, 4, 7, and 9, and $p47^{phox}$, an essential subunit of the reactive oxygen species-producing enzyme NADPH oxidase (H.U., manuscript submitted). Furthermore, PCMOs endogenously express various markers of human ESCs, namely Oct4 (including Oct4A, the isoform associated with pluripotency), Nanog, Klf4, and Myc, but lack expression of Sox2. Interestingly, induction of both *Nanog* and *Oct4* coincided with transient changes in histone modifications indicative of transcriptional (re)activation and with sensitivity to tissue-specific differentiation. Indeed, PCMOs appear to resemble in some aspects partially reprogrammed cell lines [25] in that they reactivate genes related to stem cell renewal and maintenance (e.g. *Myc*), but only few pluripotency genes (*Oct4*, *Nanog*, but not *Sox2*), and incompletely repress lineage-specific transcription factors (e.g. PU.1). These results show that in appropriate growth factor environment peripheral blood monocytes can, at least partially, be reprogrammed without exogenous introduction of pluripotency factors.

More robust regeneration of the pancreas depends largely on neogenesis from precursor cells, which can be derived from stem cells or from differentiated pancreatic duct epithelial cells [3, 4]. Bonner-Weir and colleagues convincingly demonstrated that the latter cells act as progenitors in the adult rat pancreas and can give rise to new islets after injury. Using duct-specific lineage tracing experiments they showed that following partial pancreatectomy the pancreatic ductal cells first resume proliferation and dedifferentiate to a less restricted progenitor and subsequently differentiate to form new acini and islets [4]. A similar series of events namely reversion to a less differentiated state prior to formation of the new cell type have been observed in committed B- and T-lymphoid cells from mice which can be reprogrammed to functional macrophages through expression of C/EBPα and PU.1 [48]. In fact, pancreatic ductal cells from mice can also undergo lineage switching through direct conversion in vivo by adenoviral transduction of Pdx1, Ngn3, and MafA, into endocrine β-cells [16, 55]. The generalized loss of monocyte/macrophage marker expression indicates that PCMOs, like pancreatic duct epithelial cells, represent progenitor cells with less restricted differentiation potential and that the possible formation of insulin-producing cells from monocytes, like β-cell neogenesis from pancreatic duct cells, would be a dedifferentiation rather than a transdifferentiation event [4]. The generation of insulin-producing cells from

adult circulating monocytes (whose primary function is not related to that of β-cells) could offer an option for cell replacement therapy, permitting the patient to be the donor of his own insulin-producing tissue.

Circulating monocytes have some practical advantages over other types of adult stem/progenitor cells when to be used for therapy of diabetes: (I) They are obtainable from a readily accessible body compartment by a less invasive procedure or are incurred as waste products in blood donations, and can be maintained in culture. (II) They can be applied in both autologous and allogeneic settings. (III) They may potentially avoid the autoimmune response since they are of non-β-cell origin. (IV) They have a low risk of tumorigenicity because of their limited proliferative activity and lack of hTERT expression (H.U., manuscript submitted).

The low proliferation potential, however, also represents a serious disadvantage when attempting to increase cell yields for transplantation purposes. To be clinically relevant, expansion and differentiation conditions must be optimized towards the production of large amounts of cells from one single donor, sufficient to treat one diabetic patient, or even better, several diabetics. Therefore, one main goal is to enhance the cells' proliferation potential during PCMO culture without impairing their differentiation potential towards NeoIslet cells.

29.3 In Vitro Differentiation of PCMOs to Insulin-Expressing Cells (NeoIslet Cells)

In this part we will review available data on the differentiation of insulin-expressing cells from monocytes including protocols of isolation and in vitro culture, expression of β-cell markers, and insulin secretion in vitro and in vivo. Since the generation of insulin-expressing cells has not yet been reported from other groups using monocytes as multipotent progenitors, Sections 29.3.1–29.3.3 essentially contain already published data from our own group. In Section 29.3.4 we shall devise some general strategies as to how to improve the β-cell phenotype of NeoIslets and increase their yield with soluble factors. Here, we have focussed on members of the TGF-beta/activin superfamily of growth and differentiation factors which are known to exert growth-suppressive function on monocytes and promote both definite endoderm formation and β-cell differentiation during development [36]. From this work, which is still in progress, we have included some unpublished original data.

29.3.1 General Protocol

The generation of PCMOs and NeoIslet cells from peripheral blood monocytes was described in detail by Ruhnke et al. [34]. In brief, cells are isolated from healthy donors by density gradient centrifugation and allowed to adhere to tissue culture plastics for 1–2 h in RPMI 1640 medium containing human AB serum, followed by removal of non-adherent cells by aspiration. The remaining cell population, which

typically consists of 70–80% monocytes (as determined by flow cytometry with anti-CD45 and anti-CD14), is cultured for 6 days in "dedifferentiation medium" consisting of the same medium as above supplemented with 2-mercaptoethanol, M-CSF, and IL-3. For differentiation into NeoIslet cells day-6 PCMOs are cultured for 7–10 days in RPMI 1640 medium containing fetal calf serum, epidermal growth factor (EGF), hepatocyte growth factor (HGF), nicotinamide, and low glucose. The NeoIslet cell differentiation agents were chosen according to available in vivo and in vitro data. Evidence for an important role of EGF in β-cell formation in vivo came from EGF-R-deficient mice: The most striking feature of the EGF-R(–/–) islets was that proliferation and differentiation of the neonatal EGF-R(–/–) β-cells was significantly reduced [24]. Through its receptor c-met HGF promotes glucose-dependent insulin secretion, and β-cell proliferation and survival [9, 31]. The poly(ADP-ribose) polymerase (PARP) inhibitor nicotinamide decreases proliferation and induces in vitro differentiation into insulin-secreting cells from mouse ESCs [45], adult rat hepatic oval stem cells [50], and fetal pancreatic cells. Glucose is required for pancreatic endocrine cell differentiation [15] and in vitro transdifferentiation of adult rat hepatic oval cells into pancreatic endocrine insulin-producing cells [50].

29.3.2 Profiling of β-Cell Markers in NeoIslet Cells

Treatment of PCMOs with NeoIslet differentiation medium for 4–8 days resulted in the formation of cell aggregates that resembled islets generated in vitro from pancreatic stem cells [34]. A significant fraction of cells in these clusters stained positive for insulin and glucagon. RT-PCR analysis confirmed endogenous expression of insulin, glucagon, and the glucose transporter glut-2 in NeoIslet cells but not in PCMOs [34]. Moreover, we detected elevated expression of several transcription factors involved in early β-cell differentiation such as Ngn3, Nkx6.1, and Beta2/NeuroD. High expression of NeuroD and Ngn3, the latter of which is also transiently expressed during formation of pancreatic-type endocrine cells from the biliary duct epithelium [11], combined with low expression of Pdx1 indicates that NeoIslet cells represent an early stage of endocrine cell differentiation, reminiscent of the common alpha and beta progenitors. NeoIslets also express transcription factors involved in the regulation of the insulin gene (NeuroD and MafA, the latter being absolutely required for transcription of *INS*), and of proglucagon gene transcription (c-Maf, Pax6, Cdx-2, Hnf3β, and Nkx2.2), suggesting that some NeoIslet cells have differentiated towards the α-cell phenotype. Studies by Noguchi and co-workers [27] suggest that overexpression of Pdx-1, Ngn3, Pax4, or NeuroD facilitates differentiation into insulin-expressing cells from pancreatic stem/progenitor and adult human primary duct cells. Since NeuroD was the most effective inducer in this respect [27], its expression might be a suitable surrogate marker to rapidly screen for functionally improved NeoIslet cells (see Section 29.3.4). In the course of NeoIslet cell marker analysis we also noted upregulation of the mRNA for ALK7, a

receptor serine/threonine kinase that is expressed in neuroendocrine tissues including pancreatic islets and functions as a type I receptor for activins [44]. The combination of ActRIIA and ALK7, preferred by activin AB and –B, but not activin A, is responsible for activin-mediated secretion of insulin from the β-cell line MIN6 [44]. It remains to be seen, however, whether specific stimulation of ALK7 on NeoIslets with activins can enhance glucose-stimulated insulin release from these cells.

29.3.3 In Vitro and In Vivo Functions of NeoIslet Cells

After a 4-day treatment with NeoIslet cell differentiation medium, the total insulin and C-peptide contents of pelleted cells (mean ± SD) were 0.87 ± 0.10 ng/μg protein (n = 5) and 0.89 ± 0.07 ng/μg protein (n = 5), respectively. For comparison, the insulin content of mature β-cells is approximately 44 ± 14 ng/μg protein. Increasing the glucose concentration in the NeoIslet cell medium from 3 to 22 mM stimulated insulin and C-peptide secretion [34]. The still immature phenotype of these cells in vitro may explain both the lower expression and secretion of insulin relative to that of isolated human islets. To assess the ability of human NeoIslet cells to function in vivo, their capacity to correct hyperglycaemia was investigated in the established streptozotocin diabetic mouse model. Following implantation of human PCMO-derived NeoIslet cells, correction of hyperglycaemia was observed within 2 days in the recipient, but not in control animals. The recipients remained normoglycaemic for another 8 days after transplant, a time at which cellular rejection started to occur [34]. As in NeoIslet cells, induction of β-cell-specific genes and low insulin secretion of the differentiated cells in vitro have been achieved in human bone marrow MSCs. Despite low insulin secretion, the cells were also capable of reversing hyperglycaemia when transplanted into streptozotocin diabetic mice [43]. One of several possible explanations of these findings is that the microenvironment can further enhance differentiation in vivo, which is supported by the observation that the transplanted NeoIslet cells exhibited strong immunostaining for insulin [34].

29.3.4 Strategies to Improve NeoIslet Cell Phenotype and Function

A strategy that appears to have long-term potential is to design differentiation procedures based on the ontogeny of the β-cell. The focus of this strategy is to recapitulate the molecular mechanisms governing in vivo lineage specification and normal pancreatic endocrine development/the maturation of a β-cell and to use them as a guide in directing the in vitro differentiation of embryonic or adult stem cells. A research group at Novocell Inc. has developed a five-step process that, using a differentiation procedure (growth factors and various culture conditions) that mimics the signaling that occurs during gastrulation, allowed progression from undifferentiated human ESCs through successive cell fate restrictions to definitive endoderm to

hormone-expressing endocrine cells with high efficiency [10]. Definite endoderm was generated by exposing cells to activin A and Wnt3a, followed by sequential treatment of the definite endoderm with keratinocyte growth factor, retinoic acid (RA), Noggin, and the Sonic hedgehog (Shh) inhibitor KAAD-cyclopamine to generate endocrine precursors [10]. Moreover, pancreatic endoderm derived from human ESCs generated glucose-responsive insulin-secreting cells after implantation into mice [20]. Serafimidis et al. [38] recently established an analogous protocol for the generation of endocrine pancreatic cells from mouse ESCs in combination with forced regulated expression of Ngn3. They combined embryoid body formation and activin A treatment to potentiate definite endoderm specification of ESCs. Subsequently, activin A was combined with FGF4 to induce anterior gut fates. Since pancreatic endoderm specification in vivo is mediated through RA signaling and repression of Shh signaling, ESC-derived definitive endoderm was treated with RA and cyclopamine. Expansion of the forming pancreatic progenitors was then enhanced with FGF10 and BMP4. Subsequent induction of Ngn3 expression at this stage displayed a decisive role in directing ESC differentiation towards the endocrine lineage through regulation of the Wnt, integrin, Notch, and TGF-beta signaling pathways and changes in cell motility, adhesion, the cytoskeleton, and the extracellular matrix. Interestingly, the successive application of these signals was required to generate progenitor cells that responded properly to Ngn3 induction by activation of downstream Ngn3 target genes and only those cells gave rise to insulin-positive cells upon terminal differentiation [38].

The future challenge is to adapt these differentiation procedures to PCMOs. In order to fully mimic β-cell function cells need to be equipped with a well-developed secretory apparatus for regulated hormone secretion, and it is hard to envision that such a complex structure can form in a non-endocrine cell type within only a few days during direct conversion or lineage switching. Although NeoIslet cells are capable of secreting insulin and C-peptide in a glucose-dependent manner [34], in terms of magnitude their response was by far not comparable to that of pancreatic β-cells. The secretory "hardware" may only develop from a sufficiently primitive precursor following directed endodermal differentiation to an endocrine phenotype. Therefore, the PCMO starting population has to have sufficient stem cell character to first allow for differentiation of an endoderm progenitor and ultimately an endocrine cell. Consequently, we are currently pursuing two strategies to improve the β-cell phenotype of NeoIslet cells: (I) qualitative enhancement of NeoIslet cell differentiation through factors known to promote β-cell differentiation from other stem cell/precursor types, particularly ESCs with or without ectopic expression of lineage determining transcription factors such as Ngn3, Pdx1, or Ptf1a and (II) enhancement of PCMO plasticity and proliferation potential through factors promoting self-renewal and pluripotency and/or by genetic complementation of monocytes/PCMOs with pluripotency-determining transcription factors. This will also involve avoidance of activating/differentiation stimuli (proinflammatory agents, bacterial components) which might prevent proper dedifferentiation and/or expansion of monocytes.

(I) Improvement of the NeoIslet cell phenotype: As mentioned above, TGF-beta1 acting through the TGF-beta type I receptor (also called ALK5) inhibited the development of acinar tissue and promoted the development of endocrine cells, in particular of β-cells [36]. Likewise, activins, acting through the type I receptors ALK4 and/or ALK7, both of which are expressed on NeoIslet cells, promote endoderm formation and β-cell differentiation from stem cells. Using NeuroD expression as readout (NeuroD is a direct target of Ngn3 and effectively induced insulin expression in primary duct cells [27]), we have found that addition of SB431542, a pharmacologic inhibitor of the ALK5 group of TGF-beta/activin type I receptors (ALK4/5/7) [18], to NeoIslet cell differentiation medium and PCMOs prepared according to the standard method [34] decreased NeuroD expression (Fig. 29.1). NeoIslet cells are responsive to both TGF-beta and activins as demonstrated by activation (by phosphorylation) of the intracellular signal transducer Smad2 (Fig. 29.2). Notably, only TGF-beta1 but none of the three activins (-A, -B, -AB) was able to increase NeuroD and insulin expression in NeoIslet cells. However, all three activins apparently enhanced the endoderm character of the cells as measured by upregulation of Gata4, Hnf3b, and Sox17 expression at the mRNA level. The glucagon-like peptide analog exendin-4 is known for its ability to stimulate islet cell neogenesis, β-cell replication and survival, and insulin secretion [7], while the EGF family member betacellulin has recently been shown, when coexpressed with Pdx1, to induce MSCs into the pancreatic lineage in vitro and produce islet-like spheroids capable of secreting insulin in response to glucose [23]. Indeed, we observed that both exendin-4 and betacellulin stimulated NeuroD expression

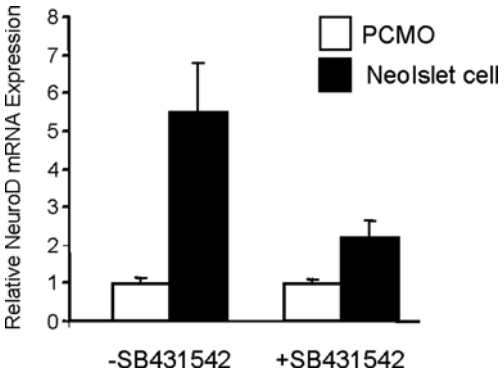

Fig. 29.1 Effect of the TGF-beta/activin type I receptor inhibitor SB431542 on NeuroD expression by NeoIslet cells. PCMOs were left untreated or were incubated in NeoIslet cell differentiation medium for 8 days with or without SB431542 (5 μM) as indicated. Following differentiation culture, cells were subjected to RNA isolation and quantitative real-time RT-PCR for Beta2/NeuroD. Five different donors were analysed with very similar results. Shown are the results from one representative donor. Data represent expression levels relative to those in PCMOs set arbitrarily at 1. Means ± SD from three wells processed in parallel

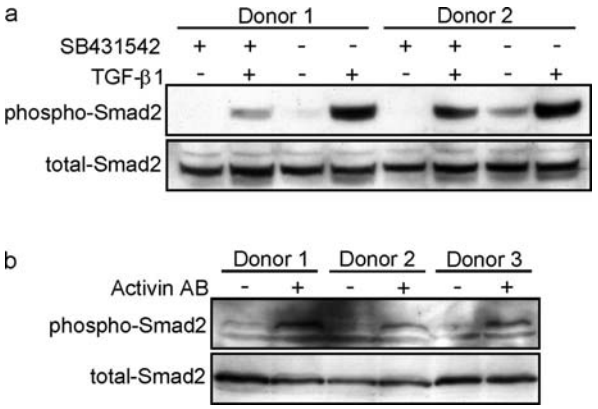

Fig. 29.2 NeoIslet cells respond to TGF-beta and activin with activation of Smad signaling. (**a**) Day-8 NeoIslet cells from two different donors were left untreated or were treated for 1 h with TGF-beta1 (5 ng/ml) in the presence or absence of the TGF-beta/activin type I receptor kinase inhibitor SB431542. Subsequently, protein lysates of the cells were fractionated by polyacrylamide gel electrophoresis and subjected sequentially to immunoblotting with antibodies specific for phospho-Smad2 and total Smad2 with intermittent stripping of the phospho-Smad2 antibody. Note the (partial) inhibition of endogenous and TGF-beta1-induced phospho-Smad2 levels by SB431542. (**b**) As in panel a, except that the NeoIslet cells from three different donors were treated with activin AB (50 ng/ml) instead of TGF-beta1 in the absence of kinase inhibitor

in NeoIslet cells and even exhibited a synergistic effect when given together. Surely, additional assays need to be performed to clarify whether these agents also induce other β-cell-specific transcription factors and enhance insulin production. The above mentioned results have encouraged us to rigorously apply protocols that try to mimic in vivo lineage specification and normal pancreatic endocrine development to both standard PCMOs and PCMOs with enhanced plasticity (see [1]). This strategy will include manipulation of other signaling pathways (Shh, Notch, FGF, RA) which has been successful in differentiation protocols for pancreatic endocrine cells from ESCs (see above).

(II) Enhancement of the stem cell phenotype and the proliferative activity of PCMOs: Activins are well known for their important role in maintaining stem cell self-renewal and pluripotency [28, 47]. We have therefore tested the impact of different activins on monocyte dedifferentiation and plasticity using Oct4A expression as readout. Both monocytes and PCMOs are highly susceptible to activin treatment as evidenced by activation (phosphorylation) of Smad2 and altered expression of respective target genes. However, addition of activin A (in the presence of serum) to monocyte → PCMO cultures was unable to increase Oct4A expression, suggesting that it cannot enhance pluripotency and self-renewal in monocytes under these conditions. This was not an unexpected finding since activin A exhibited a proendodermal differentiation effect on day-6 PCMOs (see above). As mentioned before, PCMOs express Oct4, Klf4, and Myc, but completely lack expression of Sox2. Preliminary evidence

indicates that Sox2 complementation in PCMOs further enhances plasticity as evidenced by assessment of the NeoHep phenotype (H.U., unpublished data). We are confident that these engineered PCMOs will be more susceptible to β-cell differentiation protocols and eventually exhibit higher insulin production than standard NeoIslet cells. Interestingly, we noted that addition of SB431542 to the PCMO culture medium enhanced the proliferative activity of monocytes suggesting that autocrine growth inhibition by TGF-beta or activin normally restricts further proliferation of PCMOs in vitro. It remains to be seen, however, whether transient blockade of ALK4/ALK5 signaling can be exploited to further expand PCMOs (and thereby increase NeoIslet cell yield) without compromising acquisition of pluripotency.

29.4 Perspectives and Future Directions

In several model systems it has been shown that the pancreatic endocrine phenotype can arise in vivo by lineage switching (via transdifferentiation or dedifferentiation–redifferentiation) of either non-islet pancreatic epithelial cells (ductal or acinar cells) (reviewed in Ref. [3]), duct-associated multipotent progenitors [51], or possibly extrapancreatic progenitors [52], all of which appear to have retained considerable differentiation plasticity. The same is true for peripheral blood monocytes. We have attempted to exploit the natural plasticity of circulating monocytes for reprogramming them into insulin-expressing cells via a two-step procedure, involving dedifferentiation to a stem cell-like progenitor (the PCMO), and subsequent differentiation to a (as yet immature) β-cell phenotype. Further increasing the developmental plasticity or the stem cell character of PCMOs will likely widen the spectrum for lineage switching and will therefore remain the major focus of our research. Several strategies are applied (alone or in combination) such as (i) growth factor modulation, (ii) stimulation of specific matrix–integrin interactions, (iii) treatment with chromatin-modifying agents [33], (iv) signal inhibition [39], and (v) forced expression of single or multiple pluripotency factors (to eventually achieve induced pluripotent PCMOs). We have already determined that acquisition of a plastic state during PCMO generation is a dynamic process that varies with time in culture and is transient rather than stable. Since self-renewal and pluripotency are largely regulated by the same factors, increasing pluripotency will eventually result in enhanced proliferation of PCMOs, which is a desired side effect as it will increase the amount of cells needed for transplant studies. Once this has been achieved β-cell differentiation protocols mimicking correct endoderm development, which are currently considered a necessary precedent for pancreatic cell differentiation [52], may be applied to PCMOs, with or without forced expression of β-cell fate determining transcription factors for direct conversion to β-cells. Very recently it was shown that autologous bone marrow-derived rat MSCs not only did secrete bioactive factors that establish a tissue microenvironment supporting β-cell function and survival in the pancreas but also exhibited anti-inflammatory and immunoregulatory effects on

T cells [5]. Interestingly, using very similar culture conditions as for PCMOs, we generated from murine monocytes autologous cells with therapeutic potential for the treatment of autoimmune inflammation [6] and possibly also type 1 diabetes (see Chapter 28). Combining the advantages of using peripheral blood monocytes as an easily accessible and further expandable stem cell source with sophisticated β-cell differentiation procedures might meet the demands for cell-based therapies for type 1 diabetes.

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Chapter 30
Islet Isolation for Clinical Transplantation

Tatsuya Kin

Abstract Islet transplantation is emerging as a viable treatment option for selected patients with type 1 diabetes. Following the initial report in 2000 from Edmonton of insulin independence in seven out of seven consecutive recipients, there has been a huge expansion in clinical islet transplantation. The challenge we now face is the apparent decline in graft function over time. Isolating high-quality human islets which survive and function for a longer period will no doubt contribute to further improvement in long-term clinical outcome. This chapter reviews the selection of appropriate donors for islet isolation and transplantation, describes each step during islet isolation, and discusses the scope for further improvements.

Keywords Culture · Islet purification · Organ preservation · Pancreas dissociation

Abbreviations

BMI	Body mass index
UW	University of Wisconsin
TLM	Two-layer method
CI	Class I collagenase
CII	Class II collagenase
CBD	Collagen-binding domains
IEs	Islet equivalents
FDA	Fluorescein diacetate
PI	Propidium iodide
OCR	Oxygen consumption rate
PG	Prostaglandin

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30.1 Introduction

An attractive alternative to daily insulin injections is replacement of a fully functional pancreatic β-cell to achieve a more physiological means for precise restoration of glucose homeostasis. β-cell replacement can be done by either pancreas or islet allotransplantation. Pancreas transplantation is a highly successful and well-established treatment for selected cases of type 1 diabetes, but is associated with surgical morbidity. Islet transplantation offers many advantages and has a low morbidity, but has historically been considered investigational and experimental because of limited success rates. Nevertheless, recent advances in islet isolation technology have opened the door for the reinstitution and development of new clinical islet transplantation programs around the globe, which have reported increasing success. In 2000, the Edmonton group attained insulin independence in all of seven patients by using freshly isolated islets from multiple donors and steroid-free anti-rejection therapy, a procedure now known as the Edmonton protocol [1, 2]. This protocol has set the standard worldwide for islet transplantation and now many other groups have attained similar success [3–5].

While it is clear that major advances have been made in achieving more consistent insulin independence following islet transplantation, it is also clear that the majority of islet recipients experience a decrease in graft function over time, with an insulin independence rate of only 10% at 5 years post-transplant [6]. The chronic decay in islet graft function is likely impacted by subclinical allograft rejection and recurrent autoimmunity. However, experimental studies in the absence of specific immunological destruction have indicated slowly progressive dysfunction of transplanted islets with time in nonhuman primates [7, 8], dogs [9–12], and rodents [13–15]. In addition, clinical studies in autotransplantation show that patients experience a gradual decrease in islet graft function after a sustained period of graft function, despite the absence of graft-specific immunity [16–18]. Therefore, the gradual allograft attrition can be partially attributed to nonimmunological factors.

Islets are subjected to numerous types of stress prior to transplantation. The process of islet attrition appears to begin at the time of donor brain death and continues during the islet isolation procedure. Despite many advances in technical aspects of human islet isolation, it still remains a technically demanding procedure, with several different factors influencing isolation outcome. In addition, it is difficult to isolate a sufficient number of viable islets with any regularity. Providing high-quality human islets which survive and function for a longer period will no doubt contribute to further improvement in long-term clinical outcome. The entire process of islet preparation comprises a number of steps. Among these steps are donor selection, pancreas preservation, enzymatic digestion of the pancreas, islet purification, islet culture, and islet assessment prior to transplantation. The aim of this chapter is to review each of these steps and to provide the rationale for continued efforts in islet isolation.

30.2 Donor Selection

Identifying donor-based markers of islet isolation success can provide a means of improving success of transplantation. Previous single-center retrospective studies identified several donor-related variables affecting islet isolation outcome, such as donor age, cause of death, body mass index (BMI), cold ischemia time, length of hospitalization, use of vasopressors, and blood glucose levels [19–26]. Pancreas weight has not been considered as a donor selection criterion because a value cannot be obtained prior to organ procurement. Generally, a larger pancreas contains a larger islet mass [25, 27]. Thus pancreas size can serve as a surrogate parameter for donor islet mass itself. One study developed a formula to predict pancreas weight, analyzing data from 345 deceased donors [27]. Key findings of the study are (i) males have a larger pancreas than females, (ii) pancreas weight increases with age, reaching plateau in the fourth decade, and (iii) BMI correlates with pancreas weight, but body surface area is a better predictor of pancreas weight than BMI. The finding of larger pancreas size in males is consistent with other studies [28, 29]. Recent studies reported that male donors provided a higher probability of yielding adequate islets [30, 31]. As for donor age, a positive correlation between age and islet yield is well documented [22, 25]. A juvenile donor pancreas makes it difficult to obtain an adequate number of islets [32, 33], which is partially explained by its small size. Regarding BMI, following a report indicating that BMI positively affects islet yield [34], many groups have considered BMI as an important donor factor influencing islet isolation outcome [22–25]. However, this view has led to the misconception that an obese donor is a good candidate for successful islet isolation and transplantation.

Donors with type 2 diabetes are considered unsuitable for islet isolation and transplantation because β-cell mass [35, 36] and function [37] may be decreased in type 2 diabetes. Type 2 diabetes is clinically insidious and can remain undiagnosed for many years. A negative medical history of diabetes obtained from the next of kin does not necessarily indicate the absence of glucose intolerance. Thus, it is not unexpected that a large proportion of organ donors for islets may have undiagnosed type 2 diabetes. In fact, a pancreas from an older donor with a higher BMI is not likely used for a whole organ transplant, but is preferred for islet isolation and transplantation [38, 39]. Such a donor may be on the spectrum toward type 2 diabetes. Previous studies indicated that high glycemic values during donor management were detrimental to islet recovery after isolation [19, 22, 25]. However, blood glucose levels are far too unreliable to use for the assessment of the donors' glucose metabolism in light of the pathophysiology of brain death and the pharmacology of drugs administered during the management of brain death. Although HbA_{1c} itself is not a diagnostic criterion for diabetes mellitus, its measurement in potential donors would provide useful information, since it has a high degree of specificity for detecting chronic hyperglycemia. Our islet isolation laboratory at the University of Alberta has implemented the routine measurement of donor HbA_{1c}

levels prior to islet isolation. Our current practice is that donors with $HbA_{1c} > 7\%$ are excluded for clinical islet transplantation [40].

O'Gorman and colleagues developed a scoring system based on donor characteristics that can predict islet isolation outcomes [41]. This scoring system has proven to be effective in assessing whether a pancreas should be processed for islet isolation [42]. It also allows for better management of the islet processing facility as the cost of islet isolations is high. However, using a donor score of 79 as the most appropriate cutoff value, the sensitivity and specificity for predicting successful islet isolations were only 43 and 82%, respectively. Moreover, it is not clear about the actual impact of donor score on transplantation outcome because the scoring system was developed solely based on islet isolation outcome. Similarly, other published studies dealing with donor factors do not take transplant outcome into consideration [19–25]. An older donor with a higher BMI may be a better donor with respect to successful islet isolation, but probably is not ideal for islet transplantation when the biology of islets derived from such a donor is considered. An improved scoring system which takes both the islet isolation and transplantation outcomes into account should be developed.

An attempt to use non-heart beating donor for clinical islet transplantation has been described. In experimental settings, islet yield and function derived from non-heart beating donor pancreata seem to be comparable with those from brain-dead counterparts [43]. However, in clinical settings, the results are not promising so far; all three insulin-independent recipients went back to insulin injection within 1 year after the last transplant [44].

30.3 Pancreas Preservation Prior to Islet Isolation

According to a report from the National Islet Cell Resource Center Consortium in the USA, University of Wisconsin (UW) solution is currently the standard preservation solution prior to islet isolation [45]. Recently, more pancreata are stored in histidine–tryptophan–ketoglutarate (HTK) solution, while the two-layer method (TLM) is decreasingly employed for pancreas preservation, at least in the USA.

HTK solution, originally developed for use as a cardioplegia solution, has long been used for abdominal organ preservation in Europe [46]. In 1995, Brandhorst and colleagues compared HTK and UW solutions in pancreas preservation for human islet isolation for the first time [47]. They observed that both solutions were comparable in terms of islet yield, in vitro functional viability of islets, and in vivo islet function in a mouse transplant model. Similar findings were subsequently reported by Salehi and colleagues [48]. In an experimental model performed in pigs, Stadlbauer and colleagues did not find any difference in frequency of apoptotic islet cells between pancreata preserved in UW and those in HTK [49]. At the present time, there is no evidence that HTK solution is superior to UW regarding islet isolation outcome. However, cost advantages in utilization of HTK may see further increased use of this solution for organ preservation.

The reason for decreased utilization of TLM is not clear but might be explained by recent observations in 166 and 200 human pancreata indicating no beneficial effect of TLM [50, 51]. TLM was developed in the 1980s by Kuroda, who focused on organ protection from hypoxia by supplying oxygen via perfluorocarbon during cold preservation [52]. Maintenance of adenosine triphosphate production in pancreata stored at the interface between perfluorocarbon and UW solution was observed as a result of oxygenation [53]. Tanioka and colleagues applied for the first-time TLM prior to islet isolation in a canine model [54]. Subsequently many centers introduced TLM prior to clinical islet isolation and reported improvement in islet isolation outcomes for pancreata preserved in TLM, when compared with pancreata stored in UW alone [55–57]. Of note, most of the initial studies employed a short period of TLM with continuous oxygenation at the islet isolation facilities. In an attempt to enhance the beneficial effect of TLM, our center at the University of Alberta introduced TLM for an entire period of pancreas preservation using preoxygenated perfluorocarbon. However, in contrast to the expectation, no advantages of TLM over UW were observed in terms of pancreatic adenosine triphosphate level, islet yield, in vitro functional viability, and in vivo function after clinical transplantation [50]. These findings were subsequently confirmed by other groups [30, 51]. Thus, there remains much work to be done to optimize pancreas preservation methods.

Recently, hypothermic machine perfusion has been gaining increasing acceptance as a preservation method mainly for marginal donor kidneys [58]. Hypothermic machine perfusion has several advantages over static cold storage. First, preservation solution can be continuously supplied directly to all cells. In addition, machine perfusion permits ex vivo pharmacologic manipulation of the graft. Moreover, real-time assessment of graft quality can be done by analysis of perfusate. Toledo-Pereyra and colleagues reported a canine islet autotransplantation study with 60 and 40% animal survival following hypothermic machine perfusion for 24 and 48 hours, respectively [59]. In porcine islet isolation, Taylor and colleagues showed that machine perfusion improved islet isolation outcomes when compared with static UW preservation [60]. Our center at the University of Alberta performed machine perfusion in 12 human pancreata using a LifePort^{TM} Kidney Transporter (Organ Recovery Systems, Des Plaines, IL, USA) [61]. The first four pancreata were placed on the machine, after 10 hours of static preservation in UW, for up to 24 hours; metabolic and histologic changes of pancreata were assessed. It was found that tissue energy charge was maintained during the first 3 hours in the machine perfusion and thereafter it gradually decreased. Histologic analysis revealed that tissue edema became evident at 24 hours. The next eight pancreata were processed for islet isolation after 6 hours of machine perfusion. Islet recovery and viability tended to be higher in pancreata preserved with the machine perfusion than in matched pancreata stored in static UW. These results are in accordance with the work of Leeser and colleagues who showed a feasibility of pump perfusion of human pancreata prior to islet isolation [62].

30.4 Pancreas Dissociation and Enzyme

The enzymatic dissociation of the pancreas is a critical step in islet isolation. Delivering enzyme blends to the islet–exocrine interface leads to cleavage islets. Collagen is the major structural protein constituting the islet–exocrine interface [63, 64]. Because of its tight structure and mechanical strength, collagen is not generally degraded by ordinary protease but can be efficiently degraded with high specificity by collagenase [65]. Therefore, collagenase is a key component of an enzyme product for pancreas dissociation. However, the use of collagenase alone results in an inadequate tissue digestion [66, 67]. Apparently, the presence of non-collagenase impurities is needed to enhance pancreas dissociation. Prior to the 1990s, crude collagenase, a fermentation product derived from *Clostridium histolyticum* was exclusively used for pancreas dissociation. The crude preparation from *C. histolyticum* contains two different classes of collagenase: class I collagenase (CI) and class II collagenase (CII). It also contains non-collagenolytic enzymes including amylase, cellulase, pectinase, chitinase, sialidase, hyaluronidase, lipase, phospholipase, and so on [68–70]. Composition and activity of crude preparations are exceedingly variable between different lots of a commercially available product. This variability has been recognized as a major limitation to successful pancreatic digestion [69, 71]. In the late 1990s a new enzyme blend became available from Roche (Roche Applied Science, Indianapolis, IN). This purified enzyme blend, Liberase HI, is comprised of CI, CII, and thermolysin derived from *Bacillus thermoproteolyticus* as a non-collagenolytic component. The introduction of Liberase HI has helped to reduce but not eliminate some of the lot-to-lot variability of enzyme effectiveness. The use of this product provided enhanced islet yield and function in the human and animal models, compared to the historical use of crude collagenase [72–75]. In contrast, several studies showed that Liberase is no more effective than crude collagenase in neonatal rat [76] and fetal pig [77] pancreata and induces functional damage to rat [78] and human [79] islets. Moreover, this enzyme blend still exhibits lot-to-lot variations [80, 81].

While the use of non-collagenolytic enzyme has been shown to enhance pancreas dissociation, excessive exposure of this enzyme was found to decrease islet yields through islet fragmentation and disintegration [66, 82] and to reduce islet viability [83]. Therefore, a narrow dosing window is recommended for this enzyme. A newly developed collagenase NB1 (Serva Electrophoresis GmbH, Heidelberg, Germany) contains only CI and CII, which can be blended with separately packaged neutral protease NB (Serva Electrophoresis GmbH) as a non-collagenolytic component. This type of product has several potential advantages over traditional enzyme blends. First, ratio between non-collagenolytic activity and collagenase activity can be adjusted as desired. Once the optimal ratio has been determined in a human pancreas, as has already been elucidated for the rat pancreas [82], this strategy would improve isolation outcome. Moreover, separate storage of the individual enzyme components would improve the overall stability of each enzyme activity. Finally and importantly, the non-collagenolytic component can be sequentially administered

after intraductal collagenase distention, in an attempt to avoid excessive exposure of islets to non-collagenolytic enzyme [84].

C. histolyticum possesses two homologous but distinct genes, *ColG* and *ColH*. The former encodes CI and the latter encodes CII [85, 86]. It is important to know similarities and differences between the two enzymes. CI and CII are quite different in their primary and secondary structures, but the catalytic machinery of the two enzymes is essentially identical [87]. Both enzymes have a similar segmental structure consisting of three different segments: catalytic domain, spacing domain, and binding domain [71, 88]. CI has tandem collagen-binding domains (CBD) but CII possesses a single CBD [88]. Tandem CBDs of CI may have advantages for binding to collagens in the pancreas because tandem-repeated binding domains are generally considered useful for stabilization of bindings [89]. Kinetic studies evaluating the hydrolysis of collagens by CI or CII indicate a higher catalytic efficiency of CI on collagen [90]. On the other hand, CII is characterized by the ability to attack synthetic peptide substrates at a much greater rate than CI [91]. Wolters and colleagues showed that rat pancreas digestion was more effective when both classes were used together instead of CI or CII alone [67]. van Wart and colleagues found a synergistic effect of the two enzymes on collagen degradation [92]. Wolters and colleagues concluded that CII plays a predominant role in rat pancreas dissociation whereas CI is minor in comparison [67]. Several investigators [93] and manufacturers have emphasized the view that CII is a key player in pancreas dissociation. Indeed, manufacturers have measured only CII activity in their product specification and CI activity has been ignored so far. However, the importance of CII has been challenged by a recent study demonstrating that neither CI nor CII alone is able to release islets from a rat pancreas [94]. Findings from human studies are in conflict with the classical view, too. Barnett and colleagues showed that the stability of intact CI is of great importance to the quality of the blend [80]. It is also demonstrated that a better enzyme performance is ascribed to a higher proportion of CI rather than a higher proportion of CII [81]. It is further shown that excessive CII is not effective to release islets from a human pancreas and rather a balanced CII/CI ratio is of paramount importance [95].

Cross and colleagues performed extensive immunohistological studies on binding of collagenase to collagen [96], suggesting that collagenase perfused through the duct binds to collagen inside the pancreas. Their findings also suggest that collagenase can bind to collagen without the help of non-collagenolytic enzyme activation, and that low temperature does not inhibit binding of collagenase to collagen which is in line with a previous report [97]. Another important finding from their studies is that collagenase binds to collagen located inside the islets as a result of intraductal perfusion with collagenase. This may result in islet fragmentation when the enzyme is activated.

Understanding of the structure of the islet–exocrine interface, and the nature of substrate at this interface, will be exploited to optimize pancreas dissociation. Previous studies have described the distribution of collagen types in the human pancreas. Type IV collagen is present in basement membranes associated with ducts and acini [98]. Collagen subtypes identified in the islet–exocrine interface are I, III,

IV, V, and VI [99–101]. Recently, Hughes and colleagues found that type VI collagen is one of the major collagen subtypes at the islet–exocrine interface of the adult human pancreas [102]. Type VI collagen has a high disulfide content which serves to protect the molecule from bacterial collagenase digestion [103]. It is also known that type VI collagen does not form banded collagen fibrils and is extensively glycosylated [104]. Regarding amount of collagen, it is well known that the total collagen content increases with age in most tissues [105–108]. Pancreatic collagen is affected by the normal aging process as well. Bedossa and colleagues found significantly higher collagen content in pancreata from patients over the age of 50, as compared to younger subjects [109]. The importance of pancreatic ultrastructure has been pointed out and discussed over the past two decades [99]; unfortunately, there has been little progress in this field. A better understanding of the differences in biomatrix among donor pancreata, for example, older versus younger donors, will help to improve pancreas dissociation.

In March 2007, the islet transplant community was notified of the use of a bovine brain component during the manufacturing of Liberase. To minimize the potential risk of prion disease transmission, many islet isolation centers switched to Serva collagenase, which is considered to have less risk. However, this conversion significantly affected the field. The National Islet Cell Resource Center Consortium in the USA reported that only 1.7% (3 out of 173) of islet preparations were used for clinical transplantation in 2007, a tremendous drop from 27.6% (188 out of 680) during the previous years [45]. This may be at least partially explained by the lesser effectiveness of Serva enzymes. However, some centers successively adapted this enzyme blends with or without a modification of the islet isolation protocol [110, 111]. The University of California San Francisco group achieved a high rate of islet isolation success using ~1600 units of Serva collagenase and ~200 units of neutral protease for younger donor pancreata [110].

Recently, the manufacturer of Liberase has produced an alternative collagenase manufactured in the strict absence of bovine products. Whether this new product will improve isolation outcome has yet to be demonstrated.

30.5 Islet Purification

After enzymatic digestion of a standard size pancreas (~90 g), the total packed volume of digested tissue is typically greater than 40 mL. While it is known that human liver has a capacity for adaptation and revascularization in the context of portal vein occlusion [112], the liver cannot accommodate 40 mL of tissue consisting of particles on the 100 μm scale. Substantial evidence of liver embolism, thrombosis, damage, and even death is documented in clinical settings immediately after intraportal infusion of a large amount of tissue [113–119]. Notably, there is the need to reduce tissue volume with minimum loss of islets for the safer intraportal infusion. This can be achieved by a procedure called “islet purification.”

Density-dependent separation of islets from exocrine tissue is the most simple and effective approach for islet purification. It is based on the principle that,

during centrifugation, tissue will migrate and settle to the density that is equal to its own density. Using this technique, separation can be achieved based on intrinsic differences in density between islet tissue (~1.059 g/mL) and exocrine tissue (1.059–1.074 g/mL) [120]. Theoretically, a greater difference in density between the two tissues could result in a better separation. The best separation would be expected when the islets are free from exocrine tissue and the density of exocrine tissue is well preserved. In contrast, the worst scenario would happen when the majority of islets are entrapped in the exocrine tissue (thereby a higher density of tissue) and the decreased density of exocrine tissue due to exocrine enzyme discharge or tissue swelling. There is a trade-off between purity of islets and islet mass recovered (Fig. 30.1). Obtaining an extremely high purity of islets requires sacrificing a less pure layer, which contains a considerable amount of islets. Nearly 100% of islets can be recovered if a less pure layer with a large amount of exocrine tissue is included in the final preparation, but this turns in a lower purity.

Purification of large numbers of human islets has advanced rapidly with the introduction of the COBE 2991 (COBE Laboratories Inc., Lakewood, CO, USA) [121]. The COBE 2991 cell processor, originally developed for producing blood cell concentrates, is indispensable equipment in human islet processing facilities. It allows processing of a large volume of tissue in an enclosed sterile system. Moreover,

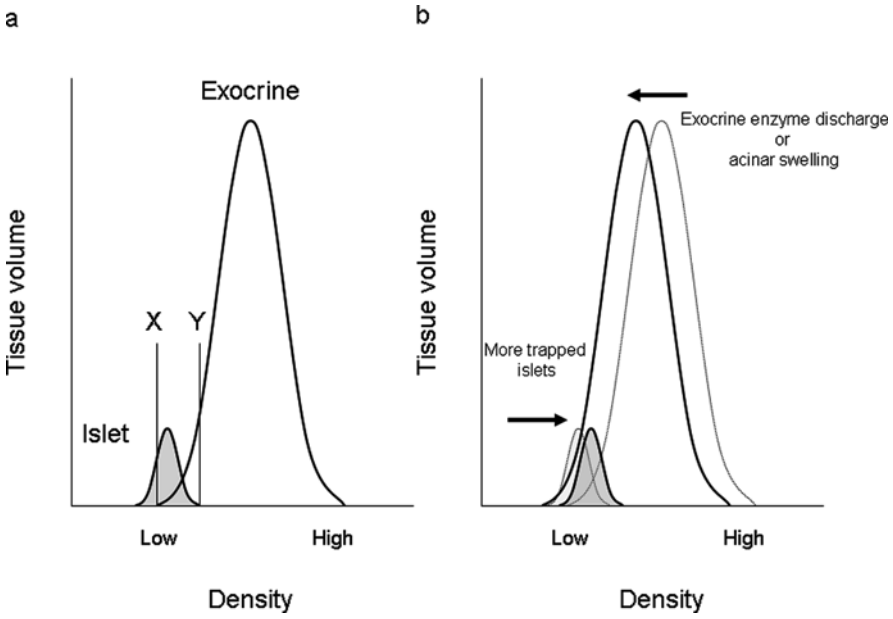

Fig. 30.1 (**a**) If the cutoff is moved down to "X" from "Y," the islet mass falls but the purity rises. (**b**) If there are more trapped islets, islet curve shifts to the right. If exocrine enzyme is discharged, exocrine curve shifts to the left. Islets represent only a small percent (<2%) of the total pancreas volume, but the islet volume in the figures was intentionally described larger for better visualization

it offers decreased operating time with an ease of generating continuous density gradients in conjunction with a two-chamber gradient maker.

Various gradient media have been developed and tested for islet purification. One of the most commonly used media is a synthetic polymer of sucrose (Ficoll; Amersham, Uppsala, Sweden)-based media. Scharp and colleagues reported that islet recovery was improved when Ficoll was dialyzed before centrifugation, to remove the low molecular weight osmotically active contaminants [122]. Olack and colleagues used Euro-Collins, an organ preservation solution, as the vehicle for dissolving the Ficoll powder [123]. Hypertonic density solutions such as Euro-Collins/Ficoll prevent edema of the exocrine tissue at low temperature and result in improved separation of islets from the exocrine tissue, when compared with standard Ficoll solution [120].

UW solution has been used for storing the pancreatic digest prior to density gradient centrifugation in an attempt to reduce acinar tissue swelling [124–126]. The beneficial effect of UW storage is ascribed to the presence of the osmotic effective substances lactobionate, raffinose, and hydroxyethyl starch. To extend this beneficial action, Huang and colleagues have introduced a mixture of UW and Ficoll-sodiumdiatrizate (Biocoll; Biochrom, Berlin, Germany) for density gradient separation [127]. They showed that their new gradient medium improved post-purification islet yield when compared with the standard medium. The UW-Bicoll purification method has been further refined by Barbaro and colleagues, who recovered 85% of islets after purification using a narrow range of density gradients [128].

Ichii and colleagues performed discontinuous density gradient purification to recover islets from the exocrine-enriched fraction obtained after the initial purification procedure [129]. This supplemental purification, so-called rescue purification,

Table 30.1 Islet recovery rate after purification

Reference	Density gradient	*n*	Pre-purification islet yield, IE	Post-purification islet yield, IE	Recovery rate[a]
Barbaro 2007 [128]	Biocoll	32	359,425	194,022	64.5 [b]
	UW-Biocoll	132	370,682	310,607	84.9[b]
Ihm 2006 [26]	Iodixanol or Biocoll	110	356,745	244,034	68.4
Yamamoto 2007 [130]	Ficoll or Ficoll followed by rescue[c]	169	454,049	306,728	67.6
Kin 2007 [81]	Ficoll	251	348,794	227,832	65.3
Kin 2008 [95]	UW-Biocoll	21	394,619	303,905	77.0
Brandhorst 2003 [131]	HBSS-Ficoll	76	463,872	245,889	53.0
Wang 2007 [132]	Biocoll	23	373,350	184,284	49.4
Nagata 2006 [133]	Ficoll	8[d]	660,770	444,426	67.3

[a]Mean post-purification IE/mean pre-purification IE × 100.
[b]Mean of each isolation's recovery rate.
[c]See Ref. [129] regarding 'rescue' purification.
[d]Non-heart beating donors.

contributed to increasing the number of islet preparations suitable for transplantation. In a subsequent report, however, the same group showed no benefit of rescue purification on isolation success [30].

Table 30.1 summarizes the islet recovery rate after purification among islet processing facilities using different gradient media [26, 81, 95, 128, 130–132]. Recovery rate varies from 50 to 85%, depending on purification methods and the quality of the pancreas. These figures clearly show that there is still room for improvement.

30.6 Islet Culture

Although there is debate as to whether freshly isolated islets are superior to cultured islets in experimental transplantation [134–136], preservation of human islets for a certain period of time by means of culture provides many benefits to clinical islet transplantation. First, islet culture provides travel time for patients living away from transplant centers. Pre-transplant culture can moreover allow attainment of therapeutic levels of immunosuppression before islet infusion. During the culture period additional quality control testing can be undertaken, including microbiological and pyrogenic tests. In addition to these practical advantages, modification or treatment of islets through culture provides a strategic opportunity to promote islet survival after transplantation. Surface modification of islets by bioconjunction during culture is one of the examples [137, 138]. Thus, a strong rationale exists for culturing islets prior to transplantation. A number of issues will be discussed below, including culture temperature, base media, and risk of islet loss during culture.

In 1977, Kedinger and colleagues transplanted cultured or fresh allo-islets into the liver of diabetic rats [139]. Rats receiving fresh islets returned to diabetic state in 8 days after transplantation. In contrast, when islets were cultured for 3–4 days prior to transplantation, graft survival was prolonged to 90 days without immunosuppression. Culture temperature is not described in the report, but this was the first attempt to alter or reduce immunogenicity of islets by in vitro culture. A few years later, Lacy and colleagues adapted room temperature for culturing islets in an attempt to prolong allograft survival [140]. They reported that culture of rat islets at 24°C resulted in a prolonged islet allograft survival in immunosuppressed recipients. Their idea of culturing islets at 24°C was based on a study performed by Opelz and Terasaki [141], who found that lymphocytes being placed in culture at room temperature lost their ability to stimulate allogeneic cells when tested in subsequent mixed lymphocyte culture. The results of Lacy's experiments support the theory that passenger leukocytes are involved in rejection of the allografts. However, the study did not demonstrate culture temperature at 24°C per se contributed to altering immunogenicity of islets. To investigate the influence of temperature, they further compared allograft survival in immunosuppressed rats receiving islets cultured at 24 versus 37°C [142]. Culture of islets at 24°C resulted in a longer allograft survival as compared to 37°C culture. When recipients were not immunosuppressed, however, culture at 24°C did not prolong graft survival as compared to fresh islets. Following

the initial study performed by Lacy and colleagues [140], many investigators have set up a culture system at 22–24°C prior to transplantation [143–151]. There are, however, little published studies specifically showing immunological superiority of 22–24°C culture over 37°C culture in an islet transplant model. A group from Germany reported a marked prolongation of rat islet allograft survival by culture at 22°C compared to 37°C culture [152]. Interestingly, the prolongation of graft survival was observed only when islets were transplanted under the renal subcapsular space; islets cultured at 22°C were rapidly destroyed at the liver. They further confirmed a similar effect in a rat to mouse xenotransplantation model [153]. It is uncertain if the strategy used in the animal models will be as satisfactory in the clinical situation. There is absolutely no information in this regard for clinical islet transplantation.

Another possible benefit of low-temperature culture is that the structural and functional integrity of islets is well preserved, likely due to a lowered metabolic rate at temperatures below 37°C. For instance, Ono and colleagues reported that rat islets cultured at 37°C possessed a higher rate of central necrosis than islets cultured at 22°C [154]. Lakey and colleagues assessed human islet recovery after 24 hours culture and they described a mean recovery rate of 73% at 22°C compared to 55% at 37°C [155]. Similarly, inadequate recovery rate at 37°C was observed in pig islets after culture compared to 22°C culture [156]. On the other hand, investigators have asserted that low-temperature culture results in impaired insulin production [157] and more apoptotic cells in islets [158]. In clinical settings, there is no consensus regarding culture temperature. Some centers have employed culture at 37°C followed by culture at 22°C prior to clinical transplantation [4]. Initially our center at the University of Alberta adapted this approach but consistently found significant loss of islets after culture (unpublished observation). Since 2003, our center has been using only 22°C during the entire period prior to transplantation [159].

Connaught Medical Research Laboratory 1066, originally designed for use with fibroblasts and kidney epithelial cells, appears to be the most widely used base medium for islet culture. Other base media used for clinical transplantation include Ham's F10 [160] and M199 [161]. Regardless of whichever base medium is employed, supplementation of medium seems to be a routine practice. Because serum contains many components that have a beneficial effect on cell survival, animal serum such as fetal calf serum is traditionally added to culture media in experimental settings. However, when islets are destined for clinical transplantation, use of animal sera has been considered unacceptable because of potential risk associated with viral or prion-related disease transmission [162]. Other potential problems of animal sera are evoking immune or inflammatory reactions in host against animal proteins [163–165], which cannot be diminished even by several washing steps [166]. Therefore adding human serum albumin as an alternative is the current standard in clinical islet culture.

One of the major concerns with culturing islets is the uncertainty of islet recovery rate after culture. There is ample evidence of a reduction in the islet mass during culture. Bottino and colleagues reported that there was at most 80% reduction in DNA content in islet preparations following 24 hours culture [167]. Another report

showed only 18% recovery rate in islet mass after 48 hours culture [168]. A retrospective study on 104 islet preparations for clinical transplantation has identified several factors associated with risk of islet loss [159]. These include longer cold ischemia time prior to islet isolation, TLM for pancreas preservation, lower islet purity, and higher proportion of larger islets in the pre-cultured preparation. One may wonder if a longer culture period leads to a greater decrease in islet mass. The retrospective study does not support this caution probably because most of the islet preparations were cultured for a short period (20 hours, median) with a narrow range (14 hours, interquartile range). Most islet processing centers employ short period culture (up to 3 days) prior to transplantation [4–6, 159]. Of note, the Brussels group cultured islets derived from several donors for up to 1 month until a critical islet mass is reached, and then infused all islet preparations into a recipient as a single transplant procedure [169]. The impact of prolonged culture period on islet loss seems to be significant as islets were combined from as many as nine donors. Importantly, two of seven recipients became insulin independent after transplantation of islets cultured for long periods.

30.7 Assessment of Islet Preparations

There is substantial heterogeneity in islet size within a human pancreas. In order to measure the quantity of islets in an islet preparation, both the number of islets and size should be taken into consideration. In 1989, a workshop was held at the 2nd Congress on International Pancreas and Islet Transplantation Association and a consensus was reached on criteria for measuring the quantity of islets [170]. The diameter of each islet within the sample aliquots is categorized within a size range: 50–100 μm, 101–150 μm, 151–200 μm, 201–250 μm, 251–300 μm, 301–350 μm, and over 350 μm. The number of islets in each category is enumerated. An "islet equivalent" is defined as an islet with a diameter of 150 μm. The number of islet equivalents (IEs) in each size class is calculated by multiplying the number of islets with a conversion factor. A conversion factor for each class is derived from the mean volume of islets in the class, divided by the volume of an islet with a diameter of 150 μm (Table 30.2).

It has been difficult to establish the amount of total IEs (or total islet volume) in human pancreas because islets are scattered in a large exocrine gland of which they represent only small percent in volume. Korsgern and colleagues [171] estimated that the number of IEs in a normal pancreas is about 500,000 IEs based on seven autopsy cases reporting islet volume of 0.5–1.3 mL [172]. Others estimated that islet volume is 2.4 mL in a normal pancreas, which is corresponding to 1,300,000 IEs [173]. In Table 30.3, islet volume data from autopsy studies are listed [174–177]. Calculated total IEs vary from 500,000 to 1,000,000 IEs, depending on the size of pancreas studied. One Japanese study [174] reported the mean pancreas weight of 122 g, which is the highest among other studies. Consequently, islet mass reported in the study is remarkably large, resulting in a calculated IE of >1,000,000 IEs.

Table 30.2 Islet equivalent conversion factors

Islet diameter range (μm)	Rank	Conversion factors
50–100	1	$[1^3 + (1+1)^3] / 54 = 0.167$
100–150	2	$[2^3 + (2+1)^3] / 54 = 0.648$[a]
150–200	3	$[3^3 + (3+1)^3] / 54 = 1.685$
200–250	4	$[4^3 + (4+1)^3] / 54 = 3.500$
250–300	5	$[5^3 + (5+1)^3] / 54 = 6.315$
300–350	6	$[6^3 + (6+1)^3] / 54 = 10.352$
>350	7	$[7^3 + (7+1)^3] / 54 = 15.833$

[a]In ref [170], this factor is reported as 1/1.50 (i.e., 0.667).

In contrast, the pancreas weight in Maclean's study is only 50% of that reported in the Japanese study [177]. Accordingly, the calculated IE from Maclean's data is almost half of the number from the Japanese study. Based on all data listed in the table, it would be reasonable to say that the average number of IEs in a normal 90 g human pancreas [27] is about 800,000 IEs.

In addition to the quantity of islets, the functional viability of an islet preparation is critical in predicting the success of islet transplantation. The viability of an islet preparation is currently assessed with the use of fluorescent stains based on dye exclusion polarity. For example, fluorescein diacetate (FDA) is a nonpolar dye and passes through the plasma membrane of living cells, whereas propidium iodide (PI) can only enter cells that have a compromised membrane. Using these two dyes together, the proportion of viable (green, FDA-positive) versus dead (red, PI-positive) cells can be assessed. FDA/PI is currently a widely used method for viability determination of the islet preparation prior to transplantation. These tests can be rapidly performed and are less labor intensive, making them attractive for use just prior to transplantation. However, there are several problems, making them

Table 30.3 Estimation of total islet equivalents in a pancreas

References	Mean age (range), year	*n*	Pancreas size (range), g or mL	Islet mass, g	Islet volume, mL	Calculated islet equivalents, IE
Sakuraba 2002 [174]	51.7 (27–69)	15	122 g (75–170)	2.03	1.92[a]	1,085,295
Westermark 1978 [175]	74.9 (66–88)	15	76 mL	NR	1.60	905,874
Rahier 1983 [176]	54 (18–86)	8	83 g (67–110)	1.395	1.32[a]	745,806
Maclean 1955 [177]	56.1 (15–81)	30	61.7 g (38.9–99.2)	1.06	1.00[a]	566,706

[a]Calculated assuming that islet density is 1.059 g/mL [120].
NR, not reported

of limited value. The main problem is that membrane integrity tests cannot distinguish between islets and non-islets. Another problem with the tests is the difficulty in assessing live/dead cells within a three-dimensional structure. Nevertheless, it is important to acknowledge that viability estimated by membrane integrity tests is predictive of some outcome measurements in clinical transplantation, according to an annual report from Collaborative Islet Transplant Registry [178].

Mitochondrial function can be used as a surrogate marker to determine islet functional capacity. Mitochondrial integrity is central to islet quality because mitochondria play a crucial role for glucose-stimulated insulin secretion [179] and islet cell apoptosis [180]. Mitochondrial activity can be evaluated using a variety of methods. These include oxygen consumption rate (OCR), detection of mitochondrial membrane potential using dyes, release of cytochrome c, and measurement of redox state. Papas and colleagues assessed OCR of human islet preparations; they also measured DNA content of the preparations in order to normalize the OCR [181]. They showed that OCR/DNA assay predicted efficacy of human islets grafted into mice. Similar to membrane integrity tests, this assay cannot offer islet specificity because oxygen is consumed by every cell in a preparation. To circumvent this limitation, Sweet and colleagues developed a flow culture system [182] that allows to measure responses of OCR in human islets against glucose stimulation. They demonstrated that glucose-stimulated changes in OCR were well correlated with in vivo function of human islet grafts [183, 184]. They also showed that glucose stimulation hardly increased OCR in non-islet tissue [184]. Given the fact that a clinical islet preparation contains a considerable amount of non-endocrine tissue, their approach would be logical and practical.

The viability of β-cells is probably most important to the outcome of transplantation. Ichii and colleagues reported a method for quantitating the β-cell-specific viability [185]. They dissociated islets into single cells, and then stained the cells with a zinc-specific dye, Newport Green (Molecular Probes, Eugene, OR, USA), and with a mitochondrial dye, tetramethylrhodamine ethyl ester. The double positive cells were quantified on a flow cytometer after dead cells were excluded using a DNA-binding dye. They showed that the β-cell-specific viability of human islet preparations was a useful marker of the outcome of a mouse transplant assay. The major limitation of this method is that the dispersed single cells are not likely representative of the original islets because a substantial fraction of cells is lost during dissociation. In addition, necrotic cells or late-stage apoptotic cells were not counted as nonviable cells, thereby leading to overestimation. Finally, several recent studies brought into question the use of Newport Green for detection of β-cells because of its low quantum yield and poor selectivity to zinc [186].

30.8 Cytoprotective Strategies During Islet Isolation

Islets are exposed to numerous types of stress induced by nonphysiological stimuli during isolation. These include ischemic stress during organ preservation and islet isolation, mechanical and enzymatic stress during digestion, and osmotic stress

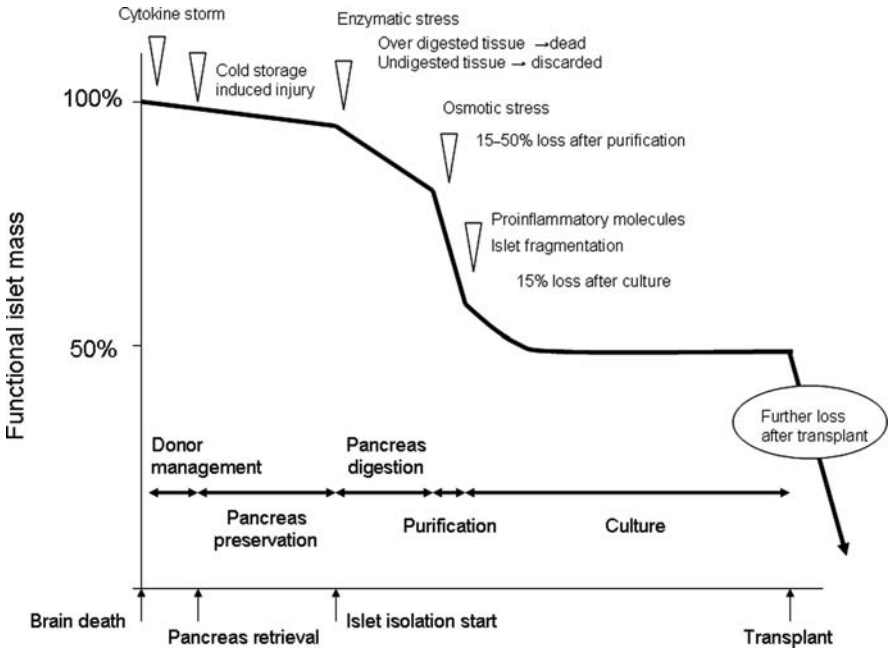

Fig. 30.2 A normal 90 g pancreas [27] contains about 800,000 IEs. The current islet isolation method yields 300,000–400,000 IEs per pancreas, indicating that 50% of islets in the native pancreas are lost during the entire process. It is difficult to assess degree of decline in functionality of islets during the islet isolation; however, major functional decline is likely to occur during the early isolation period, not during the culture periods

during purification (Fig. 30.2). A number of investigators have explored strategies to confer islet resistance to stress-induced damage. Most investigations have centered on treatment of the islets during culture. Some are focusing on modification in the isolation procedure to protect islets.

Arita and colleagues investigated the effect of beraprost sodium on dog islet isolation outcome [187]; beraprost sodium is a prostaglandin (PG) I_2 analogue which is known to exhibit a cytoprotective effect on various cell types. They digested pancreata with collagenase solution containing PG I_2 analogue. Adding PG I_2 analogue did not improve islet yield after purification. However, viability of islets was higher in PG I_2 analogue group than in control, resulting in a significant reduction in islet loss during subsequent culture.

Strategies to protect not only islets but also exocrine pancreas are of paramount importance in islet isolation. The pancreatic gland is a very sensitive organ. It easily undergoes autodigestive process, leading to damage of islets. Releasing pancreatic digestion enzymes results in a decrease in acinar tissue density, thereby greatly affecting the results of purification. Endogenous pancreatic enzymes may be released during the digestion phase. Theoretically, these enzymes have the potential

to damage islets and act through the proteolysis of collagenase, leading to a decrease in collagenase activity during the digestion process. In an attempt to inhibit the activity of these undesired enzymes, Pefabloc (Roche Applied Science, Indianapolis, IN), a serine protease inhibitor, has been used successfully to isolate pig [188], monkey [189], and human islets [190]. Pefabloc is also known to possess an anti-apoptotic effect [191–193]. This effect of Pefabloc potentially contributes to better isolation outcome, although previous studies dealing with Pefabloc did not investigate apoptosis of islets.

The use of antioxidants during islet isolation to protect islets from oxidative cell injury is a rational approach because islet cells harbor poor endogenous antioxidant defense systems [194]. Avila and colleagues delivered glutamine to the human pancreas via the duct prior to pancreas dissociation [195]. They found that glutamine treatment reduced islet cell apoptosis and improved islet yield and function. Similarly, Bottino and colleagues perfused human pancreata with a mimetic superoxide dismutase, a novel class of chemical antioxidant compounds [167]. Islet yield immediately after isolation from a treated pancreas was similar to those from a non-treated pancreas. However, in vitro islet survival was significantly improved when islets were further treated with this compound during subsequent culture.

Nicotinamide has been shown to protect islets from injury induced by cytokines [196]. Ichii and colleagues added nicotinamide into the processing medium during islet isolation [197]. They found that nicotinamide supplementation increased human islet yields. They also showed a significant increase in c-peptide levels in patients transplanted with nicotinamide-treated islets.

30.9 Conclusions

In recent years, the results of clinical islet transplantation have improved dramatically. Substantial advances in human islet isolation technology have contributed to the steady evolution of this therapy. The goal now has to be sharply focused on obtaining a large number of viable islets that provide full functional survival for the long term, thereby enhancing long-term rates of insulin independence in clinical patients. Much works remains to be done to achieve this goal; but it is clear that there is scope for significant improvements that will permit islet transplantation to be a practical therapy for more patients with type 1 diabetes.

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Chapter 31
Human Islet Autotransplantation: The Trail Thus Far and the Highway Ahead

Martin Hermann, Raimund Margreiter, and Paul Hengster

Abstract Human islet transplantation is one of the three treatment modalities besides the daily administration of exogenous insulin and pancreas transplantation, which can be applied for the treatment of type 1 diabetic patients.

Although the metabolic control achieved after islet transplantation is superior compared to exogenous insulin administration, many hurdles remain to be overcome before islet transplantation can be called a routine therapy for type 1 diabetic patients. In contrast to many other therapeutic approaches, proof of principle has been obtained for islet transplantation: As demonstrated in islet autotransplantation, the transplanted islets are not only able to survive in another organ, namely the liver, but also able to retain their functional role, in some patients even for decades. The main challenge for islet allotransplantation is, therefore, to imitate this success, thereby providing type 1 diabetic patients with a cellular therapy lasting for decades and thus circumventing the daily injections of insulin.

Keywords Allotransplantation · Pancreatitis · Total pancreatectomy · Pancreatectomy · Islet shipment · Real time live confocal microscopy · Chronic pancreatitis · Autotransplantation · Human islet allotransplantation · Human islet autotransplantation · Type I diabetes

31.1 Introduction

Type 1 diabetes is a chronic, progressive autoimmune disease resulting from the immune-mediated destruction of the insulin-producing β-cells within the pancreatic islets. One treatment option for such patients aims at replacing the β-cells through islet transplantation.

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In spite of the numerous advances in islet cell transplantation [1, 2], its transition from the stage of clinical investigation to routine clinical routine is still hindered by several yet unresolved issues [3-5]. While short-term results have been very promising, with 82% of patients maintaining insulin independence at 1 year after islet allotransplantation, long-term results show a decline in the proportion of recipients maintaining insulin independence after the first year posttransplant [1, 4]. While the 5-year post-islet transplantation graft survival is approximately 80% (as measured by C-peptide positivity), insulin independence shows a much lower rate, close to 10%, after 5 years [4]. Although the reasons for this functional decline still remain unclear, several factors can be causally linked to this deterioration ranging from alloimmune rejection, autoimmune recurrence, toxicity of immunosuppressive medications, to the inhospitability of the liver itself as a site of implantation. However, the latter possibility is challenged by the already verified long-term function of islets after autotransplantation [6, 7].

31.2 Total Pancreatectomy in Combination with Islet Autotransplantation

Chronic pancreatitis (CP) is a progressive inflammatory disease causing irreversible structural damage to the pancreatic parenchyma. Besides affecting the pancreatic exocrine function, in severe cases, the endocrine function may also be impaired leading to the onset of diabetes mellitus [8]. As in many patients CP is clinically silent, its prevalence can only be estimated, and ranges from 0.4 to 5% before the onset of clinically apparent disease. Besides heavy consumption of alcohol (150–170 g/day), pancreatic obstructions such as post-traumatic ductal strictures, pseudocysts, mechanical or structural changes of the pancreatic-duct sphincter and periampullary tumours may result in chronic pancreatitis. Of high importance is the recent recognition of a set of genetic mutations such as the loss of function mutations of pancreatic secretory trypsin inhibitor (SPINK1), which were shown to be present in CP cases that previously had been considered idiopathic (for review see [9]). Also, Sphincter of Oddi dysfunction (SOD) has increasingly been recognized as being present in CP [10].

Due to the progress in imaging techniques such as endoscopic retrograde cholangiopancreatography, magnetic resonance imaging and cross-sectional imaging, we now have a better understanding of the pathophysiology and origin of inflammation and pain in CP. Nevertheless, chronic pancreatitis still remains an inscrutable process of uncertain pathogenesis, unpredictable clinical course and difficult treatment [8, 11]. Chronic pancreatitis is associated with a mortality rate that approaches 50% within 20–25 years. Approximately 15–20% of patients die of complications associated with acute attacks of pancreatitis [8].

Complications such as biliary or duodenal stenosis, as well as intractable pain, are the current indications for surgery in patients with CP.

Surgical drainage of the duct in CP has largely been replaced by endoscopic duct drainage procedures of sphincterotomy and stent placement in the duct. Patients with CP whose pain persists after endoscopic pancreatic duct drainage are candidates for total pancreatectomy and islet autotransplantation (IAT) [12].

In the Cincinnati series of total pancreatectomy in combination with simultaneous IAT, unremitting abdominal pain refractory to high dose narcotics was the indication for surgery [13, 14]. Narcotic independence due to pain relief after total pancreatectomy and islet autotransplantation was achieved in 58–81% of the patients [6, 13]. Interestingly, in a recently performed retrospective survey, more than 95% of the patients stated they would recommend total pancreatectomy in combination with islet autotransplantation [6].

Mortality as well as morbidity associated with pancreatic resections in patients suffering from chronic pancreatitis was shown to be very low and normally leads to adequate pain control in the majority of CP patients. One drawback of surgical resection is the development of exo- and endocrine insufficiencies. Therefore, surgical resection of the pancreas is considered as a final option in the treatment of CP. Nevertheless, the addition of an islet autotransplant offers the possibility of a postoperative glucose control and should therefore always be a considerable option.

Besides being applicable to prevent surgical diabetes after extensive pancreatic resection for chronic pancreatitis, islet autotransplantation is additionally pertinent in benign tumours located at the neck of the pancreas. Even without pancreatic inflammation, extensive pancreatic resection of more than 70% of the pancreas may cause diabetes [15].

Islet autotransplantation, after extended pancreatectomy performed for the resection of benign tumours of the mid-segment of the pancreas, was shown to be a feasible option with excellent metabolic results and low morbidity. Due to the non-inflammatory nature of the pancreata, higher islet yields and, consequently, higher transplanted islet masses were achieved compared to those from organs resected for chronic pancreatitis. At a median follow-up of 5 years (range, 1–8 years), all patients ($n = 7$) had β-cell function as assessed by a positive C-peptide level. Six out of the seven patients were insulin independent [16]. Pivotal for such an approach is the unequivocal diagnosis of the benign nature of the tumour, before making the decision to perform the isolation and transplantation procedure.

The first total pancreatectomy in combination with islet autotransplantation to treat chronic pancreatitis (CP) in humans was performed 30 years ago at the University of Minnesota [5]. Besides aiming to relieve the pain of the CP patient in whom other measures had failed, the additional goal was to preserve β-cell mass and insulin secretion in order to avoid the otherwise inevitable surgical diabetes. Since then, more than 300 islet autotransplantations have been performed and reported worldwide, most of them at the University of Minnesota. With a few exceptions, the intraportal site has been predominantly applied as an implantation site for the transplanted islets [6, 19]. Since 1990 the results of autologous islet transplantation have been reported to the International Islet Transplant Registry (ITR) in Giessen, Germany [20].

Combined pancreatectomy and islet autotransplantation can be performed in adults, as well as in paediatric patients. For both patient populations, the procedures are identical and described in detail elsewhere [19, 21, 22]. Performing islet autotransplantations provides the possibility to compare the metabolic outcomes between islet autografts and islet allografts, the latter still being subject to declining function with time [1]. Besides, and prior to, the outstanding results from the Edmonton study fuelling the whole field of islet transplantation with new energy,

the “Minnesota islet autotransplantation” provided the pivotal biological “proof of principle” for the feasibility of a long-lasting successful glucose control after islet transplantation.

Islet allotransplantation shows a 5-year post-islet transplantation graft survival of approximately 80% and an insulin independence around 10% at 5 years [4]. Differences in the success of allogeneic islet transplantation among different centres illustrate the complexity of the procedure [1]. Therefore the ultimate goal, defined by insulin independence in the long term being achieved on a regular basis, has still not been achieved.

Notably, the results from islet autotransplantation obtained so far clearly show that long-term insulin independence after islet transplantation is a goal which can be realized, although also here, not on a regular basis [6, 23, 24]. In a recently published study, the outcomes of islet function over time were compared between intraportal islet autotransplant recipients at the University of Minnesota and diabetic islet allograft recipients as reported by the Collaborative Islet Transplant Registry (CITR). With regard to insulin independence, 74% of islet autotransplant recipients retained insulin independence at 2 years posttransplant vs. only 45% of the CITR allograft recipients who initially became insulin independent. Notably, 46% of the islet autotransplant patients were still insulin independent at 5 years and 28% at 10 years posttransplant [25].

31.3 What Can/Did We Learn from Islet Autotransplantations?

Three metabolic states were described in patients after islet autotransplantations: One-third of islet autotransplantation in the University of Minnesota series were long-term insulin independent, another third of the recipients became fully diabetic and the last third achieved near normoglycaemia and were therefore partially insulin independent requiring only one daily injection of insulin (Fig. 31.1a) [6].

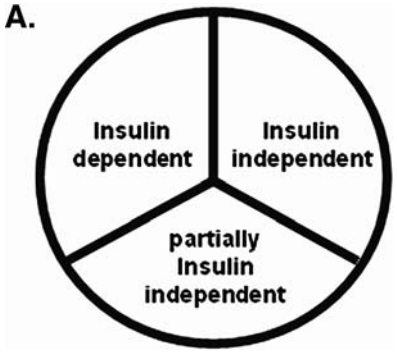

Fig. 31.1a Schematic representation of the three metabolic states described in patients after islet autotransplantations. One-third of islet autotransplantation in the University of Minnesota series were insulin independent in the long term, another third of the recipients became fully diabetic and the last third achieved near normoglycaemia and were therefore partially insulin independent requiring only one injection of insulin daily [6]

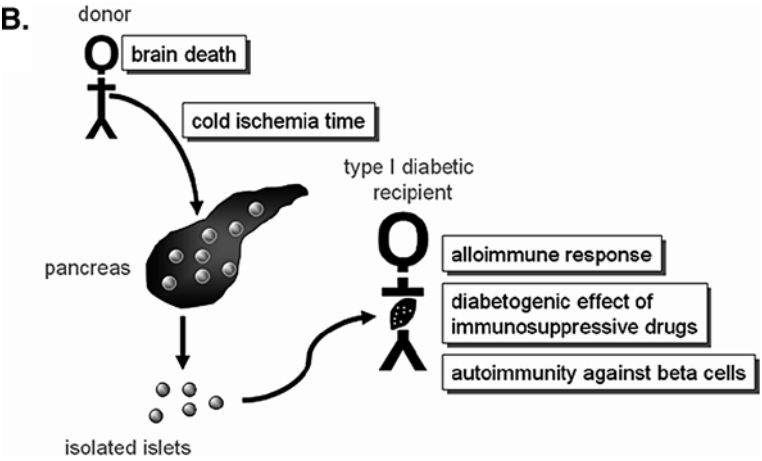

Fig. 31.1b In contrast to islet autografts, islet allografts are subject to several additional cell stress conditions. Brain death [26], longer cold ischaemia times before islet isolation from the donor pancreas [63], the patients' alloimmune response to the donor tissue, the autoimmunity against β-cells [29, 30] and the diabetogenic effect of the immunosuppressive medications [64] are the main reasons limiting long-term success of islet allotransplantation. Obviously the transport of the pancreas to the islet procurement center and the need for immunosuppression are the two main reasons limiting long-term success of islet allotransplantation

A remarkable result when comparing islet allo- with islet autotransplantation is the generally higher long-term success rate of the latter [4, 24]. There are at least three known causes (Fig. 31.1b) for organ/cell stress which are present in islet allotransplantation but not in autotransplantation, thereby possibly explaining the better long-term success rates of the latter:

1. *Brain death*: In islet allotransplantation, the organ is obtained from brain-dead patients. In animal models, brain death was shown to negatively affect islet yield as well as function due to the activation of pro-inflammatory cytokines [26].
2. *Ischaemia*: In islet autotransplantation, the organ is not subjected to prolonged cold ischaemia times which are normally present in islet allotransplantation due to the transport of the organ to the islet procurement centre. Such cold ischaemia times are known to damage the organ and impair cell viability, as well as function [27].
3. *Immunosuppression*: Besides ischaemia-associated organ damage, the need for immunosuppression in islet allotransplantation is the third major limiting cause in the long-term success of islet allotransplantation [27]. In human islet allotransplantation, immunosuppressive regimens are implemented in order to cope with both auto- as well as alloimmunity after transplantation. However, many of the immunosuppressive drugs are known to be directly β-cell toxic. Using a transgenic mouse model for conditional ablation of pancreatic β-cells in vivo, Nir and co-workers elegantly demonstrated that β-cells have a significant regenerative capacity which is prevented by the addition of the

immunosuppressant drugs Sirolimus and Tracrolimus [28]. As shown in humans, up to 15% of nondiabetic patients who received solid organ transplantation were shown to develop posttransplant diabetes as a result of calcineurin inhibitor therapy (i.e. tacrolimus) [27]. Therefore, the declining function of β-cells after human allotransplantation may also be explained by the inhibition of β-cell turnover due to the administration of immunosuppressive drugs [3].

Allograft rejection and recurrent autoimmunity, both conditions not present in islet autotransplant recipients, may additionally contribute to the decreasing insulin independence over time observed in the allogeneic setting [29, 30]. Recently it was shown that immunosuppression with FK506 and rapamycin after islet transplantation in patients with autoimmune diabetes induced homeostatic cytokines that expand autoreactive memory T cells. It was therefore proposed that such an increased production of cytokines might contribute to recurrent autoimmunity in transplanted patients with autoimmune disease, and that a therapy that prevents the expansion of autoreactive T cells will improve the outcome of islet allotransplantation [30].

Another recently published study reports that cellular islet autoimmunity associates with the clinical outcome of islet allotransplantation. In this study, 21 type 1 diabetic patients received islet grafts prepared from multiple donors, while immunosuppression was maintained by means of anti-thymocyte globulin (ATG) induction, tacrolimus and mycophenolate treatment. Immunity against auto- and alloantigens was measured before and during 1 year after transplantation. Interestingly, cellular autoimmunity before and after transplantation was shown to be associated with delayed insulin independence and lower circulating C-peptide levels during the first year after islet allotransplantation. While seven out of eight patients without pre-existent T-cell autoreactivity became insulin independent, none of the four patients reactive to both islet autoantigens GAD and IA-2 achieved insulin independence. Consequently, tailored immunotherapy regimens targeting cellular islet autoreactivity may be required [29].

An additional explanation for the lack of long-term insulin independence after islet transplantation was suggested to be the detrimental effect of hyperglycaemia on β-cell physiology. As shown in mice, increased apoptosis and reduced β-cell mass were found in islets exposed to chronic hyperglycaemia [31]. Consequently, both (auto- as well as allo-) human islet recipients usually receive insulin early on to maintain euglycaemia as much as possible. However, no study in humans has been performed so far comparing islet engraftment with and without this measure.

31.4 Still Open Issues in Islet Autotransplantation

31.4.1 Islet Mass

The timing of the pancreatectomy and simultaneous islet allotransplantation has a direct impact on islet yield. The highest islet yields and insulin independence can

be achieved when the islet autotransplantation is performed earlier in the disease course of CP [14, 32]. Interestingly, while most groups see a correlation between insulin-free status and IEQ transplanted [33], there are exceptions: One patient who received only 954 IEQ/kg remained insulin free even 4 years after transplantation [7, 34]. Considering the scarcity of available organs, such results are a crucial proof of principle showing that even very low amounts of transplanted islets may be sufficient to provide long-term insulin independence. One of the central goals for the future will be to rationalize the diversity in insulin-dependence response observed in patients. Elucidating the causes for such differences might enable us to design new therapeutic strategies, thereby allowing the successful engraftment and function of even low amounts of islets.

Interestingly islet autografts show durable function and, once established, are associated with a persisting high rate of insulin independence, although the β-cell mass transplanted is lesser than that used for islet allografts [25].

Evaluating and comparing the different outcomes after islet allo- vs. autotransplantations may help clarify the extent to which different stress parameters account for islet damage resulting in limited success rates of islet allotransplantation. There are several causes for cellular stress in islet autotransplantation.

31.4.2 Islet Shipment

Exposure of islets to a series of damaging physicochemical stresses already during explantation of the pancreas may amplify the damage caused during cold storage as well as the following islet isolation procedure. There is consensus among the major islet transplantation centres that islet yields and quality can be improved with better pancreas procurement techniques such as in situ regional organ cooling which protects the pancreas from warm ischaemic injury (for review see [35]). In addition, the development of more sophisticated pancreas preservation protocols promises to translate into an improved islet yield as well as quality.

While pancreatectomy can be performed at most hospitals, only a few centres are able to perform islet isolations. Therefore human islet autotransplantation is often limited due to the absence of an on-site islet processing facility. The setup of an islet isolation facility, designed according to the rules of good manufacturing practice, is a technically challenging, cost and time-intensive process [36, 37]. Consequently, several institutions have decided to perform transplantation of islets isolated at another centre with already established expertise. Such an “outsourcing solution” was shown to be applicable not only in human islet allotransplantation [37–39] but also in human islet autotransplantation [40, 41]. In the latter, the resected pancreata were transferred to an islet processing laboratory, which then sent back the freshly isolated islets that were transplanted into the same patient. All five patients experienced complete relief from pancreatic pain and three of the five patients had minimal or no insulin requirement, thereby demonstrating the feasibility of islet shipment for autotransplantation (median follow-up of 23 months) [41].

Although practicability as well as feasibility of islet transportation has already been proven, many questions such as the one addressing the optimal transport conditions for islets remain to be answered. While there is a worldwide consensus of how to isolate islets under GMP conditions, this is not the case for the transport of the freshly isolated islets. Many different media and transport devices have been used, ranging from 50 ml flasks, syringes and gas permeable bags [38]. Other solutions such as rotary devices avoiding detrimental cell compaction [42] may be an alternative, especially when vitality parameters such as temperature, pH or oxygen concentration are actively controlled [43]. Determining the optimal conditions for the transport of islets promises to yield better islet quality after the transport of islets and consequently an improved transplantation outcome. In addition, a gain of knowledge concerning the issues addressing the regeneration potential of freshly isolated islets may help not only to avoid unnecessary additional cellular stress but also to counterbalance it in a pre-emptive way.

In this context, the topic of islet quality assessment has to be mentioned: Similar to the transport conditions of human islets, this issue remains a matter of debate. Predicting the outcome of islet transplantation is still not possible due to the lack of reliable markers of islet potency, which might potentially be used to screen human islet preparations prior to transplantation. According to these pre-transplant criteria, islet preparations that failed to reverse diabetes were indistinguishable from those that exhibited excellent function [38].

Therefore, one of the primary challenges also in islet autotransplantation is to identify and understand the changes taking place in islets after the isolation, culture and transport. Description of such changes in living islet cells offers insights not achievable by the use of fixed cell techniques. Combining real-time live confocal microscopy with three fluorescent dyes, dichlorodihydrofluorescein diacetate (DCF), tetramethylrhodamine methyl ester perchlorate (TMRM) and fluorescent wheat germ agglutinin (WGA), offers the possibility to assess overall oxidative stress, time-dependent mitochondrial membrane potentials and cell morphology [44, 45]. The advantage of such a method resides in the fast and accurate imaging at a cellular and even subcellular level. Taking into account the use of other fluorescent dyes which can be used to visualize additional cell viability parameters such as calcium concentrations (measured with rhod-2) or apoptosis (measured with annexin-V), such an approach promises to be of great value for a better future islet assessment, post-isolation, culture and/or transport.

31.4.3 Cell Death

A significant proportion of the transplanted islet mass fails to engraft due to apoptotic cell death. Several strategies have been implemented to inhibit this process by blocking the extrinsic apoptosis inducing signals (cFLIP or A20), although only with limited impact. More recently, investigations of downstream apoptosis inhibitors that block the final common pathway (i.e. X-linked inhibitor of apoptosis protein [XIAP]) have shown promising results, in human [46–48] as well

as rodent [49] models of islet engraftment. XIAP-transduced human islets were significantly less apoptotic in an in vitro system that mimics hypoxia-induced injury. In addition, transplanting a series of marginal mass islet graft transplants in streptozotocin-induced diabetic NOD-RAG–/– mice resulted in 89% of the animals becoming normoglycaemic, with only 600 XIAP-transduced human islets [47]. Moreover, XIAP overexpression has been shown to prevent the diabetogenicity of the immunosuppressive drugs tacrolimus and sirolimus in vitro [48].

31.5 Which Are the Best Islets – Does Size Matter?

In islet allo- as well as autotransplantation, it is still a matter of debate to define the features of an ideal islet able to ensure proper long-lasting glucose homeostasis after transplantation into the liver. The central question is whether bigger islets are better suited than smaller islets.

In the early phase after transplantation, the islets are supplied with oxygen and nutrients only by diffusion. In addition, data obtained from rat islet transplantations have shown that, being in the portal vein, islets encounter a hypoxic state with an oxygen tension of 5 mmHg compared to 40 mmHg in the pancreas [50]. In a study determining whether the size of the islets could influence the success rates of islet transplantations in rats, the small islets (<125 μm) were shown to be superior compared to their larger counterparts (>150 μm). The superiority of small islets was shown in vitro, via functional assays, as well as in vivo after transplanting them under the kidney capsule of diabetic rats. Using only marginal islet equivalencies for the renal subcapsular transplantation, large islets failed to produce euglycaemia in any recipient rat, whereas small islets were successful in 80% of the cases [51]. A recent study analysed the influence of islet size on insulin production in human islet transplantation. The results convincingly showed that small islets are superior to large islets with regard to in vitro insulin secretion and higher survival rates [52]. Therefore islet size seems to be of importance for the success of human islet transplantation, and at least regarding islets it might be stated that “Small is beautiful!”

The question that remains to be answered is how to improve the transplantation outcome when using large islets. Besides applying measures that promote islet engraftment, such as the addition of the iron chelator deferoxamine which increases vascular endothelial growth factor expression [53], an alternative would be to customize large islets into small “pseudoislets” using the hanging drop technique [54].

31.6 The Role of the Surrounding Tissue: Site Matters!

To what extent is the surrounding tissue necessary or beneficial for islet function?

Besides the long-lasting functionality of autologous transplanted islets, there are at least two additional findings in islet autotransplantation that merit attention: the relatively low amounts of islets needed to achieve normoglycaemia and the impurity of transplanted islets.

In islet allotransplantation, about 850,000 islets, normally obtained from two to four pancreases, are needed to achieve insulin independence in a single type 1 diabetic patient. As a consequence, the available pool of pancreata for islet allotransplantation is limited and is therefore one of the foremost problems in islet transplantation. Interestingly islet autotransplantation has shown us that even low amounts of islets may result in long-lasting insulin independence [24, 55].

Due to extensive fibrosis, which is often present in pancreata of pancreatitis patients, the digestion process is incomplete. Theoretically, such an incomplete digestion might result in lower success rates after islet transplantation. Surprisingly, in a recent study, 8 of 12 patients who showed insulin independence after islet autotransplantation had less than 40% islet cleavage [7]. Therefore, a protective role of the tissue surrounding the islets might be postulated. Besides postulating such a protective role of the surrounding tissue, one could speculate that the digestion process may also lead to the loss of the basement membrane surrounding the islets [56] which might be detrimental as it is a well-recognized fact that the extracellular matrix provides the islets with biotrophic support [56-58].

Besides the innate surrounding tissue of the islets, the ectopic site into which the islets are implanted also seems to exert an influence on their biology: While autoislet β-cell biology can be normal (as shown by fasting glucose and haemoglobin A1c levels and intravenous glucose disappearance rates) for up to 13 years [24], there seem to be abnormalities in α-cell responsiveness to insulin-induced hypoglycaemia.

Although responses from intrahepatically autotransplanted islets to intravenous arginine were shown to be present, their responsiveness to insulin-induced hypoglycaemia was absent [59]. Similar observations were also made in islet allotransplantation: Two normoglycaemic type 1 diabetic patients who had been successfully transplanted with alloislets into the liver also failed to secrete glucagon during hypoglycaemia [59]. These findings led to a study comparing the α-cell function between autoislets transplanted either in the liver or in the peritoneal cavity of dogs. As expected from the situation in humans, the animals that received their islets transplanted into the liver did not have a glucagon response during hypoglycaemic clamps. Interestingly, in the animals that received their autoislets transplanted into the peritoneal cavity, the glucagon response was present. Both groups showed similar responses to intravenous arginine [60]. Although the underlying mechanisms are still unclear it could be said that “Site matters!”

31.7 Conclusion

The technical feasibility of islet autotransplantation has been demonstrated by several centres [14, 33 , 61]. In spite of the problems that autologous transplanted islets encounter in their new surrounding, pancreatic islet autotransplantation has prevented the onset of diabetes in pancreatectomized patients for more than two decades [62]. Therefore the biological proof of principle, for a long-lasting stable

glucose control by islets transplanted into the liver, has already been established. This success is equally surprising as well as inspiring for the more difficult task of islet allotransplantation. Understanding how autotransplanted islets can sustain their homeostasis and function in the liver, even for decades, might help us to find answers for still open questions regarding the molecular and cellular basis necessary for a successful islet allotransplantation.

Islet autotransplantation can abrogate the onset of diabetes and may therefore be considered as a valuable addition to surgical resection of the pancreas, The results obtained after islet autotransplantation have definitively provided a significant proof of principle: Islets are able to regulate glucose homeostasis over decades when transplanted into the liver.

In times like these, when the enthusiasm regarding clinical islet allotransplantation has been dampened by the inadequate long-term results, such a proof of principle is a vital beacon reminding us of the ultimate goal and prospects of islet transplantation.

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Chapter 32
Modulation of Early Inflammatory Reactions to Promote Engraftment and Function of Transplanted Pancreatic Islets in Autoimmune Diabetes

Lorenzo Piemonti, Luca G. Guidotti, and Manuela Battaglia

Abstract We acknowledge that successful long-term islet survival in the liver and immune tolerance to intrahepatic islet antigens are highly dependent upon the initial inflammatory and priming events that occur at this site. Thromboembolic and necroinflammatory events occurring in the liver early after portal vein islet transplantation are thought to reduce the total islet mass by up to 75%. The magnitude of such loss represents a major factor necessitating the extremely large number of islets needed to achieve normoglycemia. A better understanding and control of these events – including their likely support to effector immune responses – are required if we are to develop ways to prevent them, improve intrahepatic islet engraftment, and achieve long-term tolerance.

Keywords Type 1 diabetes · Pancreatic islet transplantation · Instant blood-mediated inflammatory reaction

32.1 Introduction

Despite the substantial improvements in insulin therapy thanks to new commercially available drugs, and the adoption of intensive treatment regimens able to improve glycemic control, exogenous insulin administration cannot avoid the long-term complications of diabetes and the life expectancy of diabetic patients is still shorter compared to that of the general population [1, 2].

In principle, treatment of type 1 diabetes (T1D) and many cases of type 2 diabetes lies in the possibility of finding a β-cell mass replacement capable of performing two essential functions: assessing blood sugar levels and secreting appropriate levels of insulin in the vascular bed. Currently, the only available clinical therapy capable of

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restoring β-cell mass in diabetic patients is the allogeneic/autologous transplantation of β-cells (i.e., somatic cell therapy with total pancreas, and Langherans' islets or β-cell transplantation).

Replacement of the whole gland reestablishes long-term normoglycemia, with a success rate of 80% [3], and it is especially successful in patients who undergo simultaneous pancreas and kidney transplantation. However, because of the risk of surgical complications, this procedure will never be a viable option for most T1D patients. The subjects offered this treatment are patients who have already developed many of the secondary complications, including end-stage renal failure and have a quality of life that is adequate for undergoing such a difficult treatment. Since the breakthrough made by Shapiro and colleagues [4], islet transplantation has emerged as an attractive alternative to whole pancreas transplantation. Despite advances in recent years [5], allogeneic somatic therapy is still problematic.

A nonspecific immune response mediated predominantly by innate inflammatory processes related to mechanics and site, and preexisting and transplant-induced auto- and allo-specific cellular immune responses (possibly promoted by the initial inflammation) play a major role in the loss of islets and islet function transplanted in the liver. Although significantly improved by the implementation of the Edmonton protocol, our capacity of achieving long-lasting insulin independence in T1D patients undergoing portal vein islet transplantation remains limited [5–7]. This indicates that the detrimental impact of innate and adaptive immune responses is not fully contained by the Edmonton protocol-associated regimen of generalized immunosuppression (i.e., induction with daclizumab [anti-IL-2Ralpha mAb] and maintenance with rapamycin [mTOR inhibitor] plus tacrolimus [calcineurin inhibitor]).

Prolong intrahepatic islet survival by increasing the potency of such regimen is not practicable, due to the likelihood of enhancing susceptibility to cancer and infections, and the toxicity that some of these drugs may have toward kidney functions and transplanted islets. Rather, it is intuitive that alternative strategies aimed at selectively inhibiting undesired islet-specific or nonspecific immune responses represent an ideal step toward a better management (i.e., weaning/withdrawal of generalized immune suppression) and outcome (i.e., long-lasting insulin independence) of islet transplanted T1D patients.

32.2 Defining the Site for Islet Transplantation

The liver was suggested as an optimal site for islet transplantation by Lacy and colleagues, by using a rat model of diabetes [8]. By the 1980s, successful transplantation of islet autografts was reported in humans by using infusion of cells into the patient's liver through the portal venous circulation [9–11]. Subsequently, the publication of the first case of insulin independence in a diabetic patient after islet infusion through the portal vein consecrated the liver as the site of choice for islet transplantation in humans [12]. Because of this early success, the subsequent

clinical experience of islet transplantation has been developed almost exclusively using the intrahepatic infusion through the portal vein. However, in the last years, it has becoming increasingly recognized that the liver may not be the optimal environment as a recipient site for pancreatic islets, owing not only to immunological [13, 14] but also to anatomical [15, 16] and physiological factors that likely contribute to the decline of islet mass after implantation [17–19].

The potential advantages of the intrahepatic islet transplantation include the low risk of the procedure and the delivery of insulin directly to the liver. From a clinical point of view, the process of intrahepatic infusion is currently considered safe, although there is a low risk of portal vein thrombosis and elevated portal pressure, in addition to bleeding from the percutaneous hepatic puncture site [20–23]. Numerous investigators, however, have recently addressed these complications, using high doses of heparin in conjunction with sealing the parenchymal track with thrombogenic material [24, 25].

From a metabolic point of view the process of intrahepatic infusion is considered optimal due to the fact that insulin is delivered more physiologically after intraportal transplantation [26]. In healthy individuals indeed, insulin is secreted by the pancreas into the portal venous circulation to the liver. Thus, the physiological balance between hepatic and extrahepatic insulin exposure requires portal delivery of insulin [27–29], and chronically implanted intrahepatic islets were described to be capable of restoring a pattern of insulin secretion and clearance that closely reproduces that of the native pancreas [26]. However, the argument that islets would work more physiologically after intraportal transplantation has recently received little support in the literature. It has clearly been demonstrated that intraportally transplanted islets in experimental models respond to glucose stimulation only when perfused via the hepatic artery; no response is observed after challenge via the portal vein [30]. There are also reports on alterations in islet function after intraportal islet transplantation, such as a defective glucagon response to hypoglycemia [19, 31, 32] and a defective glucose-stimulated insulin release [33].

The potential disadvantages of the intrahepatic route of islet transplantation include: (i) induction of instant blood-mediated inflammatory reaction, thrombosis, and nonantigen-specific inflammation; (ii) delayed vascularization of islets; (iii) exposure to high level of immunosuppressive agents; and (iv) glucolipotoxicity.

Intrahepatic islet infusion in men is associated with an immediate blood-mediated inflammatory reaction, thrombosis, and hepatic tissue ischemia with elevated blood liver enzymes [34–45]. Loss of as many as 50–75% of islets during engraftment in the liver [46] has been suggested to be a prime factor necessitating the very large number of islets needed to achieve normoglycemia [35]. Furthermore, the necessity for cannulation of the portal system to seed the islets leads to an increase in the portal pressure proportional to the administered islet mass [47], thus restricting the total mass that can be implanted. As a consequence, a highly purified suspension of islets is needed to transplant sufficient cells to achieve insulin independence. Because the purity of the suspension is inversely proportional to the islet yield per donor [48], fewer islets can be isolated from the already scarce donor pool, further limiting broad clinical applicability of pancreatic islet transplantation. It is

known that islets are highly vascularized. Pancreatic islets comprise only approximately 2% of the pancreas cell mass and yet consume up to 20% of the arterial blood flow [49–51]. Blood vessels within pancreatic islets are of a greater density than those in the surrounding exocrine tissue and are lined with fenestrated endothelial cells. These specialized features are responsible for the greater partial pressure of oxygen in islets as compared with acinar tissue and other organs, which is important for normal islet cell function and survival. Pancreatic islets lose this vascular supply during the isolation process [52]. In contrast to whole-organ transplantation, where organ perfusion is quickly reestablished by reconnection of arterial and venous vessels, the reestablishment of blood flow to transplanted islets requires several days and involves angiogenesis and possibly vasculogenesis. Importantly, not only are the islets avascular for several days following transplantation, but they are also less vascularized and have a lower oxygen tension than islets in the pancreas when revascularization is complete [15, 53]. Revascularization of the islet graft is estimated to require 7–14 days [54–57]. Vascular endothelial cells from both donor [58] and host [52] stimulate angiogenesis to form intragraft blood vessels in 3–5 days post-transplant, and full blood circulation is reestablished within approximately 1 week. Decreased vascular density and low revascularization have been reported in transplanted mouse [53] and human pancreatic islets [59] although studies have also shown normal vascularity [55].

The portal vein carries blood with oxygen tension slightly less than that of arterial blood, contains higher concentrations of substances from the gut that may be toxic to the islets, and the immunosuppressive agents (known to be toxic to islet cell function) are absorbed from the gut and thus their toxic effects might be magnified for islets bathed in portal blood. Recently, portal vein and peripheral blood immunosuppressant drug concentrations in islet transplant patients were measured and higher levels of both sirolimus and tacrolimus in the portal blood circulation were reported [18, 60]. These drugs may interfere with angiogenesis and may be cytotoxic to β-cells at high local concentrations.

Islets are metabolically active and require access to oxygen, glucose, and other metabolites in a hospitable environment at physiological pH, and to be free from toxic metabolites and oxygen free radicals. Chronic exposure of transplanted islets to the liver would lead to high portal vein levels of nutrients and gut hormones; the resulting hypersecretion of undiluted insulin into surrounding hepatocytes would elicit a powerful lipogenic response, overloading the nearby hepatocytes with triacylglycerol. Islets would be chronically exposed to both a uniquely high lipid environment and a high glucose environment. This combination would result in glucolipotoxicity [61]. This hypothesis is supported by liver biopsy evidence [39, 62, 63] and by the fact that fatty livers occur in 20% of subjects in association with graft dysfunction [64]. The potential pathogenic consequences of the lipid excess are suggested by the demonstration that exposure of isolated human islets to fatty acids damages β-cells and directly or indirectly results in apoptosis [65].

The recognition of these problems has renewed the interest in the search for an alternative site for implantation such as the intramuscular site and the omental pouch [66].

32.3 Main Biological Events Triggering Early Graft Failure of Transplanted Pancreatic Islets

Among the components concurring to the outcome of islet transplantation, the instant blood-mediated inflammatory reaction (IBMIR) is considered a crucial event associated with early loss of function of transplanted islets. The IBMIR is a thrombotic reaction occurring when purified human islets are incubated in ABO-compatible blood. This reaction causes morphology disruption of those islets entrapped within a thrombus [67, 68]. The IBMIR is a likely cause of both loss of transplanted tissue and the intraportal thrombosis associated with clinical islet transplantation. The IBMIR is triggered by production of tissue factor (TF) and secretion by the endocrine cells of the islets of Langerhans in the islet preparation [42]. After initial generation of thrombin, by TF-expressing islets, thrombin-activated platelets start to bind to the islet surface. Via the amplification loop involving factor XI and activated platelets [69], more thrombin is formed, generating a fibrin capsule surrounding the islets. The IBMIR occurs in clinical islet transplantation as shown by an increase in concentrations of thrombin–antithrombin complex immediately after islet infusion [42], even without clinical signs of intraportal thrombosis. Indeed, intraportal thrombosis can occur since the thrombus does not originate from the vessel wall but from the transplanted islets and it is therefore not occlusive. The IBMIR culminates in the disruption of islet morphology by infiltrating leukocytes. Polymorphonuclear cells (PMN) were found to be the predominant cell type infiltrating the islets in vitro [67, 68]. PMN appeared already 15 minutes after incubation with ABO-compatible blood, with massive infiltration occurring within 1 hour and peaked at 2 hours. Macrophages were also found to infiltrate the islets, although the number of infiltrated cells increased slightly over time [70]. B and T cells were not detected at all in the islets during the whole period of incubation, suggesting that the specific immune response is not involvedin the early phase of the IBMIR. In line with this hypothesis, immunosuppressive therapy presently used in T1D patients undergoing islet transplantation does not affect IBMIR [71]. PMN and macrophage recruitment and infiltration into transplanted islets is supposed to be a crucial event in their loss of function. It is worthwhile mentioning that islet β-cells are exquisitely susceptible to oxidative stress because of their insufficient antioxidant pool [72], a situation that points to a rapid and direct damage to the islets by infiltrating PMN and macrophages. In addition, infiltrating PMN are supposed to be directly involved in monocyte and T-lymphocyte recruitment, since it was demonstrated that PMN release chemotactic factors for T cells and macrophages [73, 74].

The mechanism(s) by which the newly transplanted islets stimulate PMN and monocyte recruitment are not completely understood. It was suggested that leukocyte infiltration is a result of complement activation [67, 68]. The anaphylatoxins C3a and C5a, released upon activation of IBMIR cascade events, were supposed to be responsible at least in part for leukocyte recruitment and infiltration into transplanted islets. However, a consistent leukocyte infiltration into the islets was also observed when both coagulation and complement activation were abrogated, clearly

indicating that mechanisms other than complement activation are responsible for leukocyte recruitment [68]. In the same context, it was hypothesized that leukocyte recruitment could be elicited by proinflammatory mediators released by the islets [75, 76]. The mechanism responsible for PMN and monocyte/macrophage recruitment could be, respectively, CXCL8 and CCL2 production. Indeed, islets have been shown to express CXCL8 and CCL2 [77, 75] and this event might trigger the inflammatory reaction at the site of transplant and may play a relevant role in the clinical outcome of islet transplantation. It was demonstrated that primary cultures of pancreatic islets expressed and secreted CCL2 and low production of CCL2 by the islets resulted as the most relevant factor for long-lasting insulin independence [75]. In addition, a significant relationship between TF and CCL2 released in vitro by the islets and plasma biochemical parameters of coagulation in patients after islet transplantation was demonstrated [36].

Overall, these results suggest that the infiltration pattern observed during the IBMIR resembles, at least in part, that detected in the reperfused organs. In this context, PMN are attracted to the graft due to upregulation and release of agents by the ischemia-induced alterations of the endothelial cells and parenchyma cells of the transplanted organ. Similarly, PMN and subsequent mononuclear leukocyte recruitment to the islets could also be due to induced specific chemotactic mediators released from islets and/or infiltrating PMN themselves. The massive infiltration by PMN probably causes direct damage to the islets, not only by functionally impairing or reducing the mass of the implanted islets but probably also by amplifying the subsequent immune responses [78].

Recently, the inflammatory reaction observed in the liver of the recipient after islet transplantation has been considered as an additional component, other than IBMIR, that could contribute, together with the subsequent immune responses, to early graft failure. In this context, intrahepatic islet infusion in humans, as well as in experimental animal models, is associated with histopathologic changes again resembling at least in part the post-ischemic organ situation. Indeed, histological evaluation of livers following portal vein islet transplant showed the presence of embolism, thrombosis, and abundant areas of liver necrosis around the transplanted islets [44, 45]. In experimental animal models of allogeneic islet transplantation, ischemia and necrosis of the liver reach a peak at 1 day after islet transplantation, are reduced by day 3, and are largely resolved by day 7 [79]. The interpretation of these observations is that islet transplant blocks the blood flow to the capillary bed resulting in acute necrosis of the surrounding liver tissue. This phenomenon is considered to be clinically relevant since no substantial dimensional differences in small liver vessels and capillaries are evident in mice and men, suggesting that the portal size difference should not be relevant at the levels of single islet microenvironment in terms of ischemia and reperfusion. Starting from day 2 after islet allotransplantation, leukocytes, mainly PMN, infiltrate the necrotic hepatic regions. Subsequently, a mononuclear leukocyte infiltrate colonizes islets, starting as peri-islets and becoming intra-islets, with progressive endocrine tissue destruction and loss of the insulin production [79] (Fig. 32.1). Again, infiltrated PMN, as demonstrated in post-ischemic situations, could have a crucial physiopathological role in directly inducing tissue damage and islet loss of function as well as in orchestrating

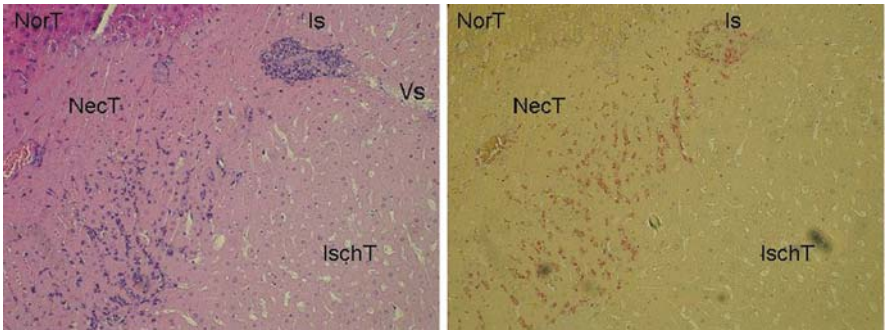

Fig. 32.1 PMN infiltration in hepatic tissue after islet iso-transplantation in diabetic wild type BALB/C mice. The islets (Is) localize in the blood vessels of portal space (Vs) 24 hours after transplantation. Intravascular thrombi are consistently found around islets. Regions with abnormal cell shape and texture (Ischemic area, IschT) or necrotic (NecT) wedge-shaped regions appear (*left panel*) in normal tissue (NorT). PMN infiltration (stained in red by naphthol AS-D chloroacetate technique for esterase) is evident into necrotic hepatic tissue and around transplanted islets (*right panel*)

subsequent mononuclear leukocyte recruitment. The mechanisms by which PMN are chemoattracted in transplanted islets could include chemotactic factors directly produced by transplanted islets as well as released by hepatic tissue. CXCL8 in patients or its murine counterpart (i.e., KC/CXCL1) in experimental models could be considered the main PMN chemoattractant released by the transplanted islets as well as by post-ischemic livers [80].

32.4 Strategies to Prevent the Instant Blood-Mediated Inflammatory Reaction

If early graft loss after intraportal islet transplantation has to be reduced, interventions can be directed against the various components of IBMIR or, ideally, against all components by a single agent, if such an agent exists. There are two fundamental approaches that could be conceived to counteract the effects of IBMIR. The first is based on systemic treatment of the recipient in order to prevent coagulation and complement activation at the transplantation site; the second is based on manipulations of the transplanted tissue to minimize its intrinsic characteristics that trigger IBMIR. The advantage of the former is its quite immediate clinical applicability; the primary disadvantage is that a systemic treatment may have generalized side effects (namely bleeding). Manipulation of the tissue prior to implantation would have the advantage of its localized effect, but the potential disadvantages are linked to the quite cumbersome technological approach needed to achieve this goal. Gene therapy approaches, as an example, have intrinsic limitations of safety and efficacy, and there is concern about the immunogenicity of viral-encoded products.

In current practice the major effort was dedicated to prevent or modulate coagulation and complement activation. A strong candidate drug to block IBMIR in clinical

islet transplantation is the low molecular weight dextran sulfate (LMW-DS; MM 5000), today available for clinical use, that inhibits both complement and coagulation activation [81, 82]. In in vitro studies, replacement of heparin by low molecular weight dextran sulfate blocked IBMIR to a greater extent. In in vivo rodent studies, treatment of the recipient with dextran sulfate significantly prolonged survival of intraportally transplanted islets [83]. Based on these evidences a clinical trial to assess the safety and effectiveness of LMW-SD on post transplant islet function in people with T1D is currently ongoing (ClinicalTrials.gov Identifier:NCT00790439).

Other anticoagulant or complement inhibitors have been shown to prevent islet damage in vitro or in vivo: melagatran, a specific thrombin inhibitor [68]; nacystelyn, a derivative of *N*-acetylcysteine [84, 85]; activated protein C [38, 86]; sCR1, a complement inhibitor [87]; TP10, the soluble complement receptor 1 [88]; low molecular mass factor VIIa inhibitor [41]; nicotinamide [89]. In general, all these strategies aimed at inhibiting the IBMIR have shown only a modest benefit in a limited series of in vivo studies in animal models. It is unlikely that an agent targeting only one component of IBMIR would block all elements of the reaction (coagulation, complement activation, production of proinflammatory mediators); therefore, the effect of a single agent on the engraftment may be limited. The identification of new strategies to reduce the detrimental effects of IBMIR should be one of the objectives of the research in the next years. For example, PMN recruitment and infiltration into transplanted islets is a key pathophysiological event responsible for direct damage of islet functionality and, at least in part, of subsequent mononuclear leukocyte infiltration and related loss of insulin production. Among chemotactic mediators, CXCL8 in transplanted patients, and the murine counterpart in experimental transplants, is supposed to be a crucial mediator in PMN recruitment into transplanted islets and thus represents, together with its receptors (CXCR1 and CXCR2), a primary therapeutic target to prevent early graft failure.

Manipulation of the tissue prior to implantation has the advantage of its localized effect. Surface heparinization of islets is an attractive alternative to soluble heparin. It provides a means to render biocompatible the islet surface when exposed to blood, thereby mimicking the protective characteristics conferred by heparan sulfate on the endothelial cells lining the vascular wall. In addition to the effects on the cascade systems and on the circulating cells, heparin coating reduces exposure of collagen and other extracellular matrix proteins on the islets that may be prothrombotic and trigger inflammation. On this basis it was demonstrated that modification of pancreatic islets with surface-attached heparin or thrombomodulin can reduce the deleterious IBMIR associated with islet transplantation [90]. Similarly, since endothelial cells (EC) readily tolerate contact with blood, a conceivable strategy to overcome IBMIR would be to create composite islet-endothelial cell grafts. This approach was recently reported. Human islets were cocultured with primary human aortic endothelial cells (HAEC) for 2–7 days to obtain 50–90% coverage [91]. Exposed to blood, HAEC-coated islets induced less activation of coagulation and complement compared to control islets with decreased platelet and leukocyte consumption and less infiltration of CD11b+ cells in clots. After transplantation to athymic nude mice, composite islet-HAEC grafts stained positive for insulin and

PECAM-1 demonstrating the presence of both islets and HAEC within the islet graft 7 weeks after transplantation. The refinement of this technique could allow introduction of composite islet-EC grafts in clinical islet transplantation, using autologous EC expanded in vitro and kept frozen until allogeneic islets become available for that specific recipient**.** Similar results were reported using composite pig islet-human EC graft [92]. More recently it was reported that addition of mesenchymal stem cells to composite islets enhanced the capacity of EC to enclose the islets without compromising the islet functionality. Moreover, the mesenchymal stem cells stimulated EC sprout formation not only into the surrounding matrices but also into the islets where intra-islet capillary-like structures were formed [93].

32.5 Strategies for Cytoprotection and Revascularization

Methods that favor islet engraftment by modulating islet cell resistance to the noxious stimuli and/or the level of inflammation at the transplant site and/or the level of vascularization may result in long-term insulin independence after transplantation of a reduced number of islets. Induction of islet cytoprotection to reduce and/or prevent the negative effects of noxious stimuli may be achieved by multiple means, including preconditioning of the graft in culture prior transplantation and/or treatment of the recipients in the peri-transplant period. Several approaches have been proposed toward this goal, including the use of a number of cytoprotective regimens via pharmacological administration, gene therapy, gene silencing, and protein transduction domains. Potential candidate molecules that have been used in experimental studies include 17-β-estradiol, nicotinamide, metal protoporphyrins, glucagon-like peptide-1 (GLP-1), which may be used during isolation, added in the culture media, or administered to the recipient as they may avoid or partially prevent the effects of oxidative stress and proinflammatory cytokines early after transplant, therefore maximizing islet engraftment.

An example of molecule useful for both pretreatment of isolated islets and recipient is α1-antitrypsin, a major protease inhibitor that inhibits the enzyme activity of neutrophil elastase and thrombin. In vitro, in the presence of α1-antitrypsin, mouse islets were protected against the effect of the cytokines, IL-1β, and IFN-γ, by means of greater viability, a 40% reduction in nitric oxide production, and greatly diminished TNF-α production. Moreover, administration of human α1-antitrypsin to recipient mice improved islet survival [94–96] and would represent a safe approach in the clinic.

Similarly it was recently reported that methylprednisolone is efficient in reducing the inflammatory status of human islets and thus has the potential to improve graft function following islet transplantation [97]. As a consequence, some groups have introduced glucocorticoid preconditioning of the islet preparation prior transplantation. In addition, in these centers all organ donors are given methylprednisolone prior procurement [97]. Moreover, antioxidant supplementation to the islet culture medium for scavenging oxygen radicals helps human islets to

reduce their inflammatory state, determined by reduction of cytokine and MCP-1 expression [98].

Several factors may improve graft angiogenesis and vascularity. Vascular endothelial growth factor-A, a well-known angiogenic factor that is secreted by islets in response to hypoxia, appears to play a significant role in angiogenesis and improvement of graft function [99, 100]. Additional growth factors, such as platelet-derived growth factor, epidermal growth factor, and fibroblast growth factor, are also thought to promote graft revascularization [101]. Overexpression of molecules known to enhance revascularization such as VEGF has been attempted, but these molecules have yet to exhibit a significant impact on islet graft survival [102, 103]. This is likely related to the fact that although vascular endothelial growth factor expression will hasten the revascularization process, it cannot provide an immediate benefit to the transplanted tissue.

Ex vivo gene transfer to isolated islets has been performed using several gene candidates including interleukin-1 receptor antagonist [104, 105], TNF-α antagonist [46, 106–108], heme oxygenase I [109, 110], insulin-like growth factor-I [111, 112], dominant negative protein kinase C[113], dominant negative MyD88 [114], nuclear factor B [115], inhibitor of B repressor [116], heat shock protein 70 [113], manganese superoxide dismutase [118], and catalase [119] for immune, inflammation, and apoptosis protection.

Several different molecules that inhibit the generation of and/or damage mediated by reactive oxygen species (ROS), including glutathione peroxidase, superoxide dismutase, and heme oxygenase-1 have tested [109, 110, 120, 121]. Although these molecules can individually protect islets during controlled in vitro challenges where ROS are specifically produced, the in vivo benefit of such an approach has only been demonstrated when glutathione peroxidase and superoxide dismutase were co-expressed in transgenic islet grafts [122].

Significant efforts have been also made to inhibit specific apoptotic triggers, either extrinsic (cFLIP and A20) or intrinsic (BCL-2 and BCL-XL). These proteins have proven to be quite effective in enhancing β-cell survival in vitro. However, reproducing the protective effect using transplanted islets has been difficult and largely unfruitful. Recently, investigations of downstream apoptosis inhibitors that block the final common pathway (i.e., X-linked inhibitor of apoptosis protein [XIAP]) have demonstrated promise in both human and rodent models of engraftment [123, 124]. The studies using XIAP strongly support the concept that inhibition of apoptosis at the level of caspases promotes β-cell survival in islet transplantation, effectively preventing cell death triggered by extrinsic and intrinsic pathways at the same time.

32.6 Modification of Transplant Site and Biomaterial-Based Strategies to Improve Engraftment

A simple and attractive approach to improve islet transplantation engraftment is to consider whether sites and techniques other than intraportal infusion into the liver are better adapted for islet implantation and survival. Although many implantation

sites have been proposed, few have found their way into the clinical setting [125]. In one clinical investigation using infusion of islets under the kidney capsule, two of three recipients showed autograft survival, measured by C-peptide secretion. However, the high transplant mass at this site relative to intraportal infusions does not justify the approach [126]. The intraperitoneal and omental pouch sites are also attractive for clinical islet cell transplantation [127] as they allow a large implantation volume and the concurrent use of transplant devices or capsules [128]. A case report of autologous islet transplantation into the brachioradialis muscle of a 7-year-old girl who had complete pancreatectomy for severe hereditary pancreatitis described C-peptide levels maintained even 2 years after transplantation [66]. The patient received conservative insulin therapy via a pump, but the maintained function of the transplant was believed to contribute to good glycemic control and prevention of hypoglycemic events.

Applications for biomaterials in improving islet engraftment by immunoisolation of the transplanted tissue through semipermeable membranes are increasing [129–131]. Three different kinds of encapsulated systems can be used for the purpose of islet transplantation: (1) perfusion chambers directly connected to the blood circulation (intravascular macrocapsules), (2) diffusion chambers in the shape of a tube or disk that can be implanted i.p. or s.c. (extravascular macrocapsules), or (3) the encapsulation of one or few islets in globular membranes (extravascular microcapsules). Intravascular macrocapsules are based on the principle of "dialysis cartridges" in which islets are seeded in the space between hollow fibers that are perfused with blood. The islets may be in a packed form or dispersed in a spacer matrix that prevents mutual adhesion and improves diffusional nutrient transport of the islets. These hollow fibers are enclosed within a larger tube, and the device is implanted into the vessels of the host by vascular anastomoses. Biomaterial used for the construction of these microcapillaries is polyacrylonitrile and polyvinylchloride copolymer, a biocompatible matrix often used in spinal cord injury. These devices permit close contact between the bloodstream and the islets, leading to efficient diffusional transport of metabolites. Encapsulation of islets in this device has been shown to induce normoglycemia in various animal models of diabetes including rats, dogs, and monkeys [132, 133]. The duration of this normoglycemia was usually restricted to several hours and success of a somewhat longer duration was exceptional. Blood clotting in the lumen of these small-diameter artificial capillaries proved to be a major obstacle in spite of intense systemic anticoagulation, which is indicative of low biocompatibility of the implant material. An increase in the diameter of the capillaries led to increased flow rate of blood and reduced the risk of thromboembolism, but not without accompanying risks plus the complications associated with vascular prosthetic surgery [134]. These considerations shifted the research focus toward extravascular macrocapsules for islet engraftment.

Extravascular macrocapsules are based on the same principles as intravascular ones but have the advantage that biocompatibility issues do not pose a serious risk to the patient. These devices have been designed in both flat sheet membranous and hollow fiber formats [135]. They can be implanted into the peritoneal cavity, the subcutaneous tissue, or under the kidney capsule. Various biomaterials have been used

to generate these devices including nitrocellulose acetate, 2-hydroxyethyl methacrylate (HEMA), acrylonitrile, polyacrylonitrile and polyvinylchloride copolymer, sodium methallylsulfonate, and alginate. Biocompatibility of these devices is seen in terms of fibrosis at the site of implantation and covering the device. Various approaches have been used to enhance the biocompatibility of these devices, including the use of hollow fiber geometry because it offers reduced surface area of contact with the host per islet. Use of a smooth outer surface and hydrogels further improves biocompatibility of these devices by the absence of interfacial tension, thus reducing protein adsorption and cell adhesion. Hydrogels also provide higher permeability for low molecular weight nutrients and metabolites. Hydrogel materials that have been used include alginate [136], agarose [137–139], polyurethane [140], chitosan–polyvinyl pyrrolidone hydrogels [141], cellulose [142], cross-linked hydrophilic poly(*N,N*-dimethyl acrylamide) with hydrophobic di-, tri-, and octamethacrylate telechelic polyisobutylene [143], and a copolymer of acrylonitrile and sodium methallylsulfonate [144]. Other approaches to address the problem of biocompatibility of these devices include membrane coating with poly-ethylene-oxide to reduce surface protein adsorption and surface hydrophobization with corona discharge. Surface fibrosis and biocompatibility remain the most significant hurdles to the successful use of both macrocapsule and microcapsule devices.

Extravascular microcapsules enclose one or a few islets and are implanted at extravascular sites for obvious reasons. Microencapsulation of islets offers several advantages over macroencapsulation: higher surface area per unit volume for better diffusive transfer of nutrients and metabolites, mechanical stability, ease in manufacturing, and easy implanting procedures. Encapsulated islets have shown improved graft function and survival compared with unencapsulated islets [145, 146]. The long-term survival and function of islets microencapsulate, however, is limited. Primary impediments to the success of microencapsulation for islet transplantation include: (1) biocompatibility, (2) inadequate immunoprotection, and (3) hypoxia. Inadequate biocompatibility is recognized by the pericapsular overgrowth on microcapsules that consist of fibroblasts and macrophages [147, 148]. Moreover, since encapsulation precludes the ingrowth of blood vessels and islet revascularization is important for long-term islet function, use of encapsulated islets has been complicated by cell death secondary to chronic hypoxia and/or decreased accessibility to nutrients and growth factors. Islets microencapsulated within an alginate-poly-(L-lysine) membrane and an agarose hydrogel membrane have been investigated for use as a bioartificial pancreas. Many groups have reported that a long-term normoglycemia in a diabetic small animal, such as a mouse or a rat, can be realized by transplanting microencapsulated islets into its peritoneal cavity. However, in clinical settings, about 10 ml of islet suspension should be injected through a catheter into the portal vein in the liver. The diameter of microencapsulated islets was several times larger than that of islets, which could result in plugged vessels if infused into the portal vein. A report documented normoglycemia in a human patient with intraportal transplantation of microencapsulated islets for a period of 9 months [149].

An emerging strategy to improve engraftment avoiding the limit of encapsulation is the use of synthetic biocompatible microporous polymer scaffold [127, 150–153]. The polymer scaffolds are porous and not intended to serve as an immune barrier. Rather, they were specifically designed to provide a solid support for islets that would allow cellular infiltration and formation of a vascular network within the transplant graft. Several basic requirements for cell transplantation on microporous scaffolds have been identified, including biocompatibility, a high surface area/volume ratio with sufficient mechanical integrity, and a suitable environment for new tissue formation that can integrate with the surrounding tissue [154, 155]. Microporous scaffolds with a high surface area/volume ratio not only have sufficient surface area to support cell adhesion but also can support nutrient transport by diffusion from surrounding tissue. Moreover, they can be fabricated from material that has sufficient mechanical properties to resist collapse while maintaining an interconnected pore structure that allows for cell infiltration from the surrounding tissue. This is important not only for integration of the engineered tissue with the host but also for development of a vascular network throughout the tissue to supply the necessary metabolites once the transplanted cells are engrafted. Encouraging reports indicate that a synthetic polymer scaffold can serve as a platform for islet transplantation and improves the function of extrahepatically transplanted islets compared to islets transplanted without a scaffold. The scaffold may also be useful to deliver bioactive molecules to modify the microenvironment surrounding the transplanted islets and, thus, enhance islet survival and function.

Camouflaging the surface of islets instead of incorporating them in a membrane barrier or in a scaffold is another approach to improve engraftment. This process involves attachment of polymeric, hydrophilic chains to the islet surface to achieve molecular coating of the islets. Surface modification of islets by bioconjugation can overcome several potential problems with islet encapsulation. The diffusional barrier of less resistance and reduced thickness can be generated around the islets, compared with an encapsulation membrane. Furthermore, fine surface coating of islets leads to reduced volume of tissue per equivalent islet, which makes transplanting islets into human subjects feasible via the portal venous route of administration [156]. Strategies for surface coating of islets essentially use linear hydrophilic polymers such as PEG with an activated functional group and a mild conjugation reaction. Surface coating of rat islets with PEG was first reported by Panza et al. [157] and was subsequently shown to be cytoprotective for porcine islet xenotransplantation in diabetic SCID mice [158]. Panza and colleagues demonstrated that the viability of islets was not compromised upon PEGylation and that islets retained the in vitro insulin response to glucose stimulation activity. Xie et al. additionally demonstrated protection in vitro against human antibody/complement-induced cytotoxicity in coated porcine islets and in in vivo islet function in the diabetic SCID mice model [158]. Xie et al. further introduced the concept of albumin shielding of islets using a disuccinimidyl derivative that is attached on one end to the islets and on the other end to an albumin moiety [158]. This concept harbors the possibility of modifying encapsulation technology to literally “build” capsules on the islet surface instead of “encapsulating” islets. Heterobifunctional PEGs can be conjugated to the

islet surface followed by attachment of another moiety on the exposed end of the PEG chains, which can then be cross-linked to each other to result in a firm microcapsule. Formation of a microcapsule in this manner will obviate many problems associated with the processing technology of microencapsulation discussed earlier [159–161].

Methods for immobilizing enzyme and glycosaminoglycans, such as urokinase and heparin, to the surface of islets utilizing layer-by-layer approach were described [162]. The surface of islets was modified with a poly(ethylene glycol)-phospholipid conjugate bearing a biotin group (biotin-PEG-lipids, PEG MW: 5000). Biotin-PEG-lipids were anchored to the cell membranes of islets, and the PEG-lipid layer on the islets was further covered by streptavidin and biotin—bovine serum albumin conjugate using a layer-by-layer method. The surface was further activated with oxidized dextran. Urokinase was anchored to the islets through Schiff base formation. Heparin was anchored to the islets through polyion complex formation between anionic heparin and a cationic protamine coating on the islets. No practical islet volume increase was observed after surface modification, and the modifications did not impair insulin release in response to glucose stimulation. The anchored urokinase retained high fibrinolytic activity, which could help to improve graft survival by preventing thrombosis on the islet surface.

32.7 Concluding Remarks

Clinical islet transplantation is currently being explored as a treatment for persons with type 1 diabetes and hypoglycemia unawareness. Although ‘proof of principle’ has been established in recent clinical studies, the procedure suffers from low efficacy and a large number of islets are needed to obtain insulin independence in clinical islet transplantation, requiring two to four cadaveric pancreases. Islet engraftment remains an unresolved problem in humans and the possibility of having clinically applicable solutions will be one of the determinants of success or failure of islet transplantation in the coming years.

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Chapter 33
Successes and Disappointments with Clinical Islet Transplantation

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Abstract Transplantation of pancreatic islets is considered a therapeutic option for patients with type 1 diabetes mellitus who have life-threatening hypoglycaemic episodes. After the procedure, a decrease in the frequency and severity of hypoglycaemic episodes and sustained graft function as indicated by detectable levels of C-peptide can be seen in the majority of patients. However, true insulin independence, if achieved, usually lasts for at most a few years. Apart from the low insulin independence rates, reasons for concern regarding this procedure are the side effects of the immunosuppressive therapy, allo-immunization, and the high costs. Moreover, whether islet transplantation prevents the progression of diabetic micro- and macrovascular complications is largely unknown. Areas of current research include the development of less toxic immunosuppressive regimens, the control of the inflammatory reaction immediately after transplantation, the identification of the optimal anatomical site for islet infusion, and the possibility to encapsulate transplanted islets to protect them from the allo-immune response. At present, pancreatic islet transplantation is still an experimental procedure, which is only indicated for a highly selected group of type 1 diabetic patients with life-threatening hypoglycaemic episodes.

Keywords Pancreatic islet transplantation · type 1 diabetes mellitus · immunosuppression · diabetic complications

33.1 Introduction

In 2000, research in the field of pancreatic islet cell transplantation was boosted by a key paper reporting insulin independence in seven out of seven patients with type 1 diabetes mellitus (T1DM) over a median follow-up of 12 months [1]. The two major

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novelties of this protocol were the administration of increased doses of pancreatic islets by infusing islets from at least two donor pancreases at separate occasions and an immunosuppressive protocol devoid of steroids. Until then, clinical outcomes had been disappointing. Of the 267 islet preparations transplanted since 1990, less than 10% had resulted in insulin independence for more than 1 year [2]. With the new protocol, success rates have increased in parallel with significant improvements in the technical procedure and medical management of islet transplantation. However, true insulin independence rates for a prolonged period of time are still very low, and patients are required to take immunosuppressive medication as long as there is evidence of remaining graft function. Moreover, islet transplantation remains a highly complex procedure, the planning and execution of which require a high degree of specialization. It also typically requires the use of at least two donor pancreases and may compete with the number of organs available for whole organ transplantation. Thus, islet transplantation is still far from representing an effective and widely available cure for T1DM. This review describes the successes and disappointments of clinical islet transplantation programmes.

33.2 The Burden of Type 1 Diabetes Mellitus

T1DM is the most common metabolic disease in childhood with incidence rates ranging from 8 to >50 per 100,000 population per year in western countries [3]. For children aged 0–14 years, the prevalence of T1DM is estimated to be at least 1 million worldwide by the year 2025 [4]. Children with T1DM usually present with a several-day history of typical symptoms such as frequent urination, excessive thirst, and weight loss, which appear when about 80% of the pancreatic β-cells are already destroyed. If those symptoms are misinterpreted, progressive insulin deficiency leads to a potentially life-threatening condition in the form of diabetic ketoacidosis. Patients with T1DM require daily subcutaneous injections of insulin in an effort to mimic the physiological release of insulin during meals and during fasting periods. The Diabetes Control and Complications Trial (DCCT) showed that intensive glycaemic control obtained by at least thrice daily insulin injections on the basis of frequent glucose measurements reduces the incidence and slows the progression of microvascular complications when compared to less intensive therapy [5]. Long-term follow-up of this trial also showed that macrovascular complications were less frequent in patients who had been in the intensive treatment arm [6].

33.3 Pathophysiology of Type 1 Diabetes Mellitus

Pancreatic tissue is composed of two cell types: acinar cells that excrete digestive enzymes into pancreatic ducts (exocrine function) and the cells contained in the islets of Langerhans that release various hormones into the blood (endocrine function). The islets of Langerhans are composed of α-cells secreting glucagon,

β-cells secreting insulin, δ-cells secreting somatostatin, and PP cells secreting pancreatic polypeptide. T1DM is an auto-immune disease which is caused by selective destruction of the insulin-secreting pancreatic β-cells. Pancreatic islets in T1DM show insulitis, which is characterized by the infiltration of predominantly CD8 positive T lymphocytes, supporting the view that β-cell destruction is a cell-mediated disease [7]. Although many individuals may have autoreactive T cells specific for β-cell autoantigens, only a selected number of people develop T1DM, as the disease results from a combination of genetic and environmental factors. The most important genes associated with an increased risk of T1DM are those located within the major histocompatibility complex human leucocyte antigen (HLA) class II region. Of the non-HLA associated genes involved in T1DM pathogenesis, the insulin gene confers the highest risk [8]. Environmental factors triggering the onset of the disease are thought to be infectious agents, dietary factors, and environmental toxins, although no unique causal factor has consistently been identified [9, 10].

The humoral response may also play a role in the destruction of β-cells in T1DM, and this may be especially important during the first year after the appearance of auto-antibodies [7]. Auto-antibodies that have been implicated in the development of T1DM target insulin, glutamic acid decarboxylase (GAD, an enzyme produced primarily by islet cells), and the transmembrane protein tyrosine kinase IA-2. The presence of a single auto-antibody usually does not predict progression to overt T1DM, but combined positivity confers a significantly increased risk. Recently, a new autoantigen was detected in the form of the zinc transporter Slc30A8. The presence of antibodies against the transporter improves the accuracy with which future occurrence of T1DM can be predicted [11].

33.4 Who May Benefit from Islet Transplantation?

In small subgroup of type 1 diabetics, glycaemic control is very difficult to obtain and patients are prone to experiencing life-threatening hypoglycaemic episodes. It is generally agreed that there is an indication for whole pancreas or pancreatic islet transplantation for these patients with so-called brittle diabetes, who may have an improvement in quality of life or may even be saved from fatal hypoglycaemia when provided with functionally active β-cells [12]. In addition, whole pancreas or pancreatic islet transplantation may be considered in patients with severe clinical and emotional problems with exogenous insulin therapy [13].

The Edmonton group has proposed two scores to quantify the severity of labile diabetes. The HYPO score quantifies the extent of the problem of hypoglycaemia by assigning scores to capillary glucose readings from a 4-week observation period in combination with a score for self-reported hypoglycaemic episodes in the previous year. The lability index (LI) quantifies the extent of glucose excursions over time and is calculated using the formula as described by this group [14].

In their 2006 guidelines, the American Diabetes Association acknowledges the advantages of islet transplantation over whole pancreas transplantation in terms of

morbidity and mortality associated with the operative procedure. However, they clearly state that islet transplantation is an experimental procedure, only to be performed in the setting of controlled research studies. As for patients who will also be receiving a kidney transplantation, simultaneous pancreas transplantation is the treatment of choice, because it may improve kidney survival and will provide insulin independence in the majority of patients [13].

33.5 Islet Transplantation: A Historical Perspective

The first evidence that islet transplantation might be considered a cure for T1DM emerged in 1972, when experiments in rodents showed that artificially induced diabetes mellitus could be reversed by transplanted pancreatic islets [15]. In the 1990s, research activity into islet transplantation greatly increased. Success rates, however, were generally low, with less than 10% of patients being insulin independent at 1 year after transplantation. More encouraging results were obtained in patients who had already had a kidney transplant, with higher rates of insulin independence and graft function as defined by C-peptide secretion [16, 17]. In 2000, a report was published describing seven T1DM patients with a history of severe hypoglycaemia and poor metabolic control who underwent islet transplantation using a modified, steroid-free immunosuppressive protocol. In addition, each patient received at least two different islet transplantations, thus the total transplanted islet mass per patient was remarkably higher than that in previous series. Over a median follow-up of 11.9 months (range 4.4–14.9), all patients were insulin free [1]. The so-called Edmonton protocol was subsequently adopted and modified by many centres. Results of a large multi-centre trial using the Edmonton protocol were published in 2006 [18]. Remaining graft function as indicated by measurable C-peptide levels and improved glycaemic control was present in 70% of patients after 2 years. The insulin independence rate was disappointingly low (14%).

33.6 Clinical Outcomes of Islet Transplantation

33.6.1 Insulin Independence and Improved Glycaemic Control

Many centres are now publishing results obtained in their islet transplant programmes [19–31]. Here, we present some of the largest reports from diverse geographic regions.

In 2005, single-centre outcomes of 65 islet transplant recipients treated according to the Edmonton protocol were reported, showing that 44 (68%) transplanted patients had become insulin independent, with a median duration of insulin independence of 15 months (IQR 6.2–25.5). A total of 5 of these subjects received only a single islet infusion, 33 received two infusions, and 6 received three infusions.

Insulin independence after 5 years was 10%. Nonetheless, after 5 years, some residual graft function could be demonstrated in about 80% of patients on the basis of detectable serum C-peptide levels. Diabetic lability and the occurrence of severe hypoglycaemia were effectively diminished [32].

Following the initial Edmonton results in 2000, a large international trial in nine centres in the United States and Europe was initiated by the Immune Tolerance Network to examine the feasibility and reproducibility of islet transplantation using the Edmonton protocol. The primary endpoint, defined as insulin independence with adequate glycaemic control 1 year after the final transplantation, was met by 16 out of 36 subjects (44%). Only five of these patients were still insulin independent after 2 years (14%). Of note, the considerable differences in results obtained by the various participating sites emphasize the need for concentration of this procedure in highly experienced centres. Again, graft function as defined by detectable C-peptide levels and associated improvements in diabetic control were preserved in a higher percentage of patients (70% after 2 years) [18].

The Groupe de Recherche Rhin Rhone Alpes Geneve pour la transplantation d'Ilots de Langerhans (GRAGIL) reported results obtained in 10 patients who received one or two islet infusions. Only 3 out of 10 patients had prolonged insulin independence after 1 year of follow-up. However, five more transplantations were considered successful, since after 1 year recipients fulfilled the pre-defined criteria of success consisting of a basal C-peptide $\geq$0.5 ng/ml, HbA1c $\leq$ 6.5%, disappearance of hypoglycaemic events, and $\geq$30% reduction of insulin needs [33].

A recent report from the Japanese Trial of Islet Transplantation showed that only 3 out of 18 recipients of islet transplantation achieved insulin independence and only for a period of 2 weeks to 6 months. Graft function was preserved in 63% after 2 years. As in the other reports, HbA1c levels decreased and blood glucose levels stabilized, with disappearance of hypoglycaemia unawareness. In this report, no information was provided about the amount of islet equivalents (IEQ; number of islets in a preparation adjusted for size of the islet, one IEQ equals a single islet of 150 μm in diameter) per kg body weight infused. Of note, in Japan all pancreata are obtained from nonheart-beating donors, since pancreata from brain-dead donors are usually allocated to whole pancreas or pancreas/kidney transplantation. In addition, the presence of brain death is frequently not examined because of cultural reasons, and invasive procedures are usually not allowed even in brain-dead donors before cardiac arrest occurs. This may lead to decreased viability of pancreatic tissue when compared with pancreata from brain-dead donors [34].

The largest registry of islet transplant data is the Collaborative Islet Transplant Registry (CITR), which retrieves its data mainly from the US and Canadian medical institutions and two European centres. In their 2008 update considering 279 recipients of an islet transplantation reported between 1999 and 2007, the registry reported 24% insulin independence after 3 years. Graft function as defined by detectable C-peptide levels after 3 years was 23–26%. The prevalence of hypoglycaemic events decreased dramatically, and mean HbA1c levels substantially improved. Predictors of better islet graft function were higher number of islet infusions, greater number of total IEQ infused, older recipient age, lower recipient HbA1c levels, whether

the processing centre was affiliated with the transplantation centre, higher islet viability, larger islet size, and the use of daclizumab, etanercept, or calcineurin inhibitors in the immunosuppressive regimens. In-hospital administration of steroids was associated with a negative outcome [35, 36].

Table 33.1 shows success rates for pancreatic islet transplantation compared with whole pancreas transplantation alone as reported by the Collaborative Islet Transplantation Registry and the International Pancreas Transplant Registry, respectively [36, 37]. Indications for pancreas transplantation alone are similar to those for islet transplantation. However, whole pancreas transplantation is usually performed simultaneously with kidney transplantation or after kidney transplantation in type 1 diabetic patients with end-stage renal disease. For simultaneous whole pancreas–kidney transplantation, favourable effects on micro- and possibly macrovascular diabetic complications have consistently been described [38]. For pancreas-after-kidney and for pancreas transplantation alone, data are less consistent, and mild or no benefits or even worsening of patient survival after these procedures have been reported [39, 40].

It is worth mentioning that islet transplantation is also performed after pancreatectomy in patients with chronic pancreatitis in order to replace endocrine pancreatic function. Patient islets are rapidly separated from the explanted pancreas and re-infused in the portal vein during or shortly after surgery. A recent report of 85 total pancreatectomy patients showed that the group of 50 patients receiving a concomitant autologous islet transplantation had a significantly lower median insulin requirement than those without concomitant transplantation, although only five patients remained insulin independent [41]. Of 173 recipients of an autologous islet transplantation post-pancreatectomy at the University of Minnesota, 55 (32%) were insulin independent and 57 (33%) had partial islet function recovery as documented by the need of only once-daily long-acting insulin and the presence of detectable circulating C-peptide levels [42]. Success rates significantly improved from 1977 to 2007. Although these results do not differ much from those reported by the CITR and may be even inferior to those from the Edmonton group, the rate of decline of insulin independence was remarkably limited. Of those with insulin

Table 33.1 Clinical outcomes of whole pancreas transplantation versus islet transplantation

	Whole pancreas transplantation	Pancreatic islet transplantation
Insulin independence after 1 year	77%	47%
Insulin dependence and detectable C-peptide after 1 year		25%
Insulin independence long term	58% (5 years)	24% (3 years)
Insulin dependence and detectable C-peptide long term		23–26% (3 years)

Data were derived from the International Pancreas Transplant Registry (until June 2004, $n = 1008$ pancreas transplantation alone) and from the Collaborative Islet Transplantation Registry (until January 2008, $n = 279$ islet transplantation alone) [35, 37]

independence, 74% remained insulin independent at 2 years, and 46% at 5 years of follow-up, which is remarkably higher than in the CITR. A reasonable explanation for these better long-term outcomes is that the absence of both the auto- and allo-immune response allowed for prolonged islet survival in these patients. The fact that these outcomes were achieved using a much lower islet mass than that used in the Edmonton protocol further highlights the strong impact of auto-immune and allo-immune injury on graft survival in type 1 diabetics receiving an infusion of allogeneic islets.

33.6.2 Long-Term Diabetic Complications

Until now, it has not been sufficiently established whether pancreatic islet transplantation can halt progression of diabetic complications or even prevent them [43, 44].

In a retrospective study, cardiovascular function was compared between a group of 17 patients who received an islet-after-kidney transplantation and a group of 25 patients with a previous kidney transplantation who were still on the waiting list for an islet transplantation or who had experienced early islet graft failure. Baseline characteristics for both groups were similar. Islet transplantation was associated with an improvement in ejection fraction and left ventricular diastolic function compared to baseline. Moreover, arterial intima–media thickness was stable in the islet transplant group, but worsened in the kidney-only group [45].

The same group reported increased kidney graft survival rates and stabilization of micro-albuminuria after islet transplantation [46]. Conversely, an uncontrolled observational study by the Edmonton group suggested an overall decline in estimated glomerular filtration rate during 4 years of follow-up after islet transplantation alone, and an increase in albuminuria in a significant proportion of patients [47]. Subsequently, Maffi et al. showed that even a mildly decreased renal function pre-transplantation should be considered a contra-indication for the currently used immunosuppressive regimen of sirolimus in combination with tacrolimus (see below), since it was associated with progression to end-stage renal disease [29].

The Edmonton and the Miami series reported ocular problems post-transplantation in 8.5 and 15% of patients, respectively. Adverse events included retinal bleeds, tractional retinal detachment, and central retinal vein occlusion [24, 32]. However, after 1–2 years, diabetic retinopathy seems to stabilize [48]. Moreover, at 1 year after transplantation, arterial and venous retinal blood flow velocity are significantly increased, possibly indicating improved retinal microcirculation [49]. The acute adverse effects on retinopathy may be due to the sudden improvement in glycaemic control after islet transplantation. The DCCT also reported initial deterioration of diabetic retinopathy in patients with pre-existing disease who were treated in the intensive insulin treatment arm as compared to those in the conventional treatment arm; however, after 1 year differences between treatment arms disappeared, and after 36 months of follow-up, intensive treatment was

consistently associated with significantly less progression of diabetic retinopathy [5]. Whether the overall effect of islet transplantation on diabetic retinopathy is beneficial in the long term remains to be proven.

Finally, two reports were published investigating effects of islet transplantation on diabetic neuropathy. Lee et al. performed nerve conduction studies in eight patients with at least 1 year of follow-up after transplantation. They concluded that peripheral neuropathy stabilized or maybe even improved, although no formal statistical analysis was provided and conclusions were based on clinical observations by a single neurologist [48]. Del Carro et al. compared nerve conduction studies in patients who had received an islet-after-kidney transplantation to patients having received a kidney transplantation only. In their interpretation of the results, they suggested that worsening of diabetic neuropathy seemed to be halted by islet transplantation, but no statistically significant differences between the two groups could be demonstrated [50].

33.6.3 Adverse Events in Islet Transplantation

Adverse events related to islet transplantation are principally related to the procedure itself and to the adverse effects of the immunosuppressive regimen. During the procedure, a large mass of β-cells is percutaneously and transhepatically injected into the portal vein. This may lead to portal vein thrombosis or thrombosis of segmental branches. On the other hand, incidence rates of up to 14% have been reported for intraperitoneal bleeding, which may require blood transfusion or even surgical intervention. This complication can be effectively prevented by sealing the catheter tract using thrombostatic coils and tissue fibrin glue [51]. Other relatively frequent procedure-related complications are abdominal pain from puncturing of the peritoneum or gall bladder and a transient rise of hepatic enzymes [52]. Post-transplantation focal hepatic steatosis occurs in approximately 20% of patients, possibly due to a local paracrine effect of insulin, but its significance with regard to graft function is not clear yet [53, 54].

T1DM patients receiving a pancreatic islet transplantation may need an additional kidney and/or whole pancreas transplantation later in life. Therefore, post-transplantation allo-immunization in roughly 10–30% of patients using immunosuppression is a cause for concern [55, 56]. Of note, up to 100% of patients develop HLA alloreactivity, with 71% having HLA panel-reactive antibodies (PRA) $\geq$ 50%, after withdrawal of immunosuppression because of islet graft failure or side effects [55, 56]. Pre- or post-transplantation alloreactivity against HLA class I and II may also be associated with reduced pancreatic islet graft survival itself [57, 58], although some authors suggested that increased PRA had no clinical significance under adequate immunosuppression [56]. As opposed to solid organ transplantation, pre-transplantation testing of PRA is currently not performed in pancreatic islet transplantation. Thus, the impact of PRA positivity on clinical outcome after islet transplantation or on future whole organ transplantation has to be further investigated.

33.7 Immunosuppressive Regimens for Islet Transplantation

As in any other immune response, activation of T cells against the islet graft involves three types of signals. Alloantigens (signal 1) in combination with co-stimulatory molecules (signal 2) presented by antigen-presenting cells (dendritic cells) trigger a T-cell response by activating three signal transduction pathways, including the calcium–calcineurin pathway. Subsequently, other molecules including interleukin-2 are released, triggering the 'mammalian target of rapamycin' (mTOR) pathway (signal 3), which initiates cell proliferation, leading to a large number of effector T cells. In addition, B lymphocytes are activated to produce alloantibodies against donor HLA antigens [59].

Following the publication by the Edmonton group in 2000 [1], the steroid-free immunosuppressive protocol this group used was adopted by many centres, although it was not the only change being introduced. Changes with regard to recipient and donor selection, the technical procedure, and the infusion of a large number of pancreatic islets from multiple donors will all have contributed to the favourable short-term results. The Edmonton immunosuppressive regimen consists of induction therapy with a monoclonal antibody against the interleukin-2 receptor (daclizumab), and maintenance therapy with a calcineurin inhibitor (tacrolimus) and an mTOR inhibitor (sirolimus). Sirolimus has been shown to display significant synergy with calcineurin inhibitors, control auto-immunity, induce apoptosis of T cells and other inflammatory cells, and induce generation of regulatory T cells (Treg). However, data have also emerged showing its potentially harmful effects on β-cell regeneration [60, 61]. The same applies for calcineurin inhibitors; although proven to be very effective in organ transplantation, they are toxic to β-cells and cause insulin resistance and diabetes mellitus. Moreover, sirolimus and tacrolimus exert direct nephrotoxic effects and they often induce the development of hyperlipidaemia and hypertension, which may further increase the risk of micro- and macrovascular complications [59]. Therefore, the combined use of sirolimus and tacrolimus to prevent acute rejection of transplanted pancreatic islets is certainly not ideal.

To increase islet transplantation success rates and diminish the often severe side effects associated with chronic use of immunosuppressive drugs [24], various centres are implementing new immunosuppressive regimens, both for the induction phase and for the maintenance phase [25, 31, 62–64]. In an attempt to promote a pro-tolerogenic state, Froud et al. tested induction therapy with alemtuzumab in three islet transplant recipients [63]. Alemtuzumab is a humanized monoclonal antibody against CD52, which is present on the surface of mature lymphocytes. Its administration leads to severe lymphocyte depletion and may favourably influence the regulatory T-cell versus effector T-cell ratio during T-cell repopulation [65]. Indeed, in these three patients, glucose metabolism seemed to be better than in historic controls, with no major infectious complications. However, other changes in the immunosuppressive regimen, such as the use of steroids on the day before islet infusion, the early switch from tacrolimus to mycophenolate mofetil (MMF) during the maintenance phase, and the use of etanercept (see below), may all have contributed to improved outcomes in this study.

Tumour necrosis factor (TNF) α is a regulator of the immune response, and its activity is inhibited by etanercept, a recombinant TNFα receptor protein. From the University of Minnesota came an interesting report of high success rates in eight patients using a protocol in which etanercept was administered as induction therapy, combined with prednisone, daclizumab, and rabbit antithymocyte globulin. Of the eight patients, five were still insulin independent after 1 year. Of note, patients received an islet graft from a single donor [25]. More centres are now using etanercept as additional induction therapy, a strategy which is supported by the fact that the CITR found an association between etanercept use and graft survival [30, 35, 66]. However, it should be pointed out that this antibody is not yet approved for transplantation therapy in the United States.

Some studies investigated the combination of etanercept induction with long-term use of subcutaneous exenatide, a glucagon-like peptide-1 (GLP-1) analogue. GLP-1 is a hormone derived from the gut, which stimulates insulin secretion, suppresses glucagon secretion, and inhibits gastric emptying [67]. Combined treatment with etanercept and exenatide in addition to the Edmonton immunosuppressive protocol was shown to reduce the number of islets needed to achieve insulin independence [30]. In addition, combined etanercept and exenatide use improved glucose control and graft survival in patients who needed a second transplantation because of progressive graft dysfunction [66]. In two studies with islet transplantation patients, exenatide reduced insulin requirements, although in one study they tended to rise again at the end of the 3-month study period, possibly due to exhaustion of β-cells [62, 63, 68]. However, these studies were very small and non-randomized. Of note, exenatide use involves the administration of twice-daily subcutaneous injections, causes severe nausea, and may lead to hypoglycaemia. Therefore, randomized controlled trials are needed to define whether its use confers additional benefit over immunosuppressive therapy alone in islet transplantation recipients [69].

Recently, an isolated case with more than 11 years of insulin independence after islet transplantation was described [70]. The intriguing question is which factors have contributed to the outcome in this particular patient. The patient had previously received a kidney transplant and was on an immunosuppressive regimen comprising antithymocyte globulin as induction therapy followed by prednisone (which was rapidly tapered), cyclosporine, and azathioprine, which was later switched to MMF. Interestingly, the authors investigated the cellular immune response and found that the patient was hyporesponsive towards donor antigens, possibly as a result of the expanded Treg pool. This may have contributed to the excellent long-term survival of the graft. Huurman et al. examined cytokine profiles and found that allograft-specific cytokine profiles were skewed towards a Treg phenotype in patients who achieved insulin independence, and that expression of the Treg cytokine interleukin-10 was associated with low alloreactivity and superior islet function [71]. The role of Tregs in allograft tolerance has long been recognized in solid organ and bone marrow transplantation, and much research is devoted to translating this knowledge into therapeutic options, which may also benefit islet transplantation [72].

Despite immunosuppressive therapy aimed at preventing rejection (i.e. allo-immunity), outcomes of islet transplantation may also be adversely influenced by *auto*-immune injury. A recent study showed delayed graft function in patients with pre-transplant cellular autoreactivity to β-cell autoantigens; in 4 out of 10 patients with recurrence of autoreactivity post-transplantation, insulin independence was never achieved. Moreover, in five out of eight patients in whom cellular autoreactivity occurred de novo after transplantation, time to insulin independence was prolonged [73]. In the international trial of the Edmonton protocol, patients with one or two auto-antibodies in the serum before the final infusion had a significantly lower insulin independence rate than those without auto-antibodies [18].

33.8 Cost-Efficacy of Islet Transplantation

So far, no study has addressed the issue of the cost-effectiveness of islet transplantation in terms of the costs per quality-adjusted life year or per micro- or macrovascular diabetic complication prevented. The GRAGIL network has estimated the average cost of an islet transplantation in the year 2000 at €77,745. These costs even slightly exceed those for a whole pancreas transplantation, mainly due to the high expenses of cell isolation [74]. A study by Frank et al. also found that pancreas processing-related costs led to higher total costs for isolated islet transplantation than for whole pancreas transplantation, even though the former was associated with less procedure-related morbidity and shorter hospital stays [20]. These high costs may be justified in patients in whom islet transplantation is deemed to be life-saving because of severe hypoglycaemic episodes. However, in other settings, they will compare extremely unfavourably to the costs of current strategies to prevent diabetic complications, such as adequate glycaemic control, blood pressure and lipid profile optimization, diet and weight loss, and angiotensin-converting enzyme inhibitor use.

33.9 Future Developments

Figure 33.1 highlights the progressive loss of pancreatic islet mass which occurs both during graft preparation and after islet infusion. This has some analogies with kidney transplantation, where the number of nephrons to start with strongly affects graft outcome. Indeed, loss of nephrons during ischemia–reperfusion injury and subsequent immune and non-immune injury eventually leads to progressive loss of renal function. Similarly, progressive decline of β-cell mass during different phases of isolation, infusion and, thereafter, as a result of the auto- and allo-immune response will eventually fail to provide prolonged insulin independence.

There are several steps in the whole procedure of islet transplantation which may be targeted in order to improve islet recovery and post-transplantation protection.

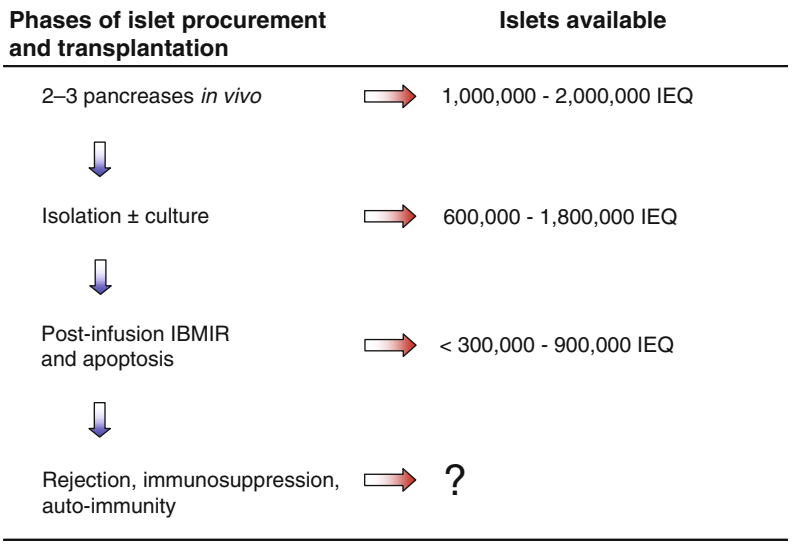

Fig. 33.1 Loss of pancreatic islet mass, from graft preparation to post-infusion degradation. IEQ, islet equivalents; IBMIR, instant blood-mediated inflammatory reaction. The IBMIR reduces islet mass by 50–70% [112]

Pre-transplantation procedures related to pancreas preservation, enzymatic digestion, purification, culture, and shipment may be further refined [75]. Islets are usually infused into the portal vein through percutaneous trans-hepatic cannulation of a portal branch. A laparoscopic technique for intra-portal islet transplantation allowing for multiple deliveries of islets into the same liver segment has also been described [76]. However, it has been recognized that the liver is not the ideal site for transplantation because of the relatively low oxygen supply in this organ, the exposure to toxins absorbed from the gastrointestinal tract, and the instant blood-mediated inflammatory reaction (IBMIR), which causes substantial islet loss shortly after infusion. Many alternative sites have been explored, including the omentum, pancreas, gastrointestinal submucosa, and muscular tissue, but these alternative approaches have so far remained experimental, with none of them being convincingly superior to the currently used method [77, 78].

Peri-transplantation care may be improved by heparinization of either the patient or, to prevent bleeding complications, the pancreatic islets themselves. In doing so, the effects of tissue factor, which is secreted by the endocrine cells of the transplanted islets and which plays a significant role in the IBMIR, are counteracted [79–81]. This may prevent the immediate and significant post-procedural islet loss. Moreover, it is now possible to visualize islets in the peri-transplantation phase using 18F-fluorodeoxyglucose positron-emission tomography combined with computed tomography in order to assess islet survival and distribution, which may also be used to evaluate alternative sites of implantation [82].

Islet encapsulation as a strategy to improve graft survival is one of the main areas in experimental research. The use of semi-permeable encapsulation material should protect the islets against the allo-immune response while at the same time allowing them to sense glucose levels and secrete insulin [83, 84].

In 2005, Matsumoto et al. performed the first islet transplantation from a living related donor in a patient who had brittle diabetes due to chronic pancreatitis. The procedure resulted in good glycaemic control and no major complications in both the donor and the recipient [85, 86]. However, results cannot be generalized to the T1DM population, as diabetic disease in the recipient did not result from an auto-immune process. Moreover, partial pancreatectomy in the donor implies major surgery with associated risks of morbidity and mortality. In the long term, donors may be at increased risk of developing diabetes mellitus themselves [87].

Another alternative source of pancreatic islets is xenotransplantation, with which some experience has been gained in humans. In 1994, a Swedish group reported xenotransplantation with fetal porcine pancreatic islets in 10 diabetic patients. Although insulin requirements did not decrease, the procedure was well tolerated and there was no evidence of transmission of porcine endogenous retroviruses (PERV) after 4–7 years of follow-up [88, 89]. More recently, xenotransplantation has been performed in China, Russia, and Mexico [90, 91]. In 2005, the group from Mexico reported a 4-year follow-up of 12 diabetic patients not taking immunosuppressive therapy who had received one to three subcutaneous implantations of a device containing porcine pancreatic islets and Sertoli cells. Sertoli cells, being immune-privileged, were added because they may confer immunoprotection to transplanted endocrine tissue. Follow-up showed a decreased insulin requirement in 50% of patients, but the decrease in HbA1c was lower than that in the 50% of patients not having a favourable response to the transplantation. Porcine C-peptide was not detectable in the urine, and the significance of this study remains to be determined. Importantly, severe ethical issues have been raised with regard to xenotransplantation as it is currently being performed. The programme in China was suspended, and the International Xenotransplantation Association has seriously objected to the Mexican and Russian studies, as they feel that the safety of the patient and of the general public (especially with regard to the spread of PERV) is not sufficiently guaranteed [92–94]. More experimental studies are needed before clinical trials in human can be initiated [95].

Possible future sources of pancreatic β-cells are mesenchymal stem cells (MSCs), which may be capable of differentiating into insulin-producing cells [96, 97]. Moreover, due to their immunomodulatory and anti-inflammatory properties, these cells may help to control the auto-immune response, thereby preventing immune injury of newly proliferating cells. *S*tudies in diabetic rats have shown improvements in glucose control after the infusion of autologous MSCs, but concern about the potential oncogenic properties of stimulated MSCs still prevents the transfer of this cell therapy into the clinic [98]. Embryonic stem cells may also serve as an alternative source for β-cell replacement [99]. Finally, transdifferentiation of adult hepatocytes and of pancreatic exocrine tissue into insulin-producing cells has

been achieved in animal and in in vitro studies; however, these approaches need to be further explored before they can be applied to humans [100, 101].

33.9.1 Novel Therapeutic Perspectives for Type 1 Diabetes Mellitus

Other therapeutic approaches for patients with T1DM are also underway. Indeed, refinement of insulin pumps in combination with continuous glucose monitoring systems may lead to better glycaemic control [102]. In the future, patients will ideally be able to use a closed-loop system consisting of a glucose sensor and an insulin pump, as well as software to automatically translate measured glucose levels into appropriate insulin doses. Moreover, trials will be conducted to test whether protection of pancreatic islets from auto-immunity will allow regeneration of these cells in the early phases of T1DM, when the β-cell pool is not yet completely destructed (see, for example, NCT00100178 at www.clinicaltrials.gov, MMF and/or daclizumab in new-onset T1DM). This appears to be a promising approach, since it is well documented that β-cells can regenerate, as observed during pregnancy and in subjects with insulin resistance [96, 97].

Intriguingly, attempts have also been made to induce tolerance. Along this line, compelling evidence has accumulated suggesting that in addition to their immunosuppressive properties, CD3-specific antibodies can induce immune tolerance especially in the context of an ongoing immune response [103]. Clinical studies have shown that this therapy may, at least partially, preserve β-cell mass in newly diagnosed type 1 diabetics [103, 104]. An alternative approach is targeting B lymphocytes, given the importance of the humoral response in the pathogenesis of T1DM and the fact that B lymphocytes also have a role as antigen-presenting cells. Promising results with a B-lymphocyte depleting monoclonal antibody have been obtained in a mouse model of diabetes [105], and in patients with newly diagnosed TIDM [106] (NCT00279305). A more drastic approach to bypass auto-immunity is autologous non-myeloablative haematopoietic stem cell transplantation, which may reset autoreactive T cells and reverse the disease in new-onset T1DM [107]. With this approach, persistent normoglycaemia was achieved for a mean of 2.5 years in 60% of patients. However, acute drug toxicity, risk of infections, and sterility may outweigh the benefits of this protocol.

Alternative approaches to the induction of tolerance include molecular biological strategies. In particular, evidence has been provided that ‘immature’ dendritic cells (DCs) can promote tolerance. To this end, CD40, CD80, and CD86 cell surface molecules were specifically down-regulated by ex vivo treating DCs from mice with a mixture of specific antisense oligonucleotides. This promoted the emergence of regulatory T cells that might possibly prevent the occurrence of diabetes [108]. Intriguingly, to circumvent the technical issues of ex vivo DC manipulation, a recent study in mice showed that the same immature phenotype can be induced by using a microsphere-based vaccine injected subcutaneously [109]. This approach

effectively prevented new-onset diabetes or even reversed it, providing the basis for testing this approach also in humans.

33.10 Conclusion

Islet transplantation is a dynamic field to which much time and resources are being devoted. If successful transplantation is defined as a transplantation after which quality of life and glycaemic control are improved, success rates of this procedure are quite acceptable. However, if success is defined in terms of long-term insulin independence or prevention of diabetes-related complications, then outcomes are outright disappointing in the first and largely unknown in the second. Moreover, only a highly selected group of patients with brittle diabetes may benefit from the procedure, which requires a high degree of expertise. At present, islet transplantation cannot be considered a standard of care for the large majority of patients with T1DM [110, 111]. As suggested by the American Diabetes Association [13], islet transplantation should still be considered an experimental procedure, to be tested in properly designed randomized controlled trials.

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Chapter 34
Islet Cell Tumours

Sara Ekeblad

Abstract Pancreatic endocrine tumours can cause hormonal symptoms by oversecretion of hormones. They are less aggressive than exocrine pancreatic cancer, but carry a variable prognosis. The tumours are either sporadic or hereditary, as part of the multiple endocrine neoplasia type 1 syndrome. Despite the rarity of these tumours, they evoke significant interest in the research community and important advances have been made over the past years. This chapter provides an overview of the tumours and recent advances in the field.

Hereditary forms of pancreatic endocrine tumours are caused by mutations in the MEN1 gene. Menin, the protein encoded by this gene, has been shown to interact with numerous transcription factors and proteins involved in cell-cycle control, shedding some light on the importance of the protein. Several genes have been shown to be up- or down-regulated, suggesting candidates to be further evaluated for a role in tumourigenesis.

Several advances have been made in prognostication; a tumour-node-metastasis system has been evaluated and seems to have prognostic value, and several new molecular prognostic markers are under evaluation. It is hoped that the tumour-node-metastasis system and other prognostic markers will be adopted in clinical routine and improve prognostication and treatment choices.

Surgery is still the only cure, but several new palliative drugs and interventions are in use or under investigation. Radiofrequency ablation is increasingly used for liver metastases, and a number of new chemotherapy drugs are being tested. Despite improvements in treatment, no clear improvement in survival has been demonstrated.

Keywords Pancreatic endocrine tumours · Multiple endocrine neoplasia type 1 · Insulinoma

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34.1 Introduction

Exocrine pancreatic cancer is a feared disease, with a usually dismal prognosis. Less known, and less common, are endocrine pancreatic tumours. These arise from the islets of Langerhans and share the endocrine phenotype of these cells. The tumours occur in approximately 1 per 100,000 in the population, representing 1–2% of all pancreatic neoplasms [1]. However, the frequency in autopsy series has been much higher, suggesting these tumours are often undetected and asymptomatic. Further, due to the indolent nature and long survival, their prevalence is higher; a recent analysis suggests up to 10% of all pancreatic tumours [2]. These tumours continue to spark significant interest in the research community. A lot of work is ongoing to better understand the nature of the tumours and to improve the outlook for patients, and there have been a number of important advances in recent years, related to understanding tumourigenesis, improving diagnosis, prognostication and treatment.

34.2 Tumour Type

Pancreatic endocrine tumours produce and secrete peptide hormones, often more than one. They can express hormones normally present in the pancreas, i.e. insulin, glucagon, somatostatin and pancreatic polypeptide, or hormones usually produced somewhere else, e.g. gastrin, vasoactive intestinal polypeptide (VIP) or adrenocorticotropic hormone (ACTH). Sometimes these hormones cause clinical symptoms, and the tumours are then called functioning (Table 34.1). These endocrine symptoms can sometimes be dramatic. Tumours not causing any identifiable endocrine symptoms are called non-functioning. Regardless of whether the produced peptides cause clinical symptoms, they can be monitored to follow tumour progression or recurrence of disease.

The most common functioning tumour is insulinoma, which secretes insulin and causes hypoglycaemia. Symptoms of hypoglycaemia include confusion, double

Table 34.1 The main functioning tumours with hormones and symptoms

Syndrome	Hormone	Symptoms
Insulinoma	Insulin, proinsulin	Hunger, confusion, double vision, agitation, tremor, tachycardia
Gastrinoma	Gastrin	Multiple peptic ulcers, dyspeptic symptoms, diarrhoea
Glucagonoma	Glucagon	Weight loss, muscle wasting, necrolytic migratory erythema
VIPoma	Vasoactive intestinal Peptide	Massive, sometimes life-threatening, secretory diarrhoea
Somatostatinoma	Somatostatin	Diffuse symptoms: hyperglycaemia, diarrhoea, weight loss
ACTH-/CRFoma	ACTH/CRF	Cushing syndrome

vision, agitation and tachycardia. Classically, symptoms develop during fasting or exercise. Patients often overeat to compensate, and obesity is not uncommon. The symptoms are not easy to read, and the patient with insulinoma often has a long history of seeking medical attention before the right diagnosis is finally made. Differential diagnoses include abuse of insulin or oral anti-diabetic drugs, Addison's disease and anorexia nervosa. Demonstration of low blood glucose and inappropriately high insulin levels after a prolonged fast (up to 72 hours) settles the diagnosis.

The second most common tumour causing an endocrine syndrome is gastrinoma [3]. These tumours produce gastrin, causing multiple peptic ulcers. Symptoms also include gastroesophageal reflux disease, and sometimes diarrhoea and malabsorption. The presence of multiple ulcers or ulcers in atypical locations leads to a suspicion of gastrinoma. Historically, gastrinoma patients died from bleeding ulcers. Now, symptoms can be effectively controlled with proton pump inhibitors (PPIs). Diagnosis of a gastrinoma is made by measurement of serum gastrin together with a gastric pH < 2, when a typical clinical picture is present. It is important that any PPIs be withdrawn before testing, ideally for 1 week, since they cause elevation of the hormone. In the absence of a clearly elevated gastrin, a basal acid output and a secretin test should be done.

Glucagonomas secrete glucagon, causing catabolism and hyperglycaemia. Patients are often severely cachexic upon presentation. They sometimes present with necrolytic migratory erythema, and it is not uncommon for the diagnosis to be first suggested by a dermatologist. Diagnosis is made by demonstration of elevated glucagon levels, usually >500 pg/mL. Lower increases can still be caused by glucagonomas, but can also be the result of several other states, e.g. pancreatitis, diabetes and renal failure.

The even more uncommon VIPoma secretes VIP, causing massive, sometimes life-threatening, diarrhoea [4]. The patient can lose dangerous amounts of water and electrolytes, and intensive care unit treatment is often required; VIPoma syndrome is sometimes called pancreatic cholera. In the presence of massive diarrhoea, the syndrome is confirmed by an increased serum VIP and, ideally, imaging of a pancreatic mass.

Somatostatinomas secrete somatostatin, an inhibitory hormone [5]. This causes more discrete symptoms, e.g. hyperglycaemia, diarrhoea and weight loss. These tumours often present later than other functioning tumours, due to the discrete hormonal symptoms. Unlike gastrinoma and insulinoma, there is no reliable provocative test to confirm a somatostatinoma.

Other rare functioning tumours secrete ACTH or corticotrophin-releasing factor (CRF) (causing Cushing's syndrome) or parathyroid hormone-related protein.

Non-functioning tumours are not responsible for any defined clinical syndrome. They can still produce and secrete hormones, which can be defective, not producing any clinical effect, or giving rise to symptoms that have not yet been understood to be part of a syndrome. Hormones that are produced by pancreatic endocrine tumours but not associated with any syndrome include pancreatic polypeptide, islet amyloid polypeptide, calcitonin and ghrelin.

Patients with non-functioning tumours often present with advanced disease, since there are no endocrine symptoms causing them to seek early medical attention. Presenting symptoms include abdominal pain and jaundice. Pathological examination of tumour tissue, from biopsy material or a surgical specimen, is especially important for non-functioning tumours to distinguish between endocrine tumour and adenocarcinoma. In the 1980s, non-functioning tumours accounted for 15–24% of pancreatic endocrine tumours [6, 7]. In recent reports the corresponding figure is about 60% [8–10]. One material shows the frequency to be as high as 74% after 2000 [11]. There can be more than one reason for this apparent increase in the frequency of non-functioning tumours. The classification of these tumours has become more stringent over the years. Previously, tumours were sometimes classified as functioning merely on the basis of immunoreactivity or elevated plasma levels of a hormone. Today, those tumours that are not causing an identifiable clinical syndrome are correctly classified as non-functioning. Another reason is an increased pathological expertise and an increased awareness of these tumours, causing a higher number of poorly differentiated pancreatic neuroendocrine tumours to be correctly classified as neuroendocrine. Thus, the increase in non-functioning tumours is likely to be related to shifting practices for classification rather than a true increase in incidence. Non-functioning tumours are also increasingly being identified en passant, because of an increase in imaging studies due to nonspecific symptoms [12].

34.3 Hereditary Syndromes

Pancreatic endocrine tumours can be either sporadic or hereditary. Hereditary forms are usually part of the rare multiple endocrine neoplasia type I syndrome (MEN1) or sometimes the even more uncommon von Hippel Lindau disease. MEN1, an autosomal dominant hereditary syndrome, was initially recognized by Wermer [13]. Patients with MEN1 develop tumours in several endocrine glands, including the parathyroids, the endocrine pancreas and the anterior pituitary. Contrary to patients with sporadic tumours, they often develop multiple tumours in the pancreas. The reason for the predominance of endocrine tumours is unknown. By definition, a person with no known affected relative is said to have the disease when he/she develops two of the above-mentioned lesions. For a person with an affected relative, only one lesion is needed for the diagnosis to be made. In 1988, the gene responsible for this syndrome was characterized as a tumour-suppressor gene and was mapped to 11q13 [5], and in 1997 the gene was cloned [14].

Today, genetic testing is done to determine whether a person with affected relatives has inherited a defective MEN1 gene or not. For a person with a defective gene, regular biochemical screening is performed to detect early signs of endocrine tumours. These tumours often secrete hormones, which can then be measured in the circulation at abnormal levels. Often, biochemical signs of tumours can be detected already in adolescence [15]. If left to its natural course, tumours often do not clinically demonstrate until middle age, and are then often metastatic at diagnosis [16].

No convincing correlation between genotype and phenotype for MEN1 has been shown, i.e. it is not possible today to predict the course of the disease based on the type of mutation found. Patients with MEN1 often develop more than one pancreatic endocrine tumour. With biochemical screening, tumours can be detected while they are still too small to reliably visualise.

The management of MEN1 patients with pancreatic tumours is debated, especially regarding early tumour surgery in asymptomatic patients. On the one hand, removing tumours before they become malignant could prove life-saving. On the other hand, pancreatic surgery can cause significant morbidity, e.g. diabetes, and it is desirable to avoid unnecessary surgery for tumours that might never have become malignant anyway. Today, it is not possible to predict which tumours will become malignant, and the debate regarding whether to operate or not continues. Measured from the date of diagnosis, patients with pancreatic tumours as part of the MEN1 syndrome often live longer than patients with sporadic tumours. This is often used as an argument for a less aggressive treatment of these tumours [17]. However, since MEN1 tumours are often diagnosed earlier in life, this does not necessarily translate in to a long life. One study showed pancreatic tumours as the number one cause of death for MEN1 patients and a median age of death from pancreatic malignancy of only 46 years [18]. MEN1 patients have also been shown to have a significantly lower 20-year survival compared to healthy age-matched controls [19]. In one large patient material, having a hereditary tumour, as opposed to a sporadic one, was not an independent predictor of a longer survival [10]. This implies that the aggressiveness of MEN1-related tumours should be decided on a case-by-case basis, based on known prognostic factors.

34.4 Tumourigenesis

The exact mechanisms involved in tumourigenesis for pancreatic endocrine tumours are not yet fully understood. Mutations in common oncogenes or tumour-suppressor genes are generally not found. The study of familial tumours, mainly MEN1, has rendered some important insights. Genes important in MEN1 tumourigenesis also play a role in a subset of sporadic cases. MEN1 is caused by mutations in the MEN1 gene, which encodes the tumours suppressor protein menin. Most MEN1-related tumours show somatic loss of the second allele, loss of heterozygosity.

Menin, a 610 amino acid protein, is localised in the nucleus and is ubiquitously expressed throughout the body. In mice models, it is homozygous lethal. The exact function of menin is still not fully known, but important discoveries have been made in recent years. The protein has been shown to interact with numerous transcription factors, e.g. JunD and NF-κB, as well as proteins involved in cell-cycle control [20]. Loss of heterozygosity on 11q, where the MEN1 gene is located, or mutations of the gene have been shown to be common also in sporadic tumours [21]. Insulinomas, however, rarely show MEN1 gene alterations [22].

Recently, newer methods have suggested several new candidate genes for involvement in tumourigenesis. Comparative genomic hybridisation has shown

chromosomal gains and losses to be frequent in these tumours [12], and microarray analysis has identified numerous genes that are over- or under-expressed [23]. These approaches do not automatically tell us which genes are important for tumourigenesis, but they do suggest candidate genes whose roles can be further evaluated using other methods.

In an era where targeted treatment of different types of cancer is becoming more and more a reality, the goal is obviously to one day have enough knowledge on which molecular events cause the transformation of these cells to be able to find targeted treatments also for these tumours. While we are certainly still far away from that, our understanding of the genes relevant to transformation is growing every day.

34.5 Radiology

Pancreatic endocrine tumours are often diagnosed by the finding of abnormally high levels of a specific hormone, corresponding with clinical symptoms. Pathological examination of tumour tissue, from biopsy material or a surgical specimen, ideally confirms the diagnosis. Radiology is important for tumour localisation and staging, and important progress has been made in this field in recent years. Computed tomography (CT) or magnetic resonance imaging (MRI) is the first choice for imaging. However, tumours are often too small to be localised with these modalities, especially insulinomas and gastrinomas. Such small tumours can be found with endoscopic or intra-operative ultrasound. The latter is extremely sensitive and is increasingly used to find very small tumours. It is especially important for tumour localisation in MEN1 patients, who often have multiple, small tumours. In MEN1 patients, the decision to operate is often based on biochemical findings, and tumours are then localised intra-operatively. The same is the case for small insulinomas. Due to the potent nature of insulin, even a tumour that is too small to localise with CT or endoscopic ultrasound can produce debilitating and even life-threatening symptoms. Intra-operative ultrasound can allow finding the tumour and performing radical surgery without having to perform a total pancreatectomy.

Eighty to ninety percent of neuroendocrine tumours express somatostatin receptors [1]. This makes them excellent candidates for somatostatin receptor scintigraphy. Radioactively labelled octreotide binds to somatostatin receptors, which are more highly expressed by these tumours cells than in surrounding tissue. Somatostatin receptor scintigraphy has been routinely used in clinical practice since the 1990s and is suggested to have a higher sensitivity for these tumours than CT and MRI [24].

Another relatively new and increasingly popular radiology modality that has proved very useful in diagnosing these tumours is positron emission tomography (PET). The standard PET used for other tumours, with fluorine-18 fluorodeoxyglucose (FDG), is usually of limited value for these tumours, since they generally have a low proliferation [25]. Instead, (11)C-5-hydroxytryptophan (5-HTP) is used. Pancreatic endocrine tumours have a high uptake of this substrate, which is used

in serotonin synthesis. 5-HTP PET has an even higher sensitivity for these tumours than CT or somatostatin receptor scintigraphy [26].

Recently, techniques that allow CT and PET images to be overlapped have evolved. This allows combining the sensitivity of the PET with the anatomical clarity of a CT image [27] and will most likely prove a big step forward in diagnosis and follow-up of these tumours.

34.6 Survival

Median survival figures of 38–104 months have been reported for patients with pancreatic endocrine tumours [6, 8–10, 28, 29]. The survival does not seem to have changed much over the last decades. One could take this to mean that no improvements have been made in the treatment of these patients, but that just might be a faulty conclusion. One might speculate that with an improvement in diagnostic tools and an increased awareness of this tumour entity among clinicians, more patients with poorly differentiated aggressive tumours, previously misdiagnosed as exocrine, are now receiving a correct diagnosis. These patients have a much shorter survival and would thus drag down survival for the group as a whole. It could thus be possible that survival actually has improved, as a result of earlier detection and improved treatment, but that an increased frequency of inclusion of more aggressive tumours leads to similar survival figures for the group as a whole.

34.7 Prognosis

The prognosis for patients with pancreatic endocrine tumours is very variable. Some patients survive for years, even with liver metastases; while others experience rapidly progressing disease almost similar to that of exocrine pancreatic cancer. Sometimes, patients with spread disease at presentation are first diagnosed as having an exocrine tumour, only to be re-diagnosed as endocrine years later when the uncharacteristically long survival leads to further investigation.

Since the prognosis is so very variable, with tumour behaviour ranging from entirely benign to highly aggressive, it is very important to try to understand the prognosis in each case. This is important both to be able to offer tailored treatment and to give the patient adequate expectations. Unfortunately, due to the rarity of the condition, there is still a lack of knowledge about prognostic factors. Morphological characteristics that normally accompany malignancy, such as atypia, pleomorphism and perineural growth, are often missing even in metastatic pancreatic endocrine tumours [30]. Production of precursor hormones or ectopic hormone production is usually considered a sign of malignancy, but is not nearly always present in malignant tumours. This lack of reliable markers of malignancy and tumour aggressiveness is frustrating to clinicians and the research community, and there is considerable work ongoing to increase our knowledge on this subject.

Factors generally believed to have an unfavourable impact on survival include the absence of primary tumour surgery, the presence of liver metastases, endocrine symptoms, tumour necrosis, a high mitotic count and/or proliferative index (Ki67) [8, 31].

Non-functioning tumours are generally perceived to carry a worse prognosis compared to functioning tumours. Clinically, it is widely accepted that non-functioning tumours usually are more aggressive than functioning ones. However, in multivariate analysis of a large patient material, the differences in survival between patients with functioning and non-functioning tumours were explained by other factors, such as Ki67 and stage, rather than the functional status of the tumour per se [10]. Thus, prediction of prognosis and decisions regarding treatment should be guided by multiple prognostic factors rather simply the fact of the tumour being functioning or non-functioning.

In 2006, a tumour-node-metastasis (TNM) staging system was proposed for these tumours (Table 34.2) [32]. TNM staging is commonly used for prognostication and treatment decisions in several types of tumours, e.g. colon cancer. The system is based on the size and invasiveness of the primary tumour and the presence of lymph node metastases and distant metastases. Two studies evaluating the clinical relevance of this system have been published [10, 33]. Both studies confirm the clinical significance of having a tumour stage IV, i.e. having distant metastases (Fig. 34.1). Further studies are needed to assess the prognostic value of the earlier stages, i.e. if the earlier stages can really differentiate between patients with different prognoses.

Pancreatic endocrine tumours are often divided into three groups according to a World Health Organization (WHO) classification system [34]: well-differentiated neuroendocrine tumours, well-differentiated neuroendocrine carcinomas and poorly differentiated neuroendocrine carcinomas. The classification is based on the number of mitoses, proliferative index (Ki67) and the presence or absence of gross invasion. While it is widely accepted that well-differentiated tumours carry a quite good prognosis and poorly differentiated carcinomas have a poor prognosis, the middle group is more difficult. In a large Swedish material, 72% of patients had well-differentiated carcinoma [10]. This is thus by far the largest group of patients, and it is also very heterogeneous. Prognosis within this group varies substantially,

Table 34.2 The new TNM classification

Stage	Description
I	Primary tumour only, <2 cm
IIa	Primary tumour only, 2–4 cm
IIb	Primary tumour only, >4 cm or invading duodenum or bile duct
IIIa	Tumour invading adjacent organs (stomach, spleen, colon, adrenal gland) or the wall of large vessels (celiac axis, superior mesenteric artery)
IIIb	Lymph node metastases
IV	Distant metastases

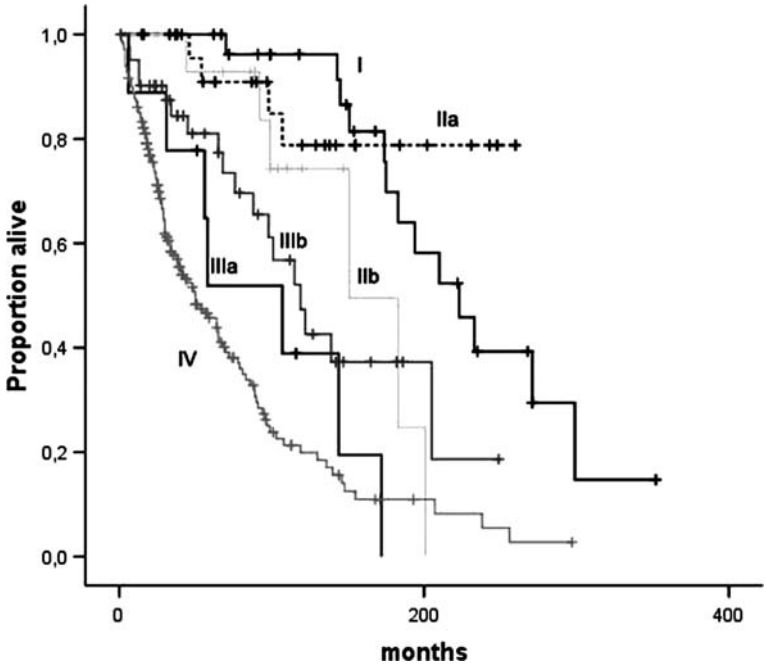

Fig. 34.1 Survival by TNM stage. Reprinted from Ekeblad et al. [10], with permission from the publisher

and it is difficult for the clinician to predict the prognosis of the individual patient with a well-differentiated carcinoma. Apart from tumour size and the presence or absence of radical surgery, a number of molecular markers are being investigated.

Ki67 is indicator for high proliferation in many tumours. In pancreatic endocrine tumours, a Ki67 index higher than 2% is commonly seen as an indicator of a more aggressive tumour [10, 35]. It is generally assumed that the higher the Ki67 index, the worse the prognosis.

Survivin is an apoptosis inhibitor and also plays a role in cell-cycle regulation. It is up-regulated in human cancers, and a high expression is in many tumour types associated with chemotherapy resistance and a poor prognosis [36]. Cancer cells with a high survivin expression simply seem to be better at surviving. A similar association in pancreatic endocrine tumours has been suggested [37].

High circulating levels of chromogranin A, a hormone frequently released by neuroendocrine tumours, have a negative prognostic value [10].

CK19 is a marker of pancreatic ductal cells and is not normally expressed by islet cells. It has been reported to be a strong predictor of poor prognosis when expressed by pancreatic endocrine tumours [38, 39], although there have also been contradictory reports [40].

Chromosomal instability, measured with comparative genomic hybridisation, seems to be associated with higher tumour burden and more advanced stage of

disease, suggesting that alterations accumulate during tumour progression [41]. Also, the level of chromosomal alterations has been shown to be a good marker of poor clinical outcome in insulinoma [22]. Losses of chromosomes 1 and 11q and gains of 9q have been shown to be present already in small tumours, suggesting that these changes take place early in the tumour progression [42].

Alkaline phosphatase in blood has also been suggested to be a marker of poor prognosis [43].

Many factors have been suggested to have prognostic value. More comprehensive evaluation in larger patient materials is needed to reach consensus on which are reliable, easy-to-use and indicate real prognostic differences and should thus be adopted in clinical routine.

34.8 Treatment

34.8.1 Management of Hormonal Symptoms

Historically, hormonal symptoms caused significant morbidity and mortality in patients with functioning tumours. Hormonal excess was the main cause of death. Several different tailored approaches were used to manage hormonal overproduction. Patients with glucagonomas were given blood transfusions or insulin, VIPoma patients obviously required replacement of volume and electrolytes, and patients with Cushing's syndrome were given adrenolytics or underwent adrenalectomy [12]. With the introduction of somatostatin analogues [44], medical care for these patients was significantly simplified. Somatostatin exerts multiple inhibitory functions on hormone secretion. It has a very short half-life, of about 2 minutes, and is thus not useful clinically. An analogue with a half-life of around 1 hour, octreotide, was introduced in the early 1980s and has revolutionised medical control of excess hormone secretion in pancreatic endocrine tumours. For example, octreotide improves diarrhoea in more than 75% of patients with VIPoma [12]. Today, longer-acting forms, that can be given once a month, are also available, further improving treatment and convenience for patients. A common problem is tachyphylaxia, meaning that after a while dose increases are necessary to obtain the same clinical response.

For patients with Zollinger–Ellison syndrome, it is not somatostatin but a much more well-known drug that has revolutionised treatment. These patients previously suffered from peptic ulcers, and often had to undergo gastrectomy. Today, symptoms are effectively controlled with PPIs, and gastric surgery no longer has a role in managing the effects of excess gastrin secretion in these patients [12]. PPI treatment can also be given intravenously. The advent of these drugs was a fantastic improvement for gastrinoma patients. However, there is a danger; since PPIs are so effective at controlling symptoms, they can also delay the diagnosis of gastrinoma, something that could lead to an increase in gastrinoma patients presenting with advanced disease [12]. If symptoms are easily treated, both the patient and the doctor are less likely to seek an uncommon but dangerous cause for the problem.

Similar drastic advances in medical management of hormone overproduction have not been seen for insulinoma patients. Somatostatin analogues are effective in around 35–55% of patients [12], but can also in some cases worsen hypoglycaemia and thus need to be used with care. The first choice of drug is instead usually diazoxide, which inhibits insulin release. This is helpful in more than half of patients, and has been used for over 20 years. Controlling hypoglycaemia still remains a challenge, and surgical removal or debulking of tumour mass is generally preferable.

With hormonal symptoms being much easier to control nowadays, more patients will live long enough to experience progressive disease and thus perhaps die from tumour progression. Thus, focus has largely turned from management of hormonal symptoms to anti-tumoural treatment.

34.8.2 Surgery

Surgery is the first choice of treatment for pancreatic endocrine tumours, both for anti-hormonal and for anti-tumoural purposes. If there are no metastases, the aim is radical removal of the primary tumour. Since pancreatic surgery is quite complicated, surgery for these tumours should preferably be performed at a specialised centre. There are substantial differences in approach compared to exocrine pancreatic tumours. Depending on tumour size and localisation, the surgeon aims for enucleation, distal pancreatic resection or a Whipple procedure, i.e. removal of the head of the pancreas, a part of the bile duct, the gallbladder and the duodenum. Local lymph node dissection should always be performed. The entire pancreas should be examined, with mobilisation of the duodenum to expose the pancreatic tail [12]. Bimanual palpation of the entire duodenum is performed in the case of a gastrinoma.

Intra-operative ultrasound is often used; always in MEN1 surgery. The reason for this is to localise possible additional tumours. Sometimes a patient is taken to surgery without a tumour having been visualised, based only on biochemical demonstration of elevated hormone levels and exclusion of other diagnoses. For MEN1 patients, an exploration of the entire pancreas should be done, and the surgeon should be aware that the largest lesion found is not necessarily the lesion causing the endocrine syndrome [12].

There is increasing discussion about the role of laparoscopic surgery for these tumours. It is performed at a few centres, mainly for small insulinomas who are considered benign [45]. Whether laparoscopic surgery can and should be used also for malignant tumours is debated, but most experts still agree laparotomy is preferable [46–48]. However, this might change in the future as laparoscopic techniques evolve.

34.8.3 Treatment of Metastatic Disease

Even when the tumour has metastasised, the removal of tumour burden, so-called debulking, can be of value to decrease hormone secretion. Some also suggests is

can improve prognosis [49, 50]. Debulking is done either by surgical resection of liver metastases or with newer techniques such as radiofrequency ablation or hepatic artery embolisation.

Radiofrequency ablation is done either intra-operatively or percutaneously. Metastases are destroyed through targeted heating. The method has been in use for around 10 years. The technique is especially suited for patients with only a few liver metastases; it works less well on diffuse liver tumour burden. A volume reduction of the metastases is seen in a majority of patients, as is alleviation of endocrine symptoms if present. Mortality is very low and morbidity acceptable [51–53]. The procedure can be performed repeatedly.

Hepatic artery embolisation has been in use since the 1980s [54]. It is performed to reduce tumour burden in patients with multiple liver metastases, as opposed to radiofrequency ablation which is more suited for fewer metastases [55]. While normal liver cells can live from portal circulation, metastases receive their blood supply from the systemic circulation. Cutting off systemic circulation with embolisation can thus selectively block tumour blood supply. It is important to follow liver enzymes after embolisation, since rare side effects include liver necrosis and cholecystitis. Embolisation can also be combined with locally delivered chemotherapy, chemoembolisation. Embolisation does produce radiologically verifiable tumour reduction and palliation of endocrine symptoms [56], but an increased survival has not yet been shown.

For select patients with liver metastases, liver transplantation can be an option.

A number of systemic treatments are available, reflecting the lack of curative treatments as well as a lack of controlled studies showing which treatment regimen is superior.

Chemotherapy with streptozotocin in combination with 5-fluorouracil (5-FU) leads to significant tumour regression in 20–63% of patients with metastatic pancreatic endocrine tumour [57, 58]. Combining streptozotocin with doxorubicin instead of 5-FU has been suggested to produce a higher response rate [59]. These combinations are mainly used in well-differentiated carcinomas, and side effects include nausea as well as dose-related nephrotoxicity.

Patients with poorly differentiated neuroendocrine carcinoma are often given a combination of etoposide and cisplatin. This produces objective responses in 42–67% of patients [60–62]. Side effects include neuro- and nephrotoxicity.

In addition to controlling hormonal symptoms, there is a discussion regarding whether somatostatin analogues also have an antiproliferative effect. Stabilisation of tumour growth has been demonstrated [63], and there have been case reports showing tumour regression [64]. Tumour stabilisation or regression, and tumour cell apoptosis, has been reported with high-dose somatostatin analogue treatment [65, 66]. The antiproliferative effect of somatostatin analogues is suggested to be due to induction of cell-cycle arrest or apoptosis as well as inhibition of angiogenesis and inhibition of secretion of factors needed for tumour growth [67]. While undoubtedly beneficial in alleviating hormonal symptom, additional studies are needed to further elucidate the clinical anti-tumoural benefit of somatostatin analogues.

Interferon α is sometimes used in the treatment of these tumours. Side effects include flu-like symptoms, bone-marrow suppression and rarely autoimmune reactions. Interferon α can lead to tumour regression in some patients [68, 69], but is not widely used for these tumours internationally. An antiproliferative effect of a novel group of interferons, interferon lambda, has been suggested [70].

A relatively new and promising addition to the treatment arsenal for these tumours is radiolabelled somatostatin analogues [71] which have been used for less than 10 years. Labelled with radioactive indium, yttrium or lutetium, they selectively target tumour tissue by binding to somatostatin receptors expressed by the tumours. Side effects are usually mild but include haematological and renal toxicity. To identify patients suitable for this treatment approach, patients undergo a somatostatin receptor scintigraphy to determine the density of receptors on their tumour. This density must be higher than that of normal tissue. Response rates of 28%, with a median time to progression of more than 36 months, have been reported for lutetium, which is generally considered the best option, although randomised studies comparing the treatments are lacking [71]. Improved quality of life has also been shown, but so far no increase in survival. With such promising treatment results, a randomised trial comparing radiolabelled somatostatin analogues with best supportive care only could be considered ethically questionable. However, studies comparing this treatment modality with, e.g. chemotherapy should be possible and could hopefully lead to more robust data regarding a potential increase in survival. Treatment with radiolabelled somatostatin analogues is limited by a restricted availability. In spite of these limitations, it is a very promising future treatment modality.

Another relatively new and interesting treatment modality is radioactive polymer microspheres, which were initially used for primary hepatocellular carcinoma and liver metastases from colorectal cancer. Yttrium-90 (^{90}Y)-embedded microspheres are administered via a hepatic artery catheter and deliver local radiation to target tumours. The high vascularisation of these tumours contributes to a favourable distribution of the microspheres, enabling delivery of a high radiation dose while sparing surrounding tissue [72]. Two recent studies have shown promising results. A partial response rate of approximately 50% was seen in a phase II study of 42 patients with neuroendocrine tumours, with 14% of patients experiencing grade 3/4 toxicities [73]. Glass or resin ^{90}Y radioembolic agents were used, both compounds generating similar response rates. A smaller study of seven patients with neuroendocrine tumours showed a partial response rate of 66%, with low toxicity [72]. Further investigation is needed, both in terms of technically optimising treatment and in terms of demonstrating superiority to other treatment modalities. Radioactive polymer microspheres could emerge as a promising treatment for patients with liver metastases from pancreatic endocrine tumours.

A number of newer systemic anti-tumoural drugs are also being investigated for pancreatic endocrine tumours. Temozolomide is an alkylating agent. It is spontaneously converted in vivo to its active metabolite, MTIC. MTIC is also the active metabolite of dacarbazine, which is used in other types of neuroendocrine tumours. Temozolomide is used for treatment of brain tumours and malignant melanoma.

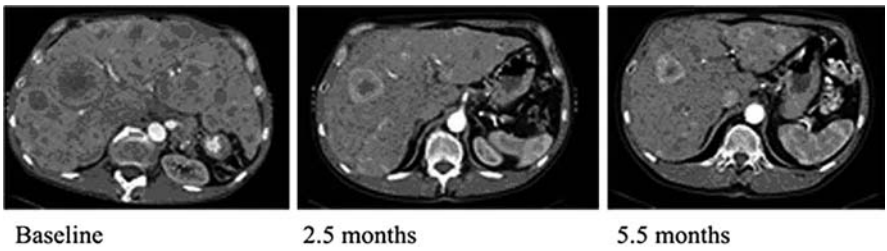

Fig. 34.2 Dramatic tumour response during treatment with temozolomide. Reprinted from Ekeblad et al. [75], with permission from the publisher

It has also shown promising results in the treatment of brain metastases from various tumours [74]. One retrospective study showed an objective radiologic response rate of 14% of patients using temozolomide as a single agent (Fig. 34.2) [75]. In a prospective study on patients with different types of neuroendocrine tumours, temozolomide plus the angiogenesis inhibitor thalidomide produced an overall radiologic response rate of 25% with a median duration of 13.5 months [76]. Unlike many chemotherapy drugs temozolomide is taken orally, which is a big advantage. Side effects include myelosuppression, fatigue and nausea [77] but the drug is better tolerated than many alternatives.

Bevacizumab is a monoclonal antibody which blocks VEGF. It is used for colorectal cancer, lung cancer and breast cancer. Encouraging results have been seen in small trials for neuroendocrine tumours, both as a single drug and in combinations [76], with responses rates around 15–20%. Trials evaluating bevacizumab in combination with other drugs are ongoing [78].

Sunitinib is a tyrosine kinase inhibitor targeting a number of receptors, e.g. VEGFR, c-KIT and RET [79], with an antiproliferative and antiangiogenic effect. It is currently used for gastrointestinal stromal tumours and renal cell carcinoma. In a large phase II study, a response rate of 13.5% was seen [80], and further studies are ongoing.

Sorafenib and vatalanib are other tyrosine kinase inhibitors being evaluated for these tumours.

The only evaluation of sorafenib so far showed a response rate of only 10% and a high incidence of side effects [78].

Thalidomide, which is taken orally, inhibits tumour necrosis factor α (TNF-α) and has an antiangiogenic effect [81]. As mentioned above, given in combination with temozolomide, it rendered a response rate of 25% [76]. A phase II trial evaluating thalidomide as a single agent in low-grade neuroendocrine tumours is ongoing.

Temsirolimus and everolimus inhibit the mammalian target of rapamycin (mTOR) pathway. Temsirolimus has not shown great promise for these tumours, but everolimus showed a 22% response rate in a phase II study. Further studies are ongoing.

Advances have been made regarding several aspects of pancreatic endocrine tumours: their genetics, prognostic factors and treatment. Much remains to be done, however, and most likely we will see significant advances in the years to come.

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