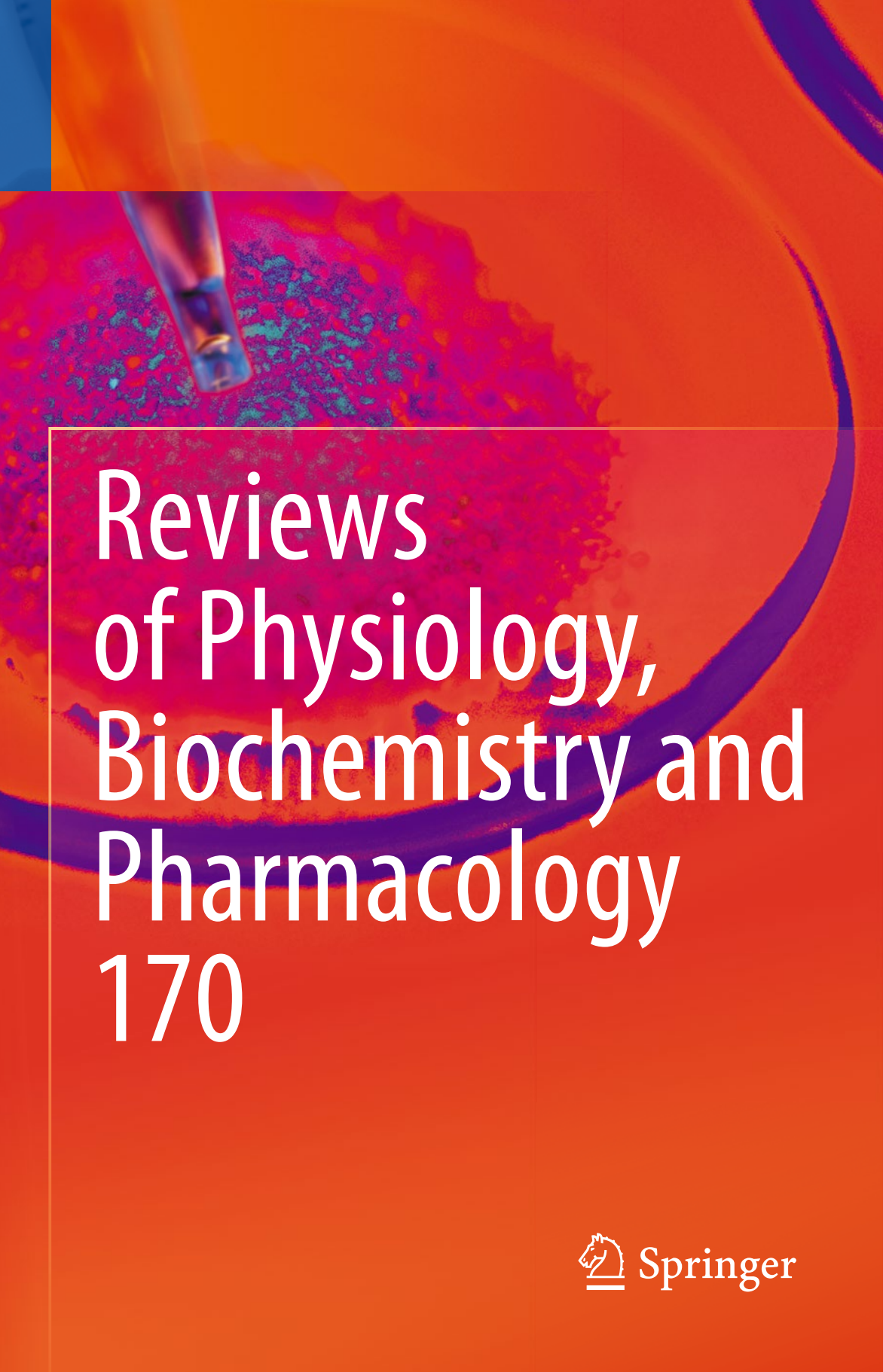
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ISSN 0303-4240 ISSN 1617-5786 (electronic)
Reviews of Physiology, Biochemistry and Pharmacology
ISBN 978-3-319-31491-4 ISBN 978-3-319-31492-1 (eBook)
DOI 10.1007/978-3-319-31492-1

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Rev Physiol Biochem Pharmacol (2016) 170: 1–36
DOI: 10.1007/112_2015_5001
© Springer International Publishing Switzerland 2016
Published online: 4 December 2015

Intrauterine Growth Retardation (IUGR) as a Novel Condition of Insulin-Like Growth Factor-1 (IGF-1) Deficiency

I. Martín-Estal, R.G. de la Garza, and I. Castilla-Cortázar

Abstract Insulin-like growth factor 1 (IGF-1) is an anabolic hormone with several biological activities, such as proliferation, mitochondrial protection, cell survival, tissue growth and development, anti-inflammatory, antioxidant, antifibrogenic and antiaging. This hormone plays an important role in embryological and postnatal states, being essential for normal foetal and placental growth and differentiation. During gestation, the placenta is one of the major sources of IGF-1, among other hormones. This intrauterine organ expresses IGF-1 receptors and IGF-1 binding proteins (IGFBPs), which control IGF-1 activities. Intrauterine growth restriction (IUGR) is the second most frequent cause of perinatal morbidity and mortality, defined as the inability to achieve the expected weight for gestational age. Different studies have revealed that IUGR infants have placental dysfunction and low circulating levels of insulin, IGF-1, IGF-2 and IGFBPs. Such data suggest that IGF-1 deficiency in gestational state may be one of the major causes of foetal growth retardation. The aim of this review is to study the epidemiology, physiopathology and possible causes of IUGR. Also, it intends to study the possible role of the placenta as an IGF-1 target organ. The purpose is to establish if IUGR could be

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The original version of this chapter was revised: The spelling of the third author's name was corrected. The erratum to this chapter is available at DOI: 10.1007/112_2016_1.

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considered as a novel condition of IGF-1 deficiency and if its treatment with low doses of IGF-1 could be a suitable therapeutic strategy.

Keywords Cell proliferation • Foetal/placental growth • GH • GH/IGF-1 axis • IGF-1 • IGF-1R • IGF-2 • IGFBP-rPs • IGFBPs • Intrauterine growth restriction • Placental lactogen • Somatostatinergic tone

Contents

Abbreviations

ALS	Acid-labile subunit
CG	Chorionic gonadotropin
CRH	Corticotropin-releasing hormone
CSF	Cerebrospinal fluid
CTGF	Connective tissue growth factor
Cyr61	Cysteine-rich protein 61
ESM-1	Endothelial cell-specific molecule
FSH	Follicle-stimulating hormone
GH	Growth hormone
GHBP	Growth hormone binding protein
GHRH	Growth hormone-releasing hormone
GnRH	Gonadotropin-releasing hormone
HPA	Hypothalamus–pituitary–adrenal gland axis
IGF-1	Insulin-like growth factor 1
IGF-1R	IGF-1 receptor
IGF-2	Insulin-like growth factor 2
IGFBP-rPs	IGFBP-related proteins
IGFBPs	IGF binding proteins
IGFs	Insulin-like growth factors
IUGR	Intrauterine growth restriction

LDL	Low-density lipoprotein
LH	Luteinising hormone
MAP	Mitogen-activated protein
NGAS	Neonatal growth assessment score
NovH	Human nephroblastoma overexpression gene
PAPP-A	Pregnancy-associated plasma protein A
PGAS	Prenatal growth assessment score
PI3K	Phosphatidylinositol-3-kinase
PL	Placental lactogen
PLGF	Placental growth factor
PSF	Prostacyclin-stimulating factor
PSG	Pregnancy-specific β-glycoprotein
TAF	Tumour adhesion factor
TSC 1	Tuberous sclerosis protein 1
TSC 2	Tuberous sclerosis protein 2

1 Insulin-Like Growth Factor 1 (IGF-1)

1.1 Introduction

Insulin-like growth factor 1 (IGF-1) is an anabolic hormone produced in several tissues, specially in the liver (Laron 2001; Le Roith 1997). IGF-1 is synthesised by the endocrine growth hormone (GH) stimulation (Sara and Hall 1990). Although IGFs were first described by Salmon and Daughaday in 1957 (Salmon and Daughaday 1957), the discovery culminated two decades later, thanks to studies performed by Rinderknecht and Humbel (Rinderknecht and Humbel 1978a,b). Finally, all these findings allowed to identify a new family of proteins composed by proinsulin, IGF-1 and IGF-2 (Le Roith 1997).

IGF-1 shares >60% homology with IGF-2 and 50% homology with proinsulin structures (Le Roith 1997). Similar to proinsulin, both hormones, IGF-1 and IGF-2, are divided into A, B, C and D domains. A and B domains are similarly bridged by two inter-domain disulphide bonds and with one internal disulphide bond in the A domain. Both domains are connected by a C domain, which, unlike proinsulin, is not proteolytically cleaved during structural maturation. In IGF-1, positions 1 to 29 are homologous to insulin B chain and positions 42 to 62 are homologous to insulin A chain. The "connecting" peptide region (C domain) has 12 amino acids and shows no homology to proinsulin C peptide (Fig. 1). Such structural similarity to insulin explains the ability of IGF-1 to bind the insulin receptor (Laron 2001; Rinderknecht and Humbel 1978a). The primary difference between IGF-1 and IGF-2 resides in their biological activity. IGF-2 is expressed predominantly in early embryonic and foetal life and IGF-1 is expressed in the adult (Laron 2001; Rinderknecht and Humbel 1978a).

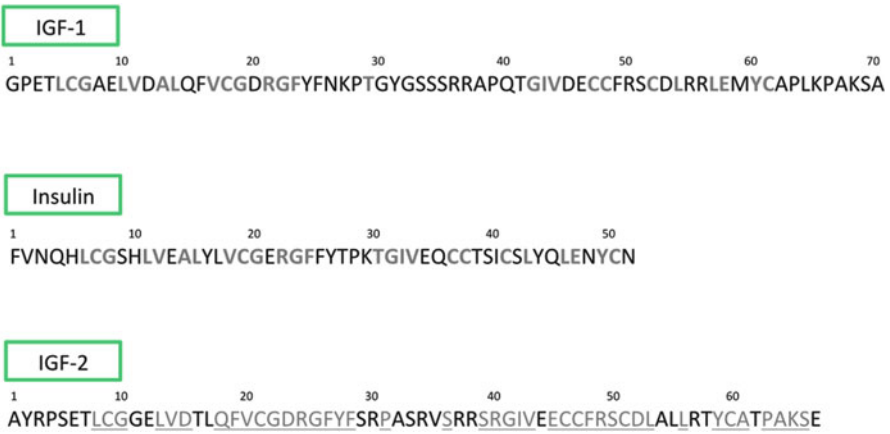

Fig. 1 Amino acid sequences of human IGF-1, IGF-2 and insulin. Homologous amino acids in IGF-1 (70 amino acids) and proinsulin and insulin (51 amino acids) are represented in *red*. Homologous amino acids in IGF-1 and IGF-2 (67 amino acids) are represented in *blue*

IGF-1, as a somatomedin, possesses insulin-like activity in the presence of insulin antibodies (Froesch et al. 1963; Zapf et al. 1978), and it is also a sulphation factor (Daughaday et al. 1972), is growth hormone dependent (Sara and Hall 1990; Daughaday et al. 1972) and acts as a mitogen (Zapf et al. 1978; Rinderknecht and Humbel 1976).

In the last decades, many evidences have provided us a wide list of IGF-1 activities, such as the following: proliferative, mitochondrial protection (Pérez et al. 2008), cell survival (Vincent and Feldman 2002), tissue growth and development (Powell-Braxton et al. 1993; Fowden and Forhead 2013), anti-inflammatory and antioxidant (García-Fernández et al. 2003, 2005), antifibrogenic (Muguerza et al. 2001) and antiaging (Puche et al. 2008; García-Fernández et al. 2008).

Because of its several physiological roles, IGF-1 activities must be strictly controlled by its association with six well-characterised binding proteins (IGFBPs 1 to 6) (Tables 1 and 2). These proteins have high affinity for IGF-1 and were identified, cloned and sequenced in the early 1990s (Jones and Clemmons 1995; Lamson et al. 1991), thanks to the development of the Western ligand blot techniques (Hossenlopp et al. 1986). IGFBPs share ≈35% sequence identity with each other, with apparent molecular mass of 24–45 kDa. They have a primary structure consisting of three different domains: the conserved N-terminal domain, the highly variable mid-region and the conserved C-terminal domain (Lamson et al. 1991; Hwa et al. 1999). The IGFBPs are produced by a variety of biological tissues and found in several biological fluids, such as follicular liquid, amniotic liquid, vitreous humour, lymph, plasma, seminal fluid, cerebrospinal fluid and gastrointestinal secretions (Rajaram et al. 1997; Binoux et al. 1991) (Tables 1 and 2). All these binding proteins are expressed by virtually all tissues, but the major source of serum IGFBPs is the liver. The IGFBPs function as carrier proteins for circulating IGFs, with higher affinity for them ($K_d \approx 10^{-10}$ M) than type I IGF receptors ($K_d \approx 10^{-8}$

Table 1 Characteristics of the IGFBP human superfamily. IGFBPs: high-affinity IGF binders

	Molecular mass (kDa)[a]	No. of amino acids[b]	IGF affinity	Modulation of IGF action	Source in biological fluids	Presence in placenta	References
IGFBP-1	25.3	234	$1 = 2$	Inhibition and/or potentiation	Amniotic fluid, serum, milk, urine, synovial fluid, interstitial fluid, seminal fluid, amniotic fluid	Labyrinth, uterine tissue, yolk sac placenta	Hills et al. (1996)
IGF BP-2	31.4	289	$2 > 1$	Inhibition	CSF, serum, milk, urine, synovial fluid, interstitial fluid, lymph follicular fluid, seminal fluid, amniotic fluid	Junctional zone, yolk sac placenta	Hills et al. (1996); Firth and Baxter (2002)
IGFBP-3	28.7	264	$1 = 2$	Inhibition and/or potentiation	Serum, follicular fluid, milk, urine, CSF, synovial fluid, interstitial fluid, seminal fluid, amniotic fluid	Junctional zone, uterine tissue	Hills et al. (1996); Firth and Baxter (2002)
IGF BP-4	26.0	237	$1 = 2$	Inhibition	Serum follicular fluid, seminal fluid, interstitial fluid, synovial fluid	Junctional zone, uterine tissue, yolk sac placenta	Hills et al. (1996)
IGFBP-5	28.6	252	$2 > 1$	Potentiation	Scrum, CSF	Junctional zone, uterine tissue	Hills et al. (1996); Firth and Baxter (2002)
IGF BP-6	22.8	216	$2 > 1$	Inhibition	CSF, serum, amniotic fluid	Uterine tissue	Hills et al. (1996); Firth and Baxter (2002)

High-affinity binder: involves greater intermolecular force between the ligand and its binder
CSF cerebrospinal fluid
[a]Predicted molecular mass (kDa) of nonglycosylated, mature protein
[b]Number of amino acids of mature protein

Table 2 Characteristics of the IGFBP human superfamily. IGFBP-rPs: low-affinity IGF binders

		Molecular mass (kDa)[a]	No. of amino acids[b]	Organ and/or tissue expression	References
IGFBP-7	IGFBP-rP1/Mac25	26.4	256	Leptomeninges	Bradham et al. (1991)
IGFBP-8	IGFBP-rP2/CTGF/CCN-2	35.5	323	Connective tissue, placenta	Myers et al. (2012); Hu et al. (1998)
IGFBP-9	IGFBP-rP3/NovH/CCN-3	36.0	329	Kidney	Holbourn et al. (2008)
IGFBP-10	IGFBP-rP4/Cyr61/CCN-1	39.5	358	Foetal and adult brain and liver; adult lung, kidney and thymus	Wang et al. (2012)
IGFBP-11	IGFBP-rP5/L56/HtrA	49.0	458	Osteoarthritic cartilage	Castilla-Cortázar et al. (2001)
IGFBP-12	IGFBP-rP6/ESM-1	18.1	165	Endothelial and epithelial cells, lung	Clemmons and Underwood (1991)
IGFBP-13	IGFBP-rP7/WISP-2/CCN-5	24.4	228	Smooth muscle, bone, uteri	Chitnis et al. (2008)
IGFBP-14	IGFBP-rP8/WISP-1/CCN-4	38.0	345	Heart, lung, smooth muscle, bone	Puche and Castilla-Cortázar (2012)
IGFBP-15	IGFBP-rP9/WISP-3/CCN-6	37.1	334	Cartilage, breast epithelium	Duan et al. (2000)

Low-affinity binder: involves less intermodular force between the ligand and its binder
[a]Predicted molecular mass (kDa) of nonglycosylated, mature protein
[b]Number of amino acids of mature protein

to 10^{-9} M), and by regulating IGF turnover, transport and tissue distribution, thus determining physiological concentrations of IGFs (Jones and Clemmons 1995; Hwa et al. 1999). For example, during normal pregnancies serum levels of IGF-1 and IGFBPs (mainly IGFBP-1) rise progressively through gestation, especially since the second trimester of pregnancy. An elevated level of IGFBP-1 in the foetal circulation is an indicator of IUGR, caused by placental insufficiency and utero hypoxia. Such binding protein is believed to restrict foetal growth by sequestering IGFs (Hills et al. 1996). Additionally they modulate IGF activities in target tissues, such as cell proliferation, differentiation, survival and migration (Jones and Clemmons 1995; Firth and Baxter 2002), being able to activate or inhibit IGF actions. Also they facilitate transport of IGFs from the vascular space to target tissues. Most IGFs in circulation are found forming complexes with IGFBPs,

especially in a ternary complex with IGFBP-3 and ALS (acid-labile subunit). The aforementioned complex serves as a reservoir for IGF and also increases the half-life of IGF-1 (Rajaram et al. 1997). In addition, IGFBPs can be associated with cell membranes or extracellular matrix, allowing them to maintain a local pool of IGF-1 (Firth and Baxter 2002).

Interestingly, another nine binding proteins arose as the so-called IGFBP-related proteins (IGFBP-rPs), which are cysteine-rich proteins with structural and functional similarities to the IGFBPs (Hwa et al. 1999). At present, there are four proteins/families that are related to the IGFBPs (Tables 1 and 2). Mac25 was originally identified as a cDNA derived from leptomeninges (Murphy et al. 1993) and was subsequently expressed in a baculovirus system. The synthesised protein was shown to bind IGFs and was renamed IGFBP-7 (Oh et al. 1996). Its expression is regulated by specific growth factors and IGFs, and it is involved in diverse biological functions, such as regulation of epithelial cell growth, stimulation of fibroblast cell growth and stimulation of prostacyclin production in endothelial cells (Hwa et al. 1999). The CCN family consists of several proteins, and it acquired its name from the first three proteins discovered: Cyr61 (cysteine-rich protein 61) (Saglam et al. 2014), connective tissue growth factor (CTGF) (Bradham et al. 1991) and the human nephroblastoma overexpression gene (NovH) (Burren et al. 1999). CTGF major function is to regulate the formation of connective tissue. This protein is also important in both physiological (tissue homeostasis) and pathological (fibrosis) conditions (Nguyen et al. 2008). Three new members of this family have been identified in Wnt-1 transformed cells: WISP-1 (Wang et al. 2012); WISP-2, which was designated CTGF-like because it was identified in primary human osteoblast cells (Myers et al. 2012); and WISP-3 (Baker et al. 2012). The CCN proteins are key signalling and regulatory molecules involved in several vital biological functions, including cell proliferation, angiogenesis, tumourigenesis and wound healing (Holbourn et al. 2008). Two other IGFBP-related proteins are L56, a potential serine protease of IGFBPs, also named HtrA (Hu et al. 1998), and endothelial cell-specific molecule (ESM-1) (Lassalle et al. 1996). The physiological role of the IGFBP-rPs in the IGF system remains undefined, but their structural relationship with IGFBP-1 to IGFBP-6 reveals the ability of some of these proteins to bind IGF-1, modulating its activity (Oh et al. 1996; Burren et al. 1999).

On the other hand, the majority of IGF-1 actions are mediated through the union of IGF-1 to its putative receptor, IGF-1R, a tyrosine kinase with an $\alpha_2\beta_2$ heterotetrameric structure that is one of the most potent natural activators of Akt pathway, closely related with cell survival, growth and proliferation (Puche and Castilla-Cortázar 2012; Annenkov 2009; Chitnis et al. 2008). Ligand binding induces phosphorylation of tyrosine residues in the intracellular domains of the β-subunits and activate the receptor. The activated IGF-1R in turn activates multiple signal transduction cascades, including the mitogen-activated protein (MAP) kinase pathway and phosphatidylinositol-3-kinase (PI3K)–Akt pathway (Duan et al. 2000). The IGF-activated pathways promote cell survival through regulation of multiple effectors (BCL-2, BAD, caspase-9, p53, etc.), cell proliferation, migration and/or differentiation (Jones and Clemmons 1995) (Fig. 2).

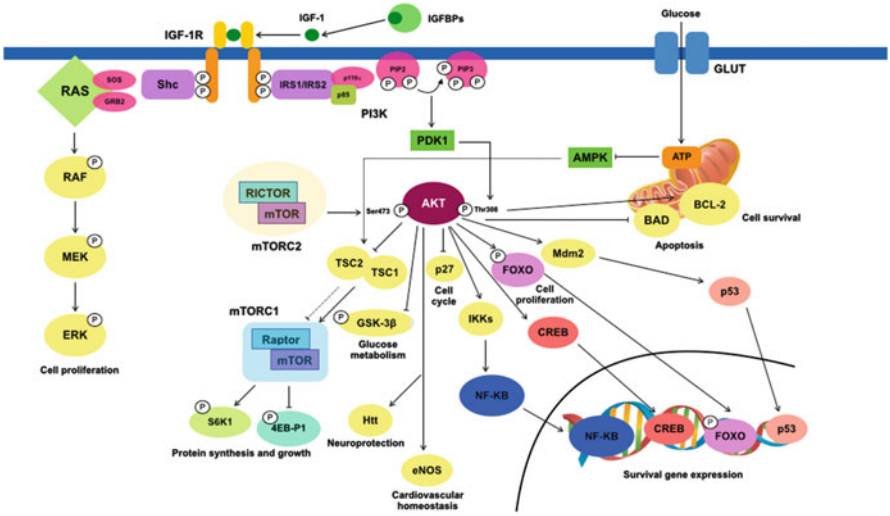

Fig. 2 The IGF-1 signalling pathway. IGF binding proteins (IGFBPs) modulate IGF-1 bioavailability. IGF-1 functions as a ligand to interact with IGF-1 receptor (IGF-1R) in the cellular membrane, which leads to autophosphorylation and recruitment of the adaptor proteins IRS-1, IRS-2 and Shc. The interaction of IRS-1 and IRS-2 with IGF-1R induces the activation of PI_3-kinase (phosphatidylinositol-3-kinase), which transforms PIP_2 in PIP_3. Akt family of kinases is activated by PDK1 and by mTOR-containing complex mTORC2 and regulates downstream signalling molecules such as TSC1 and 2 (tuberous sclerosis protein 1 and 2) and FOXO transcription factors, GSK-3β, p27, BAD and BCL-2. All these molecules are involved in several cellular processes including protein synthesis, cell proliferation, glucose metabolism, cell cycle and cell survival. In parallel, Shc activation induces the activation of the RAS/MAP kinase pathway, which increases cell proliferation. Low glucose levels activate AMPK, which activates TSC2 and inhibits mTORC1 action (*discontinue lines*)

In addition, the similarity in structure of IGF-1R and insulin receptor (≈60%) (Nissley and Lopaczynski 1991) explains that IGF-1 can also bind to the insulin receptor but with lower affinity. Ligand binding can be a secondary pathway by which IGF-1 mediates some of its metabolic functions (Rinderknecht and Humbel 1978a). Similarly, insulin can bind to the IGF-1R with a lower affinity than it does to the insulin receptor.

1.2 Physiological Activities of IGF-1

IGF-1 is an important hormone in embryological and postnatal states. Although it is mainly produced by the liver (approximately 75% of circulating IGF-1 is produced by this organ) (Ohlsson et al. 2009), virtually every tissue is able to secrete IGF-1 for autocrine and/or paracrine purposes.

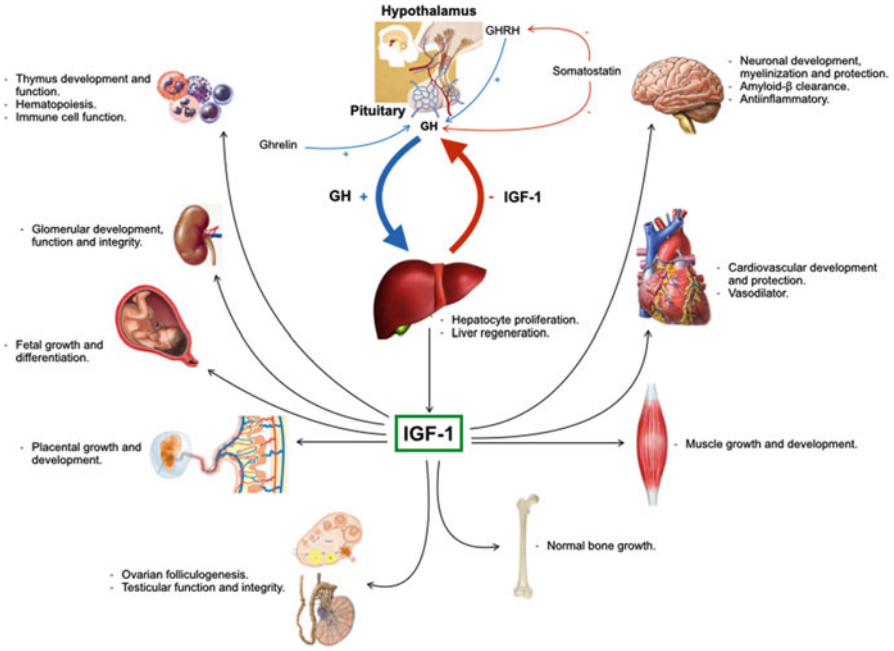

Fig. 3 Model of GH/IGF-1 axis and its target organs. Negative feedback mechanism induced by IGF-1 regulates GH/IGF-1 axis: IGF-1 inhibits GH gene expression by stimulating somatostatin secretion. GHRH (growth hormone-releasing hormone) stimulates GH secretion, which stimulates IGF-1 secretion

The secretion of IGF-1 is stimulated by growth hormone (GH), forming the GH/IGF-1 axis, where GH secretion is stimulated by growth hormone-releasing hormone (GHRH) and inhibited by somatostatin (Puche and Castilla-Cortázar 2012) (Fig. 3). Both hormones are generated in the hypothalamus as a result of neurogenic, metabolic and hormonal factors. This GH/IGF-1 axis is regulated by negative feedback mechanisms induced by IGF-1 itself: IGF-1 can inhibit GH gene expression by stimulating the secretion of somatostatin (Bertherat et al. 1995), which inhibits GH secretion. In various diseases, such as liver cirrhosis, this axis is altered: low IGF-1 serum levels and high GH levels, with the concomitant reduced somatostatinergic tone. Such disruption is reverted by the exogenous administration of IGF-1 at low doses (Castilla-Cortázar et al. 2001).

Circulating GH exist in both free and bound states by the GHBP (growth hormone binding protein – the secondary domain of the GH receptor). Hepatic GH receptor activation induces IGF-1 production, which is released in the circulation, where it is found in its free form (<1% bioactive component) and bound to IGFBPs. IGF-1 is specially bound to IGFBP-3, which binds ≈90% of the circulating hormone, increasing its half-life (Ohlsson et al. 2009).

In physiological conditions, IGF-1 activities are still being investigated, and it is being recognised as a GH-independent peptide. For example, it is known that GH and nutrition are the major factors that regulate hepatic IGF-1 expression, as well as in other organs (Clemmons and Underwood 1991). However, in some other tissues, IGF-1 expression appears to be regulated by tissue-specific trophic factors. For example, in uterus, oestrogens stimulate IGF-1 expression instead of the GH (Murphy and Friesen 1988).

IGF-1 has a wide variety of effects, but essentially, these can be divided into acute metabolic effects and long-term growth-promoting effects (Juul 2003). The acute actions of IGF-1 overlap with those of insulin on carbohydrate and protein metabolism to promote energy storage, including the stimulation of amino acid uptake into skeletal muscle, as well as the peripheral glucose uptake and regulation of insulin secretion and sensitivity (Juul 2003). On the other hand, its long-term effects are on cell proliferation, differentiation and anti-apoptosis (Jones and Clemmons 1995; Yu and Rohan 2000). Hence, IGF-1 plays a major and important role in several target organs (Fig. 3), as further described.

In the brain, IGF-1 is a potent neurotrophic and neuroprotective factor, promoting neuronal proliferation, survival and development (Gómez 2008), and it could be involved in the modulation of blood–brain barrier permeability (Carson et al. 1993). It is also one of the main factors regulating the clearance of brain amyloid-β levels with implications in Alzheimer's disease (Carro et al. 2002).

The liver is the main source of circulating IGF-1 (Ohlsson et al. 2009) and there is few data regarding local effects of this hormone in this organ (Skrtic et al. 1997). Nevertheless, it has been demonstrated that IGF-1 support hepatocyte proliferation and accelerate DNA synthesis, promoting liver regeneration (Desbois-Mouthon et al. 2006).

IGF-1 is also needed for an optimal fecundity during the reproductive period (Livingstone 2013). It increases granulose cell proliferation, steroidogenesis (Villalpando and López-Olmos 2003) and oocyte growth in most mammalian species (Silva et al. 2009; Giudice 1992; Giudice and Saleh 1995). And it also has a role on sperm number, as seen in IGF-1-deficient mice (Baker et al. 1996).

Moreover, IGF-1 has physiological roles in maintaining the normal function of the immune system, such as T lymphocytes development and function (Walsh et al. 2002), thymus development (Hadden et al. 1992) and B-cell differentiation (Landreth et al. 1992). IGF-1 regulates renal function (Bach 2012) and maintains glomerular integrity (Martin et al. 1991; Hirschberg 1996) and plays an important role in cardiovascular development and protection (Delafontaine et al. 2004), acting as a potent vasodilator (Delafontaine et al. 2004). It also controls muscle growth and development (Schiaffino and Mammucari 2011), stimulates protein synthesis in skeletal muscle (Velloso 2008), is essential for the attainment of peak bone mass during puberty, is necessary for normal bone growth (Yakar et al. 2010; Tahimic et al. 2013; Guntur and Rosen 2013) and plays a central role during muscle regeneration (Florini et al. 1996).

1.3 Role of IGF-1 During Pregnancy

Gestation can be divided into three well-defined periods. During the first period (pre-differentiation period), fertilisation, segmentation and gastrulation occur (Valsamakis et al. 2006). Once the gastrula is formed, the embryonic period begins, where proliferation and embryonic organogenesis occur. In this period, the embryo is more susceptible to damage, caused by external agents, such as alcohol, drugs, medicine, X-rays, radiation, etc. (Valsamakis et al. 2006). In the last period (foetal period), foetal organs develop both functionally and anatomically, leading to continuous foetal growth (Valsamakis et al. 2006).

All this process involves endocrine and metabolic changes in maternal pituitary gland and placental secretion (Kumar and Magon 2012). After involution of ovarian sex steroid production by the 6th week, placental oestrogen and progesterone production by the *corpus luteum* increases exponentially to term. Progesterone is important in suppressing the maternal immunologic response to foetal antigens, preparing and maintaining the endometrium to allow implantation of the embryo. Between the 8th and 10th weeks of gestation, placental production of chorionic gonadotropin (CG) rescues the *corpus luteum* from involution and maintains progesterone secretion (Kumar and Magon 2012; Freemark 2010).

During mid-gestation (13th to 28th weeks), there is a progressive increase in prolactin, secreted by the maternal pituitary gland, and placental growth hormone (pGH) levels. Several studies had found low maternal serum levels of both hormones in pregnancies associated to intrauterine growth retardation (Kumar and Magon 2012; Freemark 2010). During this period, placental lactogen (PL) is necessary for a normal production of progesterone, as seen in diverse mice strain models. Additionally, PL is responsible for the marked rise in maternal plasma IGF-1 concentration as the pregnancy approaches term (Kumar and Magon 2012).

Insulin is also an important factor for foetal metabolism, because it stimulates glucose and amino acid cellular capitation, necessary for tissue growth. Insulin deficiency in the uterus may lead to IUGR (Fowden and Forhead 2013). Inside the insulin family, IGF-1 and IGF-2 regulate cell cycle, proliferation and differentiation. Both hormones control transport capacity of the placenta and mediate stimulatory actions of insulin and thyroid hormones (Fowden and Forhead 2013). In the prenatal period, differences between GH and IGF-1 are clearly shown. During gestation, IGF-1 production is stimulated by placental GH. GH insensitivity, both in humans and in transgenic mice, has only mild retardation of growth at birth (Jameson 1999), whereas IGF-1 deficiency in gestational state reveals serious postnatal growth retardation, as has been reported both in humans and in transgenic animal models with IGF-1 gene deletion (Lupu et al. 2001; Baker et al. 1993; Liu et al. 1993; Woods et al. 1996). Interestingly, in contrast to growth hormone insensitivity, IGF-1-deficient animals are neurologically impaired, as was also reported in a single patient with a defect in the IGF-1 gene (Woods et al. 1996). Accordingly, all these data suggest that IGF-1 is necessary for normal brain development in the uterus (Randhawa and Cohen 2005).

Therefore, IGF-1 has a major role in foetal and placental growth and differentiation (Cohick and Clemmons 1993; Hiden et al. 2009; Forbes and Westwood 2010), being a major regulator of intrauterine and normal body growth (Lupu et al. 2001; Baker et al. 1993; Woods et al. 1996). IGF-1 enhances protein synthesis and inhibits proteolysis, having a key role in growth regulation both embryonically and postnatally (Fryburg et al. 1995; Clemmons 2009). It is essential for the attainment of normal body size during foetal development. Additionally, IGF-2 plays a key role in placental growth (Rajaram et al. 1997; Sferruzzi-Perri et al. 2006).

1.3.1 The Placenta as an IGF-1 Target Organ

The placenta is an intrauterine organ with central functions in pregnancy: it supplies nutrients and oxygen to the foetus and produces a range of hormones and growth factors that may affect mother, foetus or both (Hiden et al. 2009; Murphy et al. 2006). Moreover, hormones and growth factors present in maternal and foetal circulation may regulate foetal growth and placental development (Murphy et al. 2006).

Besides insulin, IGF-1 and IGF-2, several hormones (summarised in Table 3) are produced by the placenta during pregnancy, which are involved in the regulation of both foetal and placental development and growth (Hiden et al. 2009; Murphy et al. 2006). In addition to the aforementioned hormones, IGFBPs also participate in the regulation of both placental and foetal development and growth. The placenta has the ability to differentially express these proteins (Table 4). IGFBP-1 is the predominant binding protein synthesised by the placenta. It is expressed predominantly in trophoblast and decidua, where it regulates the biological activity of IGFs by modulating their interaction with IGF-1 receptor (Jones and Clemmons 1995; Rajaram et al. 1997; Gibson et al. 2001; Chard 1994; Crossey et al. 2002; Clemmons 1997; Lee et al. 1993). The other binding proteins (IGFBP-2, 3, 4, 5 and 6) are only expressed in some cells where they regulate placental development (Jones and Clemmons 1995; Rajaram et al. 1997; Clemmons 1997; Carter et al. 2006). In growth-restricted foetuses, serum and umbilical cord levels of IGFBP-1 and IGFBP-2 are increased compared to normal foetuses (Crossey et al. 2002; Street et al. 2006; Tzschoppe et al. 2015).

IGFs and insulin actions are mediated through binding to their receptors, which are expressed on distinct placental surfaces. Their expression varies with gestational age (Table 5). For example, cytotrophoblast and syncytiotrophoblast express receptors for progesterone. Such hormone is implicated in embryogenesis (Ziyan et al. 2010; Shanker and Rao 1999; Zachariades et al. 2012). These two areas of the placenta also express receptors for GnRH, which has a key role in implantation of the zygote and in endometrial, placental and foetal development (Fowden and Forhead 2013; Wolfahrt et al. 1998). They also express receptors for LH, CG and oestrogens such as oestradiol, important hormones in the development and maintenance of reproductive tissues (McCormack and Glasser 1978). Other regions of

Table 3 Placental hormones and its expression during pregnancy

Hormone	Expression in placenta	Period of gestation	Function	References
CG	Trophectoderm	Preimplantation embryo	Quality of placentation	Muyan and Boime (1997)
	Syncytiotrophoblast	1st to 12th weeks	Corpus luteum maintenance	
			Regulation of foetal testicular testosterone secretion	
PL	Syncytiotrophoblast	13th to 28th weeks	Stimulation of food intake	Handwerger and Freemark (2000)
			Regulation of growth and development	
Leptin	Syncytiotrophoblast, cytotrophoblast	1st to 40th weeks	Mother: accumulation of body fat	Ashworth et al. (2000)
			Foetus: mediate insulin's anabolic actions	
CRH	Syncytiotrophoblast, amnion, musculature of umbilical vessels, maternal decidua	Late pregnancy	Promote labour and initiation of parturition	Grammatopoulos (2008); Karteris et al. (2001)
			Vasodilation of placental vessels	
			Accelerate pulmonary maturation	
Neuropeptide Y	Cytotrophoblast	Early pregnancy until term	Stimulation of CRH from placental cells	Petraglia et al. (1989)
		Decreases after delivery	Contributes to uterine contractility	
Inhibin	Syncytiotrophoblast, cytotrophoblast	Increases during pregnancy	Control of steroidogenesis, peptide hormone and prostaglandin secretion	Petraglia (1997); Riley et al. (2000)
			Inhibition of FSH secretion	
Activin	Syncytiotrophoblast, cytotrophoblast	At term, labour	Stimulation of prostaglandins, oxytocin and FSH secretion	Petraglia (1997); Rabinovici et al. (1992)
			Modulation of cytotrophoblast proliferation and differentiation	

(continued)

Table 3 (continued)

Hormone	Expression in placenta	Period of gestation	Function	References
PSG	Syncytiotrophoblast, spongiotrophoblast	Preimplantation embryo	Prevents rejection of the foetus	Wu et al. (1999); Wynne et al. (2006)
PAPP-A	Syncytiotrophoblast, maternal decidua	Increases during pregnancy	Is the IGFBP-4 protease: increases IGF bioavailability	Lawrence et al. (1999); Sun et al. (2002)
PLGF	Trophoblast	During pregnancy	Stimulation of proliferation, migration and activation of endothelial cells	Vuorela et al. (1997)
		Increases in early stages of pregnancy	Coordinate vascularisation in the decidua and placenta	
Placental GH	Syncytiotrophoblast	13th to 28th weeks	Insulin resistance of pregnancy	Lacroix et al. (2002)
			Regulator of IGF-1	
Progesterone	Syncytiotrophoblast	6th to 8th weeks	Uterine quiescence	Shanker and Rao (1999); Freemark (2006); Iliodromiti et al. (2012)
		Increases during pregnancy and labour	Stimulation of weight gain and fat deposition	
Oestrogen	Placenta?	6th to 8th weeks	Initiation of labour	Freemark (2006); Kaludjerovic and Ward (2012); Albrecht and Pepe (2010)
			Biosynthesis of progesterone	
			Foetal adrenal maturation	
IGF-1	Syncytiotrophoblast	During pregnancy	Steroidogenesis	Hiden et al. (2009)
			Glucose and amino acid uptake	
			Foetal and placental growth, differentiation and development	
IGF-2	Trophoblast	1st to 12th weeks	Facilitates trophoblast invasion into the maternal decidua	Hiden et al. (2009)
			Key role in placental growth	

The question mark indicates that oestrogen placental expression is not yet well defined

CG chorionic gonadotropin, *PL* placental lactogen, *CRH* corticotropin-releasing hormone, PSG pregnancy-specific β-glycoproteins, *PAPP-A* pregnancy-associated plasma protein A, *PLGF* placental growth factor, *IGF-1* insulin-like growth factor-1, *IGF-2* insulin-like growth factor-2

Trimesters of pregnancy are described in weeks: 1st trimester, 1st to 12th weeks; 2nd trimester, 13th to 28th weeks; 3rd trimester, 29th to 40th weeks

Table 4 IGFBP expression in mouse placenta

Placenta	IGFBP-1	ICFRP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
Labyrinth						
Trophoblast	✓		✓			
Syncytiotrophoblast		✓				
Junctional zone						
Spongiotrophoblast		✓				
Glycogen cells		✓				
Chorionic mesoderm				✓	✓	
Uterine tissue						
Decidua	✓		✓		✓	✓
Metrial gland stroma		✓			✓	✓
Maternal vessel endothelium			✓	✓	✓	
Stromal cells	✓					
Myometrium		✓		✓	✓	✓
Yolk sac placenta						
Endoderm	✓	✓				
Mesoderm				✓		
Blood vessels						
Amnion						
Function	Inhibits IGF action	Inhibits IGF action	Major carrier of IGFs in serum	Inhibits IGF action	Potentiates IGF-1 action	Inhibits IGF action
	Stimulates smooth muscle cell migration		Reduces its affinity for IGF-1		Stimulatory effect on a variety of cell types	Inhibits IGF-2 actions preferentially
			Stimulates proliferation		Fix IGFs to extracellular matrices (bone)	

Table 5 Hormone receptor expression in placenta

Placental tissue	Hormone receptor expression
Syncytiotrophoblast	Insulin (1st trimester)
	IGF-1 (1st trimester and term)
	IGF-2 (term)
	Progesterone
	GnRH
	LH
	CG
	Oestrogens
Cytotrophoblast	Progesterone
	GnRH
	LH
	CG
	Oestrogens
Trophoblast	IGF-1 (end of gestation)
	GH
	Thyroid hormones
	PL
	CRH
Myometrium	GH
	Thyroid hormones
	PL
	CRH
Placental endothelium	Insulin (term)

the placenta, such as villous and extravillous trophoblast, also express receptors for GH, an important hormone in the physiological adjustment of gestation and control of maternal IGF-1 levels (Lacroix et al. 2002). These regions also express receptors for thyroid hormones, PL and CRH. Such hormones, respectively, regulate oxidative metabolism and energy available for gestation (Fowden and Forhead 2013; Leonard et al. 2001) and are necessary for normal production of progesterone during pregnancy (Hill et al. 1988; Freemark and Comer 1989).

Insulin receptors are expressed on the microvillous membrane of the syncytiotrophoblast in the first trimester of pregnancy, directed to the maternal circulation and, hence, maternal insulin. These receptors, at term, are mainly expressed on the placental endothelium directed to the foetal blood (Hiden et al. 2009). Insulin receptor expression suggests that, in the first trimester of pregnancy, maternal insulin regulates insulin-dependent processes, whereas, at term, it must be foetal insulin, the one that mainly controls these processes (Desoye et al. 1997). However, the IGF-1Rs are expressed in almost all placental tissues in the first trimester of pregnancy and at term (Fang et al. 1997). Nevertheless, IGF-1R expression is higher in the trophoblast than in endothelial cells at the end of gestation (Hiden et al. 2009; Abu-Amero et al. 1998). Such expression suggests that both maternal and foetal IGF-1 will affect the trophoblast compartment. Both hormones act as an

autocrine/paracrine factor in regulating early placental growth and function (Abu-Amero et al. 1998; Maruo et al. 1995). IGF-2 receptors are expressed in trophoblast and syncytiotrophoblast in first trimester of pregnancy and at term, respectively (Fang et al. 1997; Abu-Amero et al. 1998; Harris et al. 2011; McKinnon et al. 2001). Additionally, insulin and IGF-1 receptors are also expressed in resident macrophages and endothelial cells (Hiden et al. 2009). Therefore, dysregulation of insulin and IGFs may have important effects on placenta and foetus (Hiden et al. 2009), resulting in placental insufficiency and inadequate substrate supply to the developing foetus. These effects could lead to the appearance of intrauterine growth restriction (IUGR) (De Vrijer et al. 2006).

1.4 IGF-1-Deficient Conditions in Humans

As mentioned before, IGF-1 possesses a wide number of own properties (anabolic, antioxidant, anti-inflammatory and cytoprotective actions). Actually, the best-characterised conditions of IGF-1 are Laron's syndrome in children; liver cirrhosis in adults; aging, including age-related cardiovascular and neurological diseases; and, as discussed in this review, intrauterine growth restriction (IUGR).

Laron's syndrome or primary growth hormone insensitivity (GHI) was first described in 1966 by Zvi Laron et al., as a new type of dwarfism indistinguishable from genetic isolated GH deficiency. Such syndrome is characterised with unexpected high serum GH levels and the inability to synthesise IGF-1 and its binding proteins (Jameson 1999; Laron et al. 1966). Laron's syndrome was the first condition of IGF-1 described. Epidemiologically, this syndrome is closely related to an ethnic origin (>90% of cases). Clinically, patients with Laron's syndrome have growth abnormalities in uterus and in childhood; osteopenia; retardation in the maturation of dentition, organs and tissues; and a puberty delay, among other clinical manifestations (Rosenbloom 1999; Laron 1984). Animal models of GHI are available since 1997 and help us to better understand the pathophysiological changes and possible therapeutic strategies for these patients (Zhou et al. 1997).

Cirrhosis, a chronic liver disease, is characterised by low serum levels of IGF-1 and the presence of liver fibrosis, necrosis and regenerative nodules, leading to a loss of functional liver mass. The main causes are alcoholism, hepatitis B and C and fatty liver disease (Conchillo et al. 2007). Liver cirrhosis has been considered a condition of IGF-1 deficiency during adulthood, and IGF-1 has been proposed as a good indicator for functional hepatocellular capability (Caufriez et al. 1991). Nowadays, several animal models of experimental liver cirrhosis have been developed in order to better elucidate the role of IGF-1 in this pathology (García-Fernández et al. 2003; Muguerza et al. 2001; Castilla-Cortazar et al. 1997; Cemborain et al. 1998; Castilla-Cortázar et al. 2011).

Aging is a progressive, irreversible, universal and heterogeneous process of involution, characterised by a gradual loss of physiological functions that increases the probability of death. The circulating GH and IGF-1 levels progressively decline

with age (Perry 1999). Reduced GH/IGF-1 secretion in the elderly is responsible for several symptoms of aging, such as loss of muscle mass, increased adiposity, reduced bone mineral density and lower energy levels (Puche and Castilla-Cortázar 2012). Several pathologies, such as cardiovascular diseases, metabolic syndrome and neurodegenerative diseases, are correlated with aging and low circulating levels of IGF-1. Our group has demonstrated that low doses of IGF-1 restored circulating IGF-1 levels. IGF-1 replacement therapy improves insulin resistance, lipid metabolism and mitochondrial protection in aging rats (Puche et al. 2008; García-Fernández et al. 2008). Thus, IGF-1 could become a potential beneficial therapeutic strategy by improving mitochondrial function, decreasing oxidative stress and preventing insulin resistance-related pathologies. Data from transgenic mice with liver-derived IGF-1 deficiency explains the possible role of IGF-1 in vasoprotection, cardioprotection, insulin resistance, angiogenesis and neurogenesis (Puche and Castilla-Cortázar 2012).

2 Intrauterine Growth Restriction (IUGR)

2.1 Introduction

Foetal growth is a complex process involving maternal, placental and foetal factors from genetic, environmental and nutritional nature. Intrauterine growth restriction (IUGR) is an important obstetric issue defined as the inability to achieve the expected weight for gestational age (Collins et al. 2013). To define this pathology, it is really important to establish standardised curves of birth weight during foetal period and at term (Fig. 4) (Gómez-Gómez 2012). Growth-restricted foetuses/newborns are those born below the 10th percentile (weighing less than 2,500 g) according to each population (Goldenberg and Cliver 1997) and those who have an abdominal circumference less than 2.5th percentile (Valsamakis et al. 2006; Sferruzzi-Perri et al. 2006; Maulik 2006). IUGR is associated to perinatal mortality and morbidity (Kramer et al. 1990). Thereby, growth-restricted foetuses/newborns are characterised by an increased risk of clinical disorders in adult life, such as cardiovascular disease, diabetes and obesity (Hattersley and Tooke 1999; Bamfo and Odibo 2011).

There are two types of IUGR: symmetric intrauterine growth restriction, where all body parts of the baby are similarly small, and asymmetric intrauterine growth restriction, where baby's head and brain are normal, but the remaining parts of the body are smaller (Valsamakis et al. 2006).

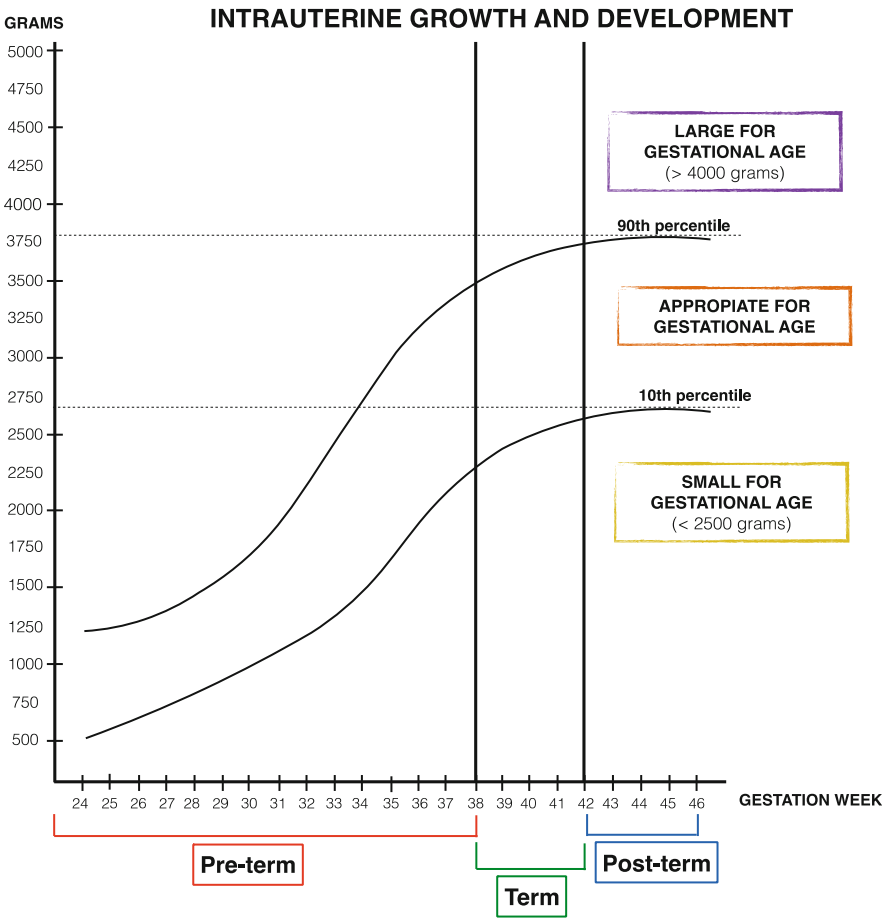

Fig. 4 Percentile curve to sort out newborns according to their weight for gestational age. Preterm babies born between 28th and 37th weeks of gestation. Term babies born between 37th and 42nd weeks of gestation. Post-term newborns born after 42nd week of gestation

2.2 Epidemiology

IUGR incidence varies according to the discrimination criteria adopted (Romo et al. 2009), but approximately 5–10% of newborns worldwide have intrauterine growth restriction (Resnik 2002). Moreover, it has been estimated that ≈20 million infants are born with low birth weight (<2,500 g) every year (WHO 2004; De Onis et al. 1998). There is a high variability depending on the geographic zone: in underdeveloped countries, IUGR affects ≈30% of pregnancies (Saleem et al. 2011), while in developed countries, it only affects ≈5% of pregnancies (Zepeda-Monreal et al. 2012; Baschat 2004; Hay et al. 2001). This variability could

Table 6 Worldwide distribution and prevalence of small for gestational age infants (shaded regions show the highest rate of small for gestational age infants)

Region		Prevalence (%)
Asia	Caucasus and Central Asia	12.9
	East Asia	5.3
	Southeast Asia	21.2
	South Asia	41.5
	West Asia	19.6
Oceania	Oceania	19.4
Africa	North Africa	8.5
	Sub-Saharan Africa	23.5
America	Latin America and the Caribbean	10.7
	Northern America	7.7
Europe	Eastern Europe	6.4
	Northern Europe	6.5
	Southern Europe	5.9
	Western Europe	6.7

be due to the higher prevalence of malnutrition and underweight at the beginning of gestation in the underdeveloped countries. In some studies, it was observed that the vast majority of small for gestational age (≈87%) and low-birth-weight babies (≈26%) were born in south Asia, southeast Asia and sub-Saharan Africa (Lee et al. 2013; Adair 1989; Isaranurug et al. 2007; Victora and Barros 2006; Victora et al. 2008; Santos et al. 2011; Gonzalez et al. 2006; Shah et al. 2008; Schmiegelow et al. 2012) (Table 6).

2.3 Physiopathology

IUGR has a multifactorial aetiology and is hard to define a specific cause. It is known that the pathology onset is due to factors of maternal, foetal and placental origin and an increase in oxidative stress (Bamfo and Odibo 2011). Moreover, different risk factors before and/or during pregnancy, as well as environmental and behavioural features, play a role in the development of the disease (Bamfo and Odibo 2011).

2.3.1 Maternal Factors

Maternal factors such as severe maternal malnutrition and underweight at the beginning of gestation and low weight gain during the gestation could be causes that promote IUGR (Mitchell et al. 2004). In addition, maternal characteristics such as age, height, nulliparity and multiparity and toxic habits (smoking, alcohol and

drug consumption, use of certain medicines, maternal stress) can increase the risk of IUGR. Alcohol crosses the placenta and could affect directly to foetal cell and tissue development and also can induce changes in mother–foetus hormonal interaction. Such changes can reschedule hypothalamus–pituitary–adrenal gland axis (HPA), leading to immunological, behavioural and cognitive deficits in the foetus (Zhang et al. 2005). The HPA axis has a key role in the implantation of the zygote and in endometrial, placental and foetal development, because it secretes several hormones such as GnRH (gonadotropin-releasing hormone), FSH (follicle-stimulating hormone) and LH (luteinising hormone) (Miller and Takahashi 2014).

Likewise, chronic maternal stress compromises normal regulation of hormonal activity during gestation, because it increases β-endorphin, glucocorticoids, catecholamines and CRH (corticotropin-releasing hormone) levels. An excess of the aforementioned hormones, in addition to an increase in cortisol levels, breaks through the placenta and can reduce foetal weight at birth. Catecholamines can also induce vasoconstriction of blood vessels causing placental hypoxia in the foetus. Hypoxia can activate HPA axis leading to an abnormal implantation of the zygote and an abnormal endometrial and placental development (Valsamakis et al. 2006; Weinstock 2005). Foetal responses to placental hypoxia include downregulation of insulin, IGF-1 and IGF-2 and increased expression of inhibitory IGFBPs (Han and Carter 2001), all of these leading to IUGR. Other risk factors that affect foetal growth could be inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematous and periodontal disease, as well as maternal vascular disease and thrombophilia. Such factors could lead to uteroplacental hypoperfusion, thus compromising foetal growth (Murphy et al. 2006; Bamfo and Odibo 2011).

Another important factor could be an increase of maternal oxidative stress. In studies with pregnant-IUGR women, an increase in oxidative stress has been observed (Biri et al. 2007). Also, these women are more susceptible to LDL (low-density lipoprotein) oxidation. LDL oxidation can lead to placental dysfunction and foetal growth retardation, as it decreases nutrient supply to the foetus (Sánchez-Vera et al. 2005). In the same way, it has been observed that in normal pregnancies, vitamin E levels (important for normal physiological function, because of its antioxidant actions) and prostacyclin levels (which have a vasodilatation action) increase progressively throughout pregnancy. On the other hand, thromboxane levels (implicated in vasoconstriction) decrease (Wang et al. 1991; Gagné et al. 2009).

Moreover, in animal models it has been observed that hypoxia induces a decrease of serum vitamin E levels and an increase in thromboxane production. These metabolic alterations would be responsible for an abnormal placental development and the decrease in steroid production. All these changes could lead to a foeto-placental vascular resistance and an increase of oxidative stress, which could be responsible for the appearance of IUGR (Parraguez et al. 2013; Majed and Khalil 2012; Sorem and Siler-Khodr 1997).

2.3.2 Foetal Factors

Foetal factors are less common. They include aneuploidies (trisomies of chromosomes 13, 18 and 21), which make up between 5 and 10% of IUGR cases; foetal malformations and congenital infections (rubella, cytomegalovirus, toxoplasmosis, etc.), which are responsible of 1.5% of IUGR cases; and inborn metabolic disorders (Bamfo and Odibo 2011).

2.3.3 Placental Factors

The placenta has two principal functions: it facilitates the exchange of nutrients, oxygen and waste products between mother and foetus and acts as an endocrine organ that integrates signals from the mother and foetus (Murphy et al. 2006). It has been estimated that the progenitor's genes account for only 20% of the variation of human birth weight. Nevertheless, the majority of the variation (62%) is due to the intrauterine environment. Thus, a suitable placental growth is essential for normal foetal development. For example, an adequate trophoblastic invasion is necessary. Trophoblastic tissue is metabolically active and produces hormones, absorbs nutrients and eliminates waste products (Bamfo and Odibo 2011). Therefore, anatomical abnormalities of the placenta, such as an abnormal insertion of the umbilical cord and placental thrombosis, decrease uteroplacental blood flow during pregnancy and consequently oxygen and nutrient transport (Murphy et al. 2006). Placentas from IUGR pregnancies have been shown to have poor invasion of the trophoblastic cells into the maternal decidual tissues, particularly the maternal spiral arteries (Setia and Sridhar 2009; Brosens et al. 2002). Studies looking into the pathological process of IUGR have pointed to an abnormal placental function as a common mechanism. However, it is known that the placental dysfunction is often gradual and it can occur much earlier than any demonstrable IUGR (Voigt and Becker 1992), making the resolution of this hypothesis difficult. Also, it was observed that approximately 20–30% of dichorionic twin pregnancies present IUGR, as they share placentas and such could lead to the appearance of stress in uteroplacental circulation, compromising development and growth of both foetuses (Bamfo and Odibo 2011).

The placenta, as a key organ for foetal growth, has a major role in amino acid transport, the most important nutrient for foetal life. During pregnancy, there is an active transport across the placenta from the maternal to the foetal circulation. The concentration of free amino acids in the placental tissue is higher than the concentration both in foetal and maternal plasma. In IUGR pregnancies, the concentrations of most essential amino acids (valine, leucine and isoleucine) decreased in foetal tissues but are significantly higher in maternal tissues. Such observation is a result of a maladaptation to pregnancy, suggesting the key role of amino acid transport. Several studies in animals showed a significantly reduced uptake of oxygen, glucose and essential amino acids in IUGR pregnancies. Also, studies in vitro in humans showed a reduced uptake of leucine and lysine, suggesting a reduced

activity of cationic amino acid transporters. Together, these data suggest the key role of amino acid transport in foetal development and its deficiency in IUGR pregnancies (Avagliano et al. 2012).

It is known that the placenta plays an important role in the production and transport of growth hormones that are critical for foetal growth and placental development (Murphy et al. 2006). It has been described that decreased levels of PL, which induces early embryonic growth and production of IGF-1 and insulin (Murphy et al. 2006), are associated with reduced foetal size. The same happens when oestradiol levels decrease, but in neither cases values are predictive, so the role of both hormones in the disease's pathophysiology is unknown (Markestad et al. 1997). In fact, detailed hormonal relationships of the mother–placenta–foetus unit are not known.

IGFs also control growth directly, where circulating IGF-1 appears to be virtually independent of foetal GH secretion (Randhawa and Cohen 2005). However, under this condition, placental GH may take this role as the prime regulator of maternal serum IGF-1 during pregnancy (Verhaeghe et al. 2000), being of particular interest the positive expression of IGF-1R in placenta (Reece et al. 1994) and the lower expression of placental-derived IGF-1 during IUGR (Koutsaki et al. 2011). In general, the endocrine milieu of the human foetus with growth retardation is also characterised by low circulating levels of insulin, IGF-1, IGF-2 and IGFBP-3 and high levels of GH and IGFBP-1 (Tzschoppe et al. 2015; Setia and Sridhar 2009; De Zegher et al. 1997). At this point, a study in zebrafish demonstrated that knockdown of IGFBP-1 significantly alleviated the hypoxia-induced growth retardation and developmental delay. Consistently, overexpression of IGFBP-1 caused growth and developmental retardation under normoxia conditions (Kajimura et al. 2005).

2.4 Clinic Course of IUGR

IUGR is the second most frequent cause of perinatal morbidity and mortality, only preceded by prematurity (Valsamakis et al. 2006). IUGR newborns could suffer numerous clinical disorders, such as hypoglycaemia, breathing difficulties that could cause neonatal asphyxia, hypothermia, ventricular haemorrhage and polycythaemia (Maulik 2006; Bamfo and Odibo 2011). All these clinical disorders can lead to consequences during early life, which could affect estatural and weight development and may also affect neurological development, resulting in behavioural anomalies, immature sleep patterns, diminution of visual fixation, decrease in overall activity, alteration of early mother–child interaction, alteration of motor skills and hyperactivity (Maulik 2006). It has been observed that children born small for gestational age had between 5 and 7 times increased risk to develop cerebral palsy, compared with those whose weight at birth was normal. It is still unknown whether this abnormal growth is the cause or the consequence of this disability (Jacobsson et al. 2008; Dahlseng et al. 2014).

In addition, newborns with IUGR had an increased risk during adulthood of suffering other clinical disorders, such as cardiovascular disease, insulin resistance, diabetes and hypertension, all of them related to metabolic syndrome (Valsamakis et al. 2006; Maulik 2006). As previously stated, kidney growth is under IGF-1 control; and a reduced IGF action, parallel to increased cortisol levels, results in a smaller number of glomeruli (Vehaskari et al. 2001). Alterations in the renin–angiotensin system are also frequent, probably downstream to activation of the HPA axis. These changes together with compensatory responses for the reduced kidney function probably account for the predisposition to adult hypertension (Vehaskari et al. 2001).

In the last years, a role for an altered GH/IGF axis in foetal programming in IUGR is being proposed, constituting the so-called thrifty phenotype hypothesis (Setia and Sridhar 2009), with an already proven inverse association between IGF-1 levels at 9 months and 17 years. Under this perspective, GH/IGF-1 axis may be programmed early in life. This foetal programming could be involved in, at least, two pathological conditions in later life, insulin resistance and hypertension. Firstly, children with IUGR show an impaired GH/IGF-1 axis, which might be contributing to reduced insulin sensitivity and IGF-1 resistance, as higher basal and GH-induced IGF-1 levels are required to achieve a growth velocity similar to that of other children. Secondarily, this alteration leads to a compensatory hyperinsulinemia to counteract insulin antagonistic effects of GH (Woods et al. 2002) and an impaired regulation of glucose transporter-4 expression by insulin in muscle and adipose tissue (Jaquet et al. 2001).

Moreover, some studies have shown that women who had given birth to newborns small for their gestational age (birth weight lower than 2,500 g) have an increased risk of mortality, due to cardiovascular alterations, such as ischaemic heart disease. This risk is 7 times higher than in women who had given birth to newborns normal for their gestational age (Smith et al. 2001).

2.5 Diagnosis

Diagnosis of IUGR is based upon clinical exploration and specific tests (Maulik 2006). When suspected, a complete medical history of the mother should be done, including the evaluation of risk factors such as medication use, recent infections, toxic exposure, smoking, alcoholism or drug consumption (Maulik 2006). The medical history must be completed with physical exploration of abdominal circumference size and uterine fundal height (Maulik 2006). If still suspected, an umbilical uterine arterial Doppler could be performed in order to establish the diagnosis, which allows to detect placenta insufficiency (Gheita et al. 2011). Ultrasound biometry allows to obtain parameters about foetal development such as foetal abdominal circumference, foetal head circumference and foetal femur length (Bamfo and Odibo 2011; Gheita et al. 2011).

However, at present, a suspected diagnosis of IUGR is made based on diverse criteria established by Gardosi, who defined personalised growth charts that improve detection of IUGR and help to distinguish slow growth foetuses (Chard et al. 1992). Deter et al. (1992) also established diverse criteria used to detect growth anomalies: prenatal growth assessment score (PGAS) and neonatal growth assessment score (NGAS) (Deter et al. 1992).

3 Conclusions and Perspectives

Intrauterine growth restriction (IUGR) is a relevant obstetric pathology. This disease is considered the second most frequent cause of perinatal morbidity and mortality, only preceded by prematurity, having a multifactorial aetiology. In recent years the understanding and characterisation of the pathophysiology and specific causes of the disease has become essential in order to reach a useful and successful therapeutic strategy.

Insulin-like growth factor 1 (IGF-1) is an anabolic hormone with a major role in foetal and placental growth and development. IGF-1 is produced by almost every tissue, including the placenta. The placenta is a metabolically active intrauterine organ. It secretes several hormones (IGF-1, IGF-2, GH, PL, etc.) and facilitates the exchange of nutrients, oxygen and waste products between mother and foetus. It is why the placenta plays an important role in foetal and embryonic development. Hence, suitable placental growth is essential for normal intrauterine development.

Several studies in IGF-1-deficient animals showed the key role of IGF-1 in foetal growth, liver cirrhosis, aging, vasoprotection, cardioprotection, insulin resistance, angiogenesis and neurogenesis. Also, several studies in humans have revealed that IUGR infants have low circulating levels of insulin, IGF-1 and IGF-2 and an abnormal placental function. Together, these data postulate that the mere IGF-1 deficiency in the gestational state may produce serious intrauterine growth retardation. Thus, it can be established that IGF-1 low levels could compromise oxygen and nutrient transport across the placenta, producing an abnormal placental growth and environment, leading to an abnormal foetal growth and development. Therefore, IUGR could be considered as a novel condition of IGF-1 deficiency, where replacement therapy at low doses with this hormone could be a beneficial and useful therapeutic strategy.

Our group has demonstrated that low doses of IGF-1 in IGF-1-deficient animals can restore physiological IGF-1 levels and improve insulin resistance, lipid metabolism and mitochondrial protection. Low doses of IGF-1 in these animals can have several beneficial hepatoprotective, neuroprotective, antioxidant and antifibrogenic effects. In consequence, treatment of IUGR, a novel condition of IGF-1, with low doses of IGF-1 prior to birth, where the foetus and placenta are growing and developing, could be beneficial, restoring circulating IGF-1 levels, and could improve the characteristics of the pathology.

Our perspective is to design and appropriate IGF-1-deficient mouse model to determine the pathophysiology of IUGR and to observe if low doses of IGF-1 during pregnancy could restore IGF-1 levels in both mother and foetus and if the administration of such hormone could improve foetal growth. Also, our perspective is to design a multicentric study between several hospitals in Monterrey (Nuevo Leon, Mexico) where we would try to administrate low doses of IGF-1 to pregnant mothers with possible IUGR and see how this treatment would affect both mother and foetus.

Acknowledgements The authors would like to express our gratitude to MCs Gabriel Amador Aguirre, Ing. Karl Steinmetz, Dr. Mariano García-Magariño Alonso, Dr. Luis Espinosa Sierra and Joshua S. Williams for their invaluable help. This work was possible thanks to the financial help of "Fundación de Investigación HM Hospitales" and "Tecnológico de Monterrey".

Conflict of Interest The authors declare that they have no conflict of interest.

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Rev Physiol Biochem Pharmacol (2016) 170: 37–66
DOI: 10.1007/112_2015_5002
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Published online: 9 February 2016

CFTR: A New Horizon in the Pathomechanism and Treatment of Pancreatitis

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Conflict of Interest Statement: The authors have no conflict of interest to declare.

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Abstract Cystic fibrosis transmembrane conductance regulator (CFTR) is an ion channel that conducts chloride and bicarbonate ions across epithelial cell membranes. Mutations in the *CFTR* gene diminish the ion channel function and lead to impaired epithelial fluid transport in multiple organs such as the lung and the pancreas resulting in cystic fibrosis. Heterozygous carriers of *CFTR* mutations do not develop cystic fibrosis but exhibit increased risk for pancreatitis and associated pancreatic damage characterized by elevated mucus levels, fibrosis, and cyst formation. Importantly, recent studies demonstrated that pancreatitis causing insults, such as alcohol, smoking, or bile acids, strongly inhibit CFTR function. Furthermore, human studies showed reduced levels of CFTR expression and function in all forms of pancreatitis. These findings indicate that impairment of CFTR is critical in the development of pancreatitis; therefore, correcting CFTR function could be the first specific therapy in pancreatitis. In this review, we summarize recent advances in the field and discuss new possibilities for the treatment of pancreatitis.

Keywords CFTR • Cystic fibrosis • Epithelial transport • Pancreas • Pancreatitis

Contents

1 Basics of CFTR

1.1 Biosynthesis and Degradation

The cystic fibrosis transmembrane conductance regulator (CFTR) protein is a cyclic AMP (cAMP)-regulated chloride (Cl^-)/bicarbonate (HCO_3^-) channel, expressed in the apical plasma membrane (PM) of secretory epithelia in the airways, pancreas, intestine, reproductive organs, and exocrine glands (Riordan 2008). CFTR consists of two homologous halves, each containing a hexa-helical membrane-spanning

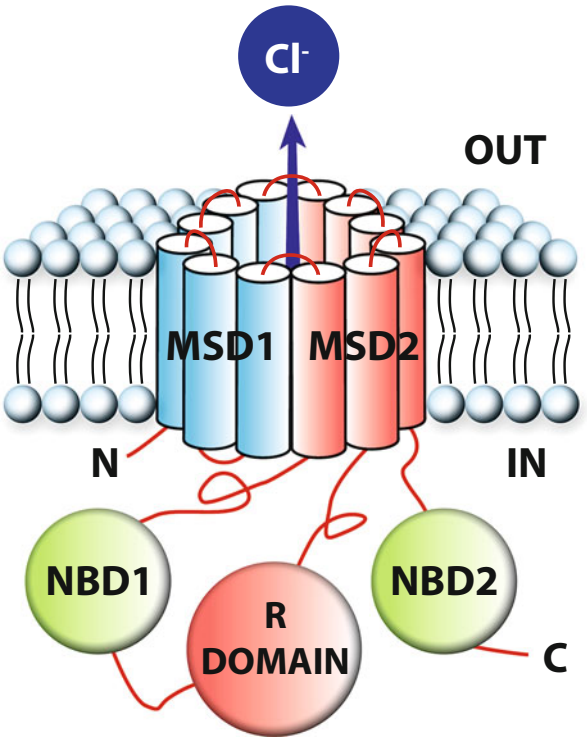

Fig. 1 Schematic structure of CFTR. The CFTR Cl^- channel consists of two homologous halves, each containing a hexa-helical membrane-spanning domain (MSD1 and MSD2) and a nucleotide-binding domain (NBD1 and NBD2). The two halves are connected by the R domain

domain (MSD1 and MSD2) and a nucleotide-binding domain (NBD1 and NBD2) (Fig. 1). The two halves are connected by the R domain (Riordan 2005). The NBDs contain conserved ATP-binding sequences: Walker A and B motifs, classifying CFTR as a member of the ATP-binding cassette (ABC) transporter family. Structural, biochemical, and functional evidence suggest that the two NBD domains interact and the ATP-binding site of one NBD is complemented by the ABC signature motif of the other (Riordan 2005).

While NBD1 folds largely cotranslationally, the native fold of NBD2 as well as CFTR is attained posttranslationally (Lukacs and Verkman 2012). Assembly of MSD1, NBD1, R domain, and MSD2 is necessary and sufficient to form the minimal folding unit of CFTR (Du and Lukacs 2009). These and other observations support the cooperative domain folding model and ensure the dynamic conformational coupling between the cytosolic NBDs and the pore-forming MSDs in the native molecule and provide a structural explanation for the cooperative domain *unfolding*, caused by cystic fibrosis (CF) mutations (Du and Lukacs 2009).

Despite interactions with several cytosolic and endoplasmic reticulum (ER) chaperones (heat shock proteins Hsp70 and Hsp90; co-chaperones, Hdj2 [DNAJ1], HsBp1, Hop, and p23; small Hsps, calnexin, and calreticulin), only 30–60% of the newly synthesized nascent CFTR attains folded conformation, presumably due to the metastable nature of NBD1 and NBD2, the slow kinetics of domain assembly, and the highly efficient ER quality control (Kim and Skach

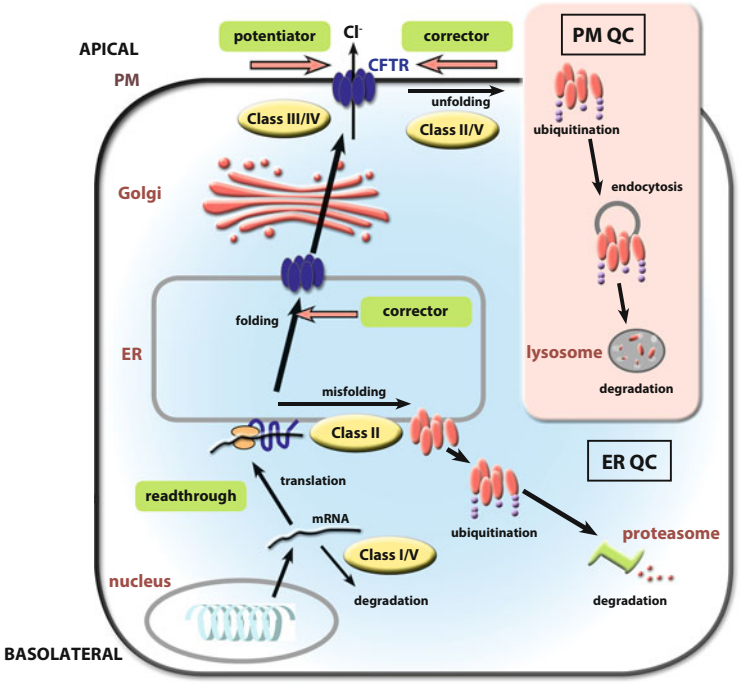

Fig. 2 Biosynthesis and maturation of CFTR. Only a fraction of newly synthesized and cotranslationally core-glycosylated channels undergoes conformational maturation in the endoplasmic reticulum (ER). The natively folded channel is then transferred by vesicular transport to the *cis*/*medial* golgi, where CFTR is complex-glycosylated before being delivered to the plasma membrane (PM). Unfolded CFTR, similar to other damaged proteins, is rapidly removed from the PM upon ubiquitination by the PM quality control (QC) system. CFTR ubiquitination accelerates internalization, impedes recycling, and facilitates preferential sorting toward lysosomal degradation. The six major classes of mutations are highlighted in *yellow* and treatment options in *green*

2012). The conformational maturation of CFTR is promoted by cytosolic ATP and the conjugation of *N*-glycan chains at the ER (Lukacs and Verkman 2012). Approximately 40–70% of the synthesized core-glycosylated channels are eliminated in the ER by the ubiquitin-proteasome system (UPS). The native CFTR that bypasses multiple checkpoints in the ER is packaged into COPII-coated transport vesicles and undergoes complex glycosylation in the *medial* Golgi before being delivered to the PM (Fig. 2) (Farinha et al. 2013).

At the PM, CFTR is constitutively internalized followed by efficient recycling from the endosomes back to the PM, which renders the channel metabolically stable (half-life [$T_{1/2}$] of ~12–14 h). In contrast, the most common mutation, p.F508del, has severalfold accelerated PM turnover ($T_{1/2} \sim 2$ h) relative to its wild-type counterpart similar to other conformationally damaged PM proteins (Okiyoneda et al. 2011). Damaged PM proteins are usually removed following their ubiquitination that confers accelerated internalization, impeded recycling, and

preferential sorting toward lysosomal degradation (Okiyoneda et al. 2011). Thus, it is plausible to assume that wild-type CFTR harboring various structural defects due to cellular stress and aging, similarly to that of the p.F508del mutant, is recognized by components of the protein homeostasis (proteostasis) machinery and is targeted by a ubiquitin- and ESCRT-dependent mechanism for lysosomal proteolysis. Multiple E3 ubiquitin ligases (CHIP, gp78, NEDD4, and c-Cbl) in cooperation with chaperones/co-chaperones (Hsc70, Hsp90, and co-chaperones, e.g., Hdj2 [DNAJ1], Bag1, HOP, and Aha1) have been implicated in the removal of damaged p.F508del and wild-type CFTR from the PM (Apaja and Lukacs 2014). Supporting the ubiquitin-dependent degradation of wild-type CFTR, the deubiquitinating enzyme (DUB) Usp10 stabilizes the channel at the PM by facilitating its recycling (Bomberger et al. 2009).

Presently, six major classes of mutations are distinguished based on their cellular pathophysiology (Fig. 2) (Zielenski 2000). Severely reduced cell surface CFTR expression is associated with (a) class I mutations which include frameshift, splicing, or nonsense mutations that introduce premature termination codons; (b) class II mutations, which lead to misfolding and impaired protein biogenesis at the ER; (c) class V mutations which result in reduced synthesis due to promoter or splicing abnormalities; and (d) class VI mutations that destabilize the channel in post-ER compartments and/or at the PM. CFTR function is selectively compromised by class III and IV mutations that can impair the gating and channel pore conductance, respectively.

1.2 Physiological Functions

In epithelial tissues, transport of anions by CFTR represents the rate-limiting step for anion (Cl^- and HCO_3^-) secretion, which ultimately controls transepithelial fluid secretion and hence hydration of the epithelial luminal surfaces (Fig. 3). In addition, through CFTR's ability to conduct HCO_3^-, as well as to regulate Cl^-/HCO_3^- exchangers belonging to the SLC26A family (Lee et al. 2012), CFTR also controls the pH of the secreted fluid. CFTR, therefore, controls both the amount and composition of epithelial secretions such as pancreatic juice, sweat, and airway surface liquid which play vital physiological roles in the innate defense of the lungs, digestion of foods, reproduction, and body temperature regulation.

A major interest in the channel stems from the fact that loss of function mutations in the gene encoding CFTR result in the inherited disease CF, one of the most common, life-shortening genetic diseases in the Caucasian population. At the other end of the spectrum and affecting far more people globally, overactive CFTR causes clinically important secretory diarrheas induced by toxins from pathogenic bacteria, such as *Vibrio cholerae*.

CFTR gating is regulated by the R domain which contains multiple PKA and PKC phosphorylation sites and which physically interacts with NBD1 (Baker et al. 2007; Gadsby et al. 2006; Hwang and Sheppard 2009; Chong et al. 2013)

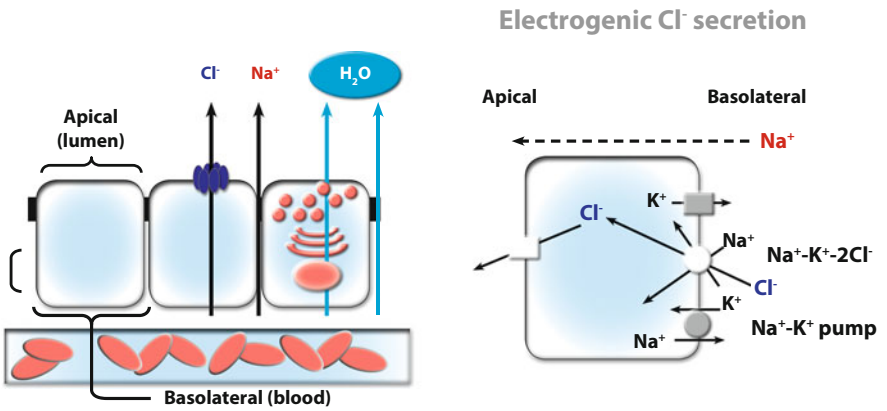

Fig. 3 Mechanism of transepithelial fluid secretion in the gastrointestinal tract. In epithelial tissues CFTR is usually expressed on the apical PM mediating the rate-limiting step for anion (chloride and bicarbonate) secretion, which ultimately controls transepithelial fluid secretion and hence hydration of the luminal surface. During electrogenic Cl^- secretion, Cl^- is transported into the cells via the basolateral membrane by the $Na^+/K^+/2Cl^-$ cotransporter that is followed by the Cl^- transport through the apical membrane via the CFTR channel

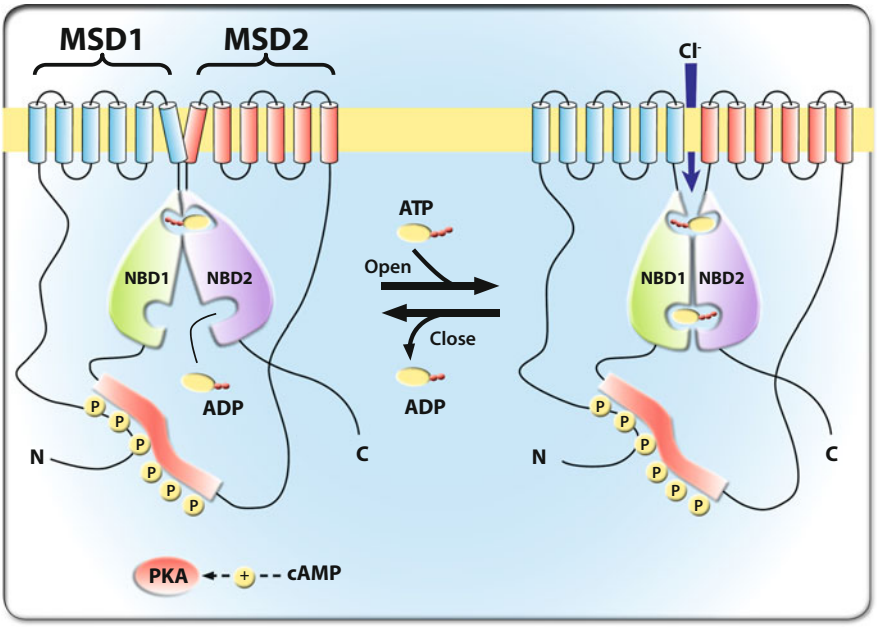

Fig. 4 Regulation of CFTR gating. CFTR gating is regulated by the R domain which contains multiple PKA and PKC phosphorylation sites and which physically interacts with NBD1. CFTR uses the energy of ATP binding and hydrolysis to drive ligand-induced conformational changes in the protein that lead to the regulated opening and closing (gating) of the channel "pore"

(Fig. 4). Uniquely, CFTR uses the energy of ATP binding and hydrolysis to drive ligand-induced conformational changes in the protein that lead to the regulated opening and closing (gating) of the channel "pore." In this way, CFTR gating tightly "regulates" the flow of anions in and out of the cell, with the anion being transported down its prevailing electrochemical gradient by a purely facilitative diffusional process.

For the channel to open, it must be first phosphorylated by PKA, and then ATP must also bind to CFTR's NBDs. Extensive biochemical, electrophysiological, structural, and molecular modeling studies support a scheme whereby following PKA phosphorylation of the R domain; CFTR gating is induced by ATP binding and dimerization of its two NBDs (Mense et al. 2006). This event is transmitted to the MSDs that form the "pore" of the channel through a complex and dynamic interaction between their cytoplasmic linker loops (coupling helices) and the dimerized NBDs (He et al. 2008). ATP (ligand) binding therefore drives the structural rearrangement of the transmembrane helices that result in the opening of the channel pore (Fig. 4). CFTR is also an ATPase (Cheung et al. 2008); hydrolysis of ATP and the release of ADP and P_i disrupt the NBD dimer thereby terminating channel opening. Further rounds of channel opening can continue if ATP binds again, but the gating cycle can be stopped by the removal of phosphate groups from the R domain by intracellular phosphatases (Gadsby et al. 2006; Hwang and Sheppard 2009). It is apparent then that CFTR gating is a complex process involving a variety of intracellular factors that regulate multiple inter- and intra-domain interactions within the protein.

1.3 Genetic Mutations

Genetic variations in *CFTR* that affect membrane levels or channel activity lead to various pancreatic phenotypes (Ooi and Durie 2012). CF with pancreatic insufficiency develops when both *CFTR* alleles harbor "severe" mutations resulting in loss of CFTR function. CF can also present with pancreatic sufficiency provided a severe mutation on one *CFTR* allele is compounded by a "mild" or "variable" mutation on the other allele, with some preservation of CFTR function. Whereas CF patients with pancreatic insufficiency do not develop acute pancreatitis, about 15–20% of pancreatic sufficient CF patients may present with acute or recurrent acute pancreatitis. The combination of a severe and a mild *CFTR* mutation can also result in idiopathic recurrent acute or chronic pancreatitis, and some of these patients exhibit abnormal sweat Cl^- or nasal potential difference (NPD) tests. Thus, a clear diagnostic line between CF-related pancreatitis and CFTR-related idiopathic chronic pancreatitis cannot be drawn. Finally, heterozygous carrier status for a CF-causing mutation increases the risk for recurrent acute and chronic pancreatitis. The discovery that *CFTR* variants are overrepresented in chronic pancreatitis was first published in 1998 (Sharer et al. 1998; Cohn et al. 1998) and was confirmed by a large number of follow-up studies (e.g. Weiss et al. 2005; Cohn

et al. 2005; Bishop et al. 2005; Noone et al. 2001; Ockenga et al. 2000; Truninger et al. 2001). However, many of these were limited by the use of small cohorts, the lack of screening unaffected controls, and incomplete analysis of the *CFTR* gene resulting in some controversy regarding the extent of disease risk, the role of non-CF-causing variants and the significance of common polymorphic variants, and their associated haplotypes. Recently published analyses of large German (660 cases), French (253 cases), and North-American (984 cases) cohorts (Masson et al. 2013; Rosendahl et al. 2013; LaRusch et al. 2014) confirmed earlier findings and strengthened the consensus on the role of *CFTR* variants in chronic pancreatitis.

Heterozygous carriers of the severe p.F508del *CFTR* mutations are overrepresented in patients with chronic pancreatitis (~7%) relative to healthy controls (~3%). Thus, CF carrier status confers a small risk for chronic pancreatitis (odds ratio ~2.5). Other types of severe mutations are less frequently found in chronic pancreatitis but seem to be associated with similar disease risk as p.F508del.

Heterozygous carriers of the mild *CFTR* mutations p.R117H are enriched among cases with chronic pancreatitis (~2.5%) relative to controls (~0.6%). Mutation p.R117H appears to cause comparable or even slightly higher risk (odds ratio ~4) than the severe p.F508del mutation, for reasons that are not readily apparent. With respect to the CF phenotype, the penetrance of p.R117H is influenced by the length of the poly-T tract in intron-8 (legacy numbering); however, such conclusion cannot be drawn for chronic pancreatitis on the basis of available data. Other mild or variable CF mutations are rare in chronic pancreatitis but are likely to confer similar risk as p.R117H.

Compound heterozygous genotypes composed of severe and mild *CFTR* alleles are strong risk factors for chronic pancreatitis and may be considered causative (Masson et al. 2013). The odds ratio of 16 described for a large German cohort is likely a conservative estimate for disease risk (Rosendahl et al. 2013). Due to some uncertainty which mutations can be considered mild, the reported frequency of compound heterozygotes in chronic pancreatitis cases has been highly variable. After exclusion of T5 allele carriers (see below), neutral mutations as given in the CFTR2 database (http://cftr2.org), and mutations of unknown clinical significance, the prevalence of compound heterozygotes was 1.4% in a German cohort (Rosendahl et al. 2013) and 2.4% in a French cohort (Masson et al. 2013). Compound heterozygosity for two mild *CFTR* mutations is rare and probably increases pancreatitis risk strongly, although solid data are lacking.

Non-CF-causing *CFTR* variants are not overrepresented in chronic pancreatitis cases. A recent study suggested that some non-CF-causing variants (e.g., p.R75Q, p.L997F) may be risk factors for chronic pancreatitis due to a selective defect in HCO_3^- conductance (LaRusch et al. 2014). While functional measurements seem to support this contention, a genetic association between these variants and chronic pancreatitis cannot be confirmed (Masson et al. 2013; Rosendahl et al. 2013; LaRusch et al. 2014; Martinez et al. 2014).

Common polymorphic *CFTR* alleles do not modify pancreatitis risk. There are two adjacent polymorphic regions (poly-T and poly-TG tracts) in intron-8 which can alter inclusion of exon-9 (legacy numbering) during splicing (Chu et al. 1993;

Cuppens et al. 1998; Groman et al. 2004). A short poly-T tract (c.1210-12T[5], legacy name T5) in combination with a longer poly-TG tract (c.1210-34TG[12], legacy name TG12) results in more transcripts lacking exon-9 that give rise to inactive CFTR protein. A T5 allele combined with a severe *CFTR* mutation may cause CF and increases the penetrance of the mild p.R117H mutation. Studies on smaller cohorts reported enrichment of the T5 allele in chronic pancreatitis (Weiss et al. 2005; Bishop et al. 2005; Noone et al. 2001; Arduino et al. 1999); however, more recent analyses on larger cohorts did not find such overrepresentation (Rosendahl et al. 2013; LaRusch et al. 2014). When analyzed separately, the distribution of the TG12 allele between chronic pancreatitis cases and controls was also equal (Rosendahl et al. 2013). Whether or not the T5-TG12 complex allele confers a small risk in chronic pancreatitis remains to be determined. Curiously, small but measurable differences were reported for the distribution of the TG10 and TG11 alleles between chronic pancreatitis cases and controls, either analyzed separately or as part of conserved haplotypes (Rosendahl et al. 2013; Steiner et al. 2011). In two studies, TG10 appeared to increase disease risk slightly, while TG11 seemed to be protective. Since the two studies used overlapping cohorts and the results clearly contradict the expected functional effects of the TG10 and TG11 tracts, cautious interpretation of the findings is warranted until independent confirmation (Table 1).

Chronic pancreatitis is a complex genetic disease, and mutations in different risk genes are often found in cases. Transheterozygosity of *CFTR* variants with mutations in *PRSS1*, *SPINK1*, and *CTRC* was reported (Weiss et al. 2005; Noone et al. 2001; Rosendahl et al. 2013; LaRusch et al. 2014; Masson et al. 2013). The combined effects of multiple pathogenic mutations result in strongly amplified disease risk, best documented for the co-occurrence of *SPINK1* and *CFTR* mutations. There is no compelling evidence for epistasis between *SPINK1* and *CFTR* mutations; the two susceptibility genes seem to act independently in determining the pancreatitis phenotype (Rosendahl et al. 2013). *CFTR* mutations can also contribute to the increased clinical penetrance of anatomical risk factors for chronic pancreatitis such as pancreas divisum (Bertin et al. 2012).

Table 1 The effect of CFTR mutations on the risk for chronic pancreatitis

CFTR alleles	Pancreatitis risk	Examples
Severe/mild	High risk, causative	p.F508del/p.R117H
Mild/mild	Likely high risk	p.R117H/p.R117H
Severe/–	Low risk, 2.5-fold	p.F508del/–
Mild/–	Low risk, 4-fold	p.R117H/–
Non-CF causing	No risk	p.R75Q, p.L997F
T5	No risk	
TG12	No risk	
T5-TG12	Limited data	

2 Pancreatic Damage in CF

2.1 Experimental Observations in CF Models

A CF animal model that recapitulates the human findings provides a powerful tool to study disease pathophysiology and design therapies. There are five main CF animal models with pancreatic manifestations (Table 2).

2.1.1 Mice

CF mouse models were generated by disrupting the endogenous *Cftr* gene in embryonic stem cells using the homologous recombination technology (Snouwaert et al. 1992; Clarke et al. 1992). In general, intestinal disease is the hallmark of CF mouse models with minimal or no pathological changes in the pancreas. The lack of pancreatic disease may be due to developmental/structural differences in the rodent pancreas, relatively low CFTR expression with higher expression of alternative anion channels, modifier genes, and/or the short life-span of CF mice (Gray et al. 1994; Olivier et al. 2015). This model is an important tool to study the intestinal disease in CF without the confounding effects of pancreatic disease.

2.1.2 Rats

Recently, a CF rat model was generated using zinc finger endonuclease technology (Tuggle et al. 2014). Animals demonstrated CFTR channel dysfunction in airway epithelia, histological abnormalities in the ileum, and absence of vas deference, all characteristics of CF. The CF rats did not have any evidence of pancreatic involvement within the first 6 weeks of life.

2.1.3 Pigs

The CF porcine model was generated by disrupting the *CFTR* gene in pig fibroblasts followed by somatic cell nuclear transfer (Rogers et al. 2008). CF pigs have multisystem disease that recapitulates the human disease and exhibit a severe pancreatic phenotype. The pancreatic lesions start in utero and progress over time in this model (Abu-El-Haija et al. 2012), in concordance with the previous autopsy studies of humans with CF (Andersen 1938). The newborn CF pig pancreas has acinar cell loss, duct proliferation, dilated acini/ducts with eosinophilic material, expansion of interlobular connective tissue, and scattered inflammatory cell aggregates (Rogers et al. 2008; Abu-El-Haija et al. 2012). Mucous cell metaplasia is a late finding. In older animals, the exocrine pancreatic lesions progress and the pancreas is mostly replaced by fat and fibrosis. The islets are morphologically

Table 2 Characteristics of pancreatic lesions in CF animal models and humans

	In utero	Birth	Juvenile/adult
Mouse	Unknown	No or minimal disease; high mortality at weaning if intestinal obstruction is left untreated	No or minimal disease
Rat	Unknown	Unknown but probably normal	Normal pancreas histology at 22–44 days of age
Pig	Inflammation, dilated acini/ducts with eosinophilic periodic acid-Schiff (PAS)-positive material, acinar cell loss	Acinar atrophy progresses; acini/ducts are filled with eosinophilic material; duct cell proliferation, mucus cell metaplasia, increased loose connective tissue; normal islet morphology but abnormal glycemic response and decreased insulin secretion; high mortality from meconium ileus and intestinal obstruction/perforation, ameliorated in gut-corrected animals	Acinar cell loss, dilated ducts with inspissated material, duct cell proliferation, mucus cell metaplasia, fatty infiltration and fibrosis; normal islet morphology but animals develop spontaneous hyperglycemia
Ferret	Unknown	Minimal disease; dilation of most acini and ductules with inspissated, eosinophilic secretions, no inflammation; high mortality from intestinal disease, gut-corrected CF ferrets are available	Rapid destruction of the pancreas with progressive inflammation, acinar atrophy, fibrosis, islet cell loss
Zebrafish	Not applicable	Acinar cell loss starts at 14–16 days postfertilization (dpf), neutrophil infiltration is present and seems to have an impact on acinar damage, high mortality	Acinar atrophy, duct dilatation with PAS-positive material, fibrosis; disorganized islets appearing smaller and more numerous than those in wild type
Humans	Pancreatic phenotype correlates with the severity of *CFTR* mutation. First lesions identified ~ 17 weeks of gestation as deposits of PAS-positive material within some acini and ductules. Pancreatic lesions progress to include degeneration of acinar	Depending on the severity of *CFTR* mutation, pancreatic lesions progress to acinar atrophy, fatty infiltration, and fibrosis	Depending on the severity of *CFTR* mutation, pancreatic lesions progress to acinar atrophy, fatty infiltration, and fibrosis. Partial islet cell loss is a late finding. With increased age, CF-related diabetes develops. Life expectancy is ~37 years

(continued)

Table 2 (continued)

	In utero	Birth	Juvenile/adult
	cells and lack of zymogen granules, dilatation of acini and ducts filled with inspissated PAS-positive material and ductular proliferation. Inflammation is mild to moderate. Islets are normal morphologically		

intact but functionally abnormal (Uc et al. 2015). As in humans (Kopelman et al. 1985), the pancreatic fluid is acidic, low in volume and high in protein, and concentrated in CF pigs at birth (Uc et al. 2012). The proinflammatory, complement cascade, proapoptotic, and profibrotic pathways are activated in CF pig pancreas and likely contribute to the destructive process (Abu-El-Haija et al. 2012).

2.1.4 Ferrets

CF ferrets were generated using homologous recombination as described for CF pigs. The pancreatic lesions are mild in CF ferrets at birth compared to CF pigs (dilation of most acini and ductules with inspissated, eosinophilic secretions). Interestingly, the exocrine pancreas undergoes rapid destruction over the first month of life (Sun et al. 2010), leading to acinar loss, fibrosis, and pancreatic insufficiency. Even though CF ferrets have only mild exocrine pancreatic disease at birth, they still manifest significant problems in glucose tolerance and insulin secretion (Olivier et al. 2012). Fifteen percent of ferrets are pancreatic sufficient and have normal pancreas, suggesting a possible role of genetic modifiers. The CF ferret model is emerging as an important tool to investigate the early pathogenesis of CF-related diabetes.

2.1.5 Zebrafish

Recently a zebrafish model of CF has been described (Navis and Bagnat 2015). The development of the pancreas is normal initially, followed by rapid destruction during larval life via a process that involves neutrophil infiltration. The zebrafish model along with pig and ferret models underlines the importance of inflammation in pancreatic damage.

Phenotypic differences among the various CF models may be due to species-specific CFTR processing, genetic and environmental influences, and effects of

other ion channels. These animal models offer a unique opportunity to study the pathogenesis of CF pancreatic disease and various therapeutic approaches.

2.2 Clinical Characterization of Pancreatic Function in CF

The consequences of mutations in the *CFTR* gene have been demonstrated by pancreatic function studies which show that CF patients have low-flow secretions with a high protein concentration, which can precipitate in the duct lumina causing obstruction and damage. These changes begin in utero, and after delivery the process of small duct obstruction leading to large duct obstruction continues. At birth and for several months afterward, there is a release into the blood stream of proteins originating in the pancreas. An example of this is immunoreactive trypsinogen (IRT) which serves as the basis for the neonatal screening test for CF. Interestingly, despite the wholesale destruction of the exocrine pancreas occurring, the infant is asymptomatic; the reason for this is yet to be determined. Eventually, this process results in severe inflammatory changes, obstruction of ducts by mucus and calcium-containing debris, destruction of acini, and generalized fibrosis. Contrary to popular belief that the pancreas is entirely nonfunctioning at birth, the high IRT does show that some exocrine pancreatic tissue is still present and this may have a bearing on possible small molecule therapy targeted at the remainder of the pancreas which may rescue enough tissue to cause viability of the remaining pancreas.

One of the most remarkable observations is that genetic factors exquisitely influence the degree of pancreatic disease and its rate of progression. Large studies of CF patients resulted in their classification as pancreatic insufficient (PI) or pancreatic sufficient (PS). PI patients comprise of 85% of all CF patients and have maldigestion as defined by evidence of steatorrhea following 72-h fat balance studies; these patients require pancreatic enzyme replacement therapy with meals. In contrast, PS patients have evidence of pancreatic damage (as shown by the high neonatal IRT test) but retain sufficient endogenous exocrine pancreatic function to sustain normal digestion.

Exocrine pancreatic status is directly linked to genotype (Kristidis et al. 1992). Analysis of particular CFTR mutations in patients with these pancreatic phenotypes (PI vs. PS) revealed two categories of alleles: “severe” and “mild.” Patients who are homozygous or compound heterozygous for severe alleles belonging to classes I, II, III, or VI exhibit pancreatic insufficiency, whereas a mild class IV or V allele sustains pancreatic function in a dominant fashion, even if the second mutation is severe. This observation finds plausible explanation since all known mild alleles belong to class IV or class V, all of which are (or predicted to be) associated with some residual channel activity at the epithelial apical membranes. However, this classification system is not entirely consistent as there are some class I mutations with a stop codon at the end of the gene which are in fact PS. A small proportion (2–3%) of patients carrying severe mutations on both alleles are PS at diagnosis, but

most experience gradual transition from PS to PI. A few missense mutations (e.g., p.G85E) confer a variable pancreatic phenotype.

While mild mutations confer sufficient CFTR function to prevent complete pancreas destruction, many PS patients have reduced exocrine pancreatic capacity and are at an increased risk of pancreatitis. Recurrent acute and chronic pancreatitis is a relatively infrequent complication of CF first reported by Shwachman in 1975 (Shwachman et al. 1975). In this retrospective study, only 0.5% of CF patients had pancreatitis. More recently Durno et al. reported that in a cohort of over 1000 patients, followed over a period of 30 years, the incidence was 1.7% (Durno et al. 2002). All the patients with pancreatitis were PS. In fact, this subgroup of PS patients appears to be highly susceptible to pancreatitis, since almost one in five was affected by this complication. In the largest study to date of CF PS patients, Ooi et al. in a seminal paper determined the association between severity of CFTR genotype and the risk of pancreatitis (Ooi et al. 2011). They examined a large cohort of 277 PS patients from two CF centers of which 62 had well-documented pancreatitis. Using a novel pancreatic insufficiency prevalence score, the mutations were divided into three main groups, severe, moderate-severe, and mild. They found that the proportion of patients who developed pancreatitis was significantly greater for genotypes in the mild group than the moderate-severe group. Thus, the more mild mutations are associated with increased risk of pancreatitis.

The sweat Cl^- and NPD results in patients with pancreatitis ranged from the values for healthy controls and obligate heterozygotes to the values for CF patients with PS and PI. Median sweat Cl^- and NPD results in patients with no mutation or one mutation were clustered with values obtained in controls and obligate heterozygotes. In contrast, in patients with pancreatitis carrying *CFTR* mutations on both alleles, median ion transport values were intermediate between those of the controls and obligate heterozygotes and those of PS CF patients. Some individual values overlapped with the CF patients, and in 21% of patients, the diagnosis of CF could be confirmed by abnormal ion channel measurements. Thus, CFTR-mediated ion channel abnormalities are influenced by the number or severity of the *CFTR* mutations and show a range of abnormalities similar to those in patients with mild or severe classic CF at one extreme and controls and obligate CF heterozygotes on the other. This continuum of electrophysiological abnormalities is not surprising as PS patients have a 17% risk of developing pancreatitis and many of these presentations are in adulthood.

Similar observations have been made in individuals with other CF-like phenotypes, such as men with infertility due to congenital bilateral absence of the vas deferens, who are known to carry a high frequency of *CFTR* mutations (Wilschanski et al. 2006). A relatively large population was examined, and similar to the patients with idiopathic pancreatitis, a wide range of electrophysiological abnormalities was observed. Abnormalities of CFTR function correlated closely with the number and severity of *CFTR* mutations.

3 Effects of Pancreatitis-Inducing Factors on CFTR

The two most common causes of pancreatitis are alcohol abuse and biliary disease. Since only a small fraction of alcoholics and patients with biliary disease ever develops pancreatitis, it is evident that there is individual susceptibility. Other intrinsic (genetic) and extrinsic factors (e.g., smoking, diet) must also be responsible for the induction of the disease. Furthermore, the importance of nonoxidative ethanol metabolites such as fatty acid ethyl esters as well as cytotoxic fatty acids in acute pancreatitis is now being increasingly recognized (Huang et al. 2014).

3.1 Ethanol and Fatty Acids

The potential role of CFTR in pancreatitis is highlighted by the fact that genetic deletion of the apical Cl^- channel increased the severity of alcohol-induced acute pancreatitis in the mouse (Maléth et al. 2015). Furthermore, patients with alcoholic acute pancreatitis had lower levels of CFTR in their pancreatic ducts than control subjects (Maléth et al. 2015). Using numerous complimentary techniques, we demonstrated that ethanol and fatty acids dose-dependently reduced CFTR expression and activity in pancreatic ductal epithelial cells and inhibited secretion of fluid and HCO_3^- (Maléth et al. 2015; Judák et al. 2014). The oxidative ethanol metabolite acetaldehyde had no such effects. The decrease in CFTR expression and PM density in response to ethanol, palmitoleic acid, or palmitoleic acid ethyl ester administration was caused by accelerated channel turnover at the apical membrane and by aberrant protein folding (Maléth et al. 2015). The inhibition of CFTR by ethanol and fatty acids was associated with a sustained increase in concentrations of intracellular Ca^{2+} and cAMP, depolarization of mitochondrial membranes, and depletion of ATP. Supplementation with ATP almost completely prevented inhibition of CFTR activity by ethanol and fatty acids (Judák et al. 2014). These findings are in accord with those of Yamamoto et al. (Yamamoto et al. 2003), who found that 0.3–30-mM ethanol augmented, whereas 100-mM ethanol inhibited secretin-stimulated pancreatic ductal fluid secretion in the guinea pig. Notably, ethanol and fatty acids also evoke a rise in the cytosolic Ca^{2+} concentration and cause depolarization of the mitochondrial membrane and ATP depletion in pancreatic acinar cells, which served as the basis for the above described studies (Criddle et al. 2006; Criddle et al. 2004).

3.2 Bile Acids

Bile acids are probably the most important components of the bile which are predominantly synthesized in the liver. In humans, the most abundant primary

bile acids are chenodeoxycholate and cholate that are conjugated with taurine or glycine.

Pancreatic ductal HCO_3^- secretion is likely to be markedly reduced in patients with biliary acute pancreatitis (Takács et al. 2013). Furthermore, under experimental conditions, luminal administration of a low dose of chenodeoxycholate (0.1 mM) stimulated ductal HCO_3^- secretion (Venglovecz et al. 2008) which was caused by significantly elevated apical Cl^-/HCO_3^- exchange activity in guinea pig ducts (Venglovecz et al. 2008) and human CFPAC-1 pancreatic duct cells (Ignáth et al. 2009). This stimulatory effect was dependent on Ca^{2+} signaling and CFTR expression; however, it is unlikely to be the result of increased Cl^- conductance, since chenodeoxycholate administration did not activate CFTR Cl^- channel activity in guinea pig pancreatic duct cells. High concentrations (1 mM) of chenodeoxycholate administered from the basolateral or luminal membranes strongly inhibited pancreatic ductal HCO_3^- secretion by causing severe mitochondrial damage (Venglovecz et al. 2008; Maléth et al. 2011). The consequent ATP depletion will affect ATP-dependent transporters like the Na^+/K^+ pump, K^+ channels, and CFTR.

3.3 Smoking

Data on the effects of smoking on CFTR in the pancreas are scarce and contradictory. Pancreatic CFTR mRNA expression in rats exposed to chronic smoke inhalation was increased in animals that developed inflammatory signs upon cigarette smoke exposure compared with animals not showing morphological pancreatic damage (Wittel et al. 2006). In another study, it has been shown in human subjects that cigarette smoking impairs secretin-stimulated pancreatic ductal secretion which implicates the role of CFTR in the process (Kadiyala et al. 2013). Our preliminary data also support this notion since cigarette smoke extract dose-dependently inhibited pancreatic ductal CFTR activity and HCO_3^- secretion in guinea pigs. In fact, the latter results are in accord with those found in other organs such as the airway (Bagheri-Hanson et al. 2014) or intestinal epithelial cells (Raju et al. 2013). It seems that CFTR dysfunction due to smoking is primarily an acquired phenomenon and is not affected by the presence of heterozygous *CFTR* mutations (Raju et al. 2014). The interpretation of these results, at least with respect to the compounds responsible for the inhibitory effect of CFTR, is difficult as cigarette smoke is estimated to contain more than 7000 chemical components. Nevertheless, several constituents of cigarette smoke have been implicated in the inhibitory effects on CFTR such as acrolein (Raju et al. 2013), cadmium, and manganese (Hassan et al. 2014). Moreover, these effects were ameliorated by the use of various antioxidants like *N*-acetylcysteine (a known scavenger of acrolein) or alpha-tocopherol, suggesting that free radical-induced damage may be responsible for the inhibition of CFTR by cigarette smoke (Rab et al. 2013).

Interestingly, CFTR is also prominently involved in maintaining the antioxidant glutathione levels in the basal epithelial lining fluid which is also decreased by cigarette smoke (Gould et al. 2012). Thus, cigarette smoke may further reduce the antioxidant defense system in a vicious circle. Cigarette smoke exposure has also been reported to induce CFTR internalization and insolubility (Clunes et al. 2012). Taken together, these data strongly suggest that CFTR could be a therapeutic target in smoking-related diseases. In fact, drugs which increase CFTR function (the CFTR potentiator ivacaftor and the phosphodiesterase 4 inhibitor roflumilast) were found to have beneficial effects on cigarette smoke-induced Cl^- channel inhibition (Lambert et al. 2014; Sloane et al. 2012).

4 CFTR Dysfunction in Pancreatitis

4.1 Acute Pancreatitis

An association between CFTR and the pathogenesis of acute pancreatitis has been presumed for a long time. Impaired CFTR expression in *Cftr*$^{-/-}$ and p.F508del *Cftr* mice resulted in continuous overexpression of proinflammatory cytokine genes and more severe acute pancreatitis upon cerulein hyperstimulation (DiMagno et al. 2005, 2010). The *Cftr*$^{-/-}$ and p.F508del *Cftr* mice displayed elevated pancreatic edema, neutrophil infiltration, and increased mRNA expression of multiple inflammatory mediators. However, induction of acute pancreatitis in *Cftr*$^{-/-}$ mice did not cause acinar cell injury but rather decreased acinar cell apoptosis with mild exocrine pancreatic insufficiency (DiMagno et al. 2005; DiMagno et al. 2010). Although the authors focused on the alterations in acinar cells function, CFTR is expressed in the pancreatic duct, and thus, the increased severity of pancreatitis is most likely due to compromised ductal fluid and HCO_3^- secretion. Recently, we investigated the role of CFTR in the pathogenesis of acute alcohol-induced pancreatitis and found markedly reduced in vivo pancreatic fluid secretion in control and *Cftr*$^{-/-}$ mice treated with ethanol and fatty acids (Maléth et al. 2015). The *Cftr*$^{-/-}$ mice also displayed more severe acute pancreatitis induced by i.p. injection of ethanol and palmitic acid, including more extensive necrosis (Maléth et al. 2015). The role of CFTR in acute pancreatitis was further demonstrated in a study examining the scaffolding protein Na^+/H^+ exchanger regulatory factor-1 (NHERF-1) in ductal function (Pallagi et al. 2014). Deletion of *Nherf1* reduced expression of CFTR at the apical membrane of the duct and consequently reduced fluid and HCO_3^- secretion. Furthermore, it resulted in more severe acute pancreatitis induced by cerulein hyperstimulation or sodium taurocholate in mice (Pallagi et al. 2014). These findings have potential clinical relevance, since we detected markedly decreased CFTR protein and mRNA expression in small pancreatic ducts in tissue samples from patients diagnosed with alcohol-induced acute pancreatitis (Maléth et al. 2015).

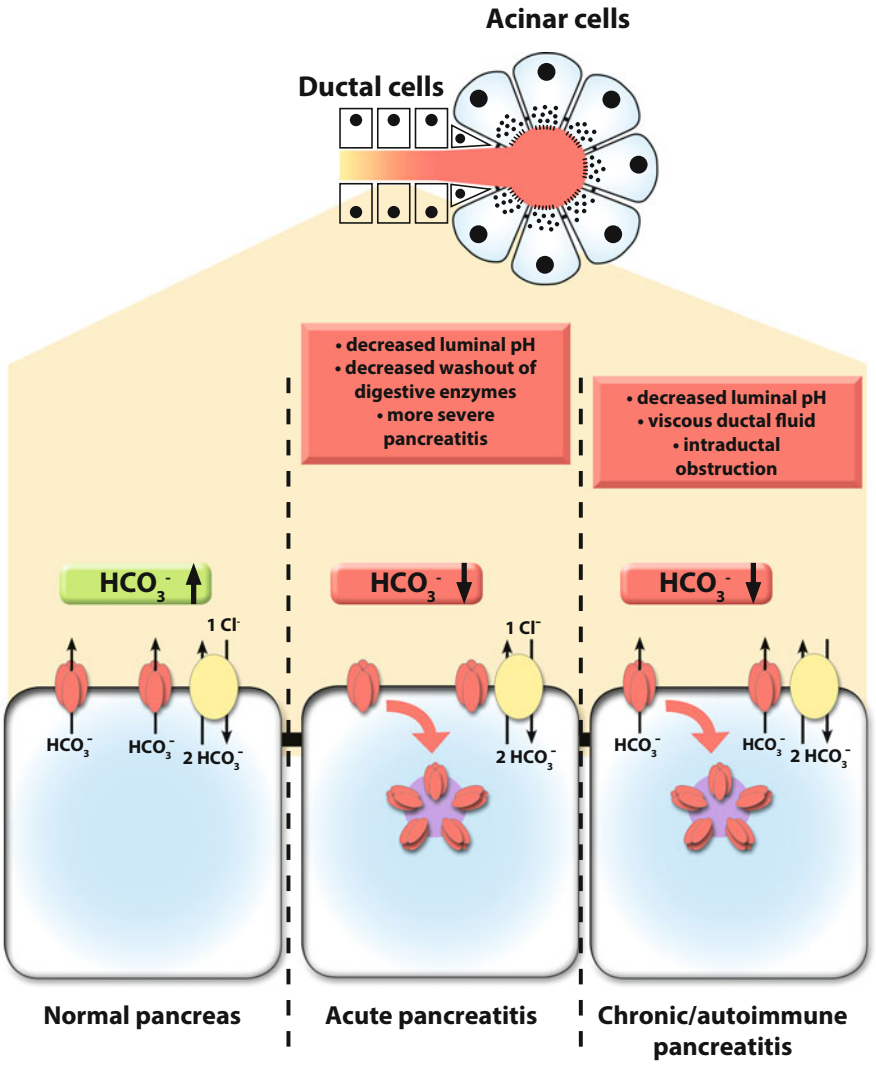

Fig. 5 CFTR dysfunction in pancreatitis. Under physiological conditions (*left*), CFTR (*red*) is expressed on the luminal membrane of small inter/intralobular pancreatic ducts with the SLC26A6 Cl^-/HCO_3^- exchanger (*yellow*). This expression and the close connection of the two proteins are required for the maintenance of the alkaline luminal pH. During acute pancreatitis (*middle*) the function of CFTR is inhibited, and its expression is decreased leading to impaired bicarbonate and fluid secretion and consequently decreased luminal pH. The washout of the activated digestive enzymes is insufficient. Overall, these changes together will increase the severity of acute pancreatitis. In chronic or autoimmune pancreatitis (*right*), CFTR expression is markedly decreased leading to the described consequences. The more viscous, protein-rich ductal fluid promotes the formation of intraluminal protein plugs. The intraductal obstruction will result in pancreatic atrophy and exocrine pancreatic insufficiency

Together, these observations suggest that diminished CFTR function, due to either reduced expression or activity, may have crucial importance in the pathogenesis of pancreatitis (Fig. 5).

4.2 Chronic Pancreatitis

CFTR dysfunction due to mislocalized protein expression in pancreatic ductal cells has been observed in alcoholic, obstructive, autoimmune, and idiopathic chronic pancreatitis (Maléth et al. 2015; Ko et al. 2010). In addition to the direct effects of toxic factors (see section III), indirect mechanisms such as inflammation and necrosis likely contribute to impaired CFTR expression. Importantly, the decreased expression of CFTR observed in chronic pancreatitis is likely the cause of impaired ductal function during pancreatitis (Ko et al. 2010). Aberrant expression of CFTR leads to diminished fluid and HCO_3^- secretion due to a reduction in the interrelated activity of both the electrogenic $1Cl^-/2HCO_3^-$ exchanger SLC26A6 and CFTR. This leads to decreased intraluminal pH, decreased washout of the digestive enzymes, and more viscous protein-rich ductal fluid (Ko et al. 2012) (Fig. 5). These changes promote the formation of intraluminal protein gel or plugs, which are early histological features of chronic pancreatitis (Sarles et al. 1965). Intraductal obstruction can lead to pancreatic atrophy, ductal mucinous hyperplasia (Allen-Mersh 1985; Hegyi and Petersen 2013), goblet cell metaplasia, and perhaps pancreatic stone formation (Ko et al. 2012).

5 Future Perspectives

5.1 Current Treatment Options

In the 26 years since the discovery of the gene that causes CF, our understanding of how mutations in *CFTR* cause the varied pathophysiological manifestations of this disease has increased substantially. This knowledge has led to the possibility of new therapeutic approaches aimed at the basic defect. This section will summarize the current state of mutation-specific therapy and will focus on orally bioavailable potentiators, correctors, and suppressors of *CFTR* gene mutations.

5.1.1 Gene Therapy

After the *CFTR* gene was discovered, there was considerable enthusiasm that gene therapy could be rapidly developed. Indeed, CF has had among the highest number of gene therapy trials. Several vector systems have been tested in human trials

including adenoviruses, adeno-associated viruses, and cationic lipids. Despite optimism from in vitro and initial in vivo studies, the trials have hitherto been unsuccessful. The most ambitious gene therapy study in the United Kingdom which randomized 140 patients using a cationic lipid-based vector showed a modest improvement in lung function (Alton et al. 2015).

5.1.2 Mutation Class-Specific Therapy

An alternative to gene therapy are compounds that affect synthesis, trafficking, and channel function of CFTR. The classification of *CFTR* mutations shown in Fig. 2 enabled researchers to consider targeting distinct groups of patients.

5.1.3 Class I Mutations

This class of mutations includes premature termination codons (PTCs) or nonsense codons. Nonsense mutations are responsible for about 10% of CF cases worldwide. However, in Israel nonsense mutations are the cause of CF in most patients (Kerem et al. 1997). Because such mutations produce little functional CFTR, these patients usually have a severe CF phenotype. The increased understanding of ribosomal function, the process of translation, and small molecules that change the interaction between the ribosome and mRNA have led to the identification of several agents that are capable of suppressing PTCs. This has resulted in a novel strategy to treat CF and other genetic disorders caused by PTCs by restoring full-length protein expression. Aminoglycoside antibiotics were the first drugs demonstrated to suppress PTCs in disease-causing mutations, allowing the translation of full-length proteins (Hermann 2007). In 1996, Howard et al. (Howard et al. 1996) described PTC suppression by the synthetic aminoglycoside Geneticin (G418) to restore function in HeLa cells expressing nonsense codons. This pivotal work was extended to four nonsense mutations of the *CFTR* gene that were expressed in the human airway cell line IB3-1. In this study the commonly used aminoglycoside, gentamicin, was incubated with these cells and full-length protein was produced (Bedwell et al. 1997). The preclinical studies mentioned above have led to a number of clinical trials designed to test both proof of principle and efficacy in patients with genetic diseases caused by PTCs (Table 3).

As stated earlier, about 60% of CF patients in Israel carry PTCs or class I mutations. Either nasal or intravenous administration of gentamicin improved NPD (Wilschanski et al. 2000, 2003; Clancy et al. 2001; Sermet-Gaudelus et al. 2007). In all of these studies, there was a variability of response with some patients not responding to gentamicin. Linde et al. (Linde et al. 2007) showed that this variability may be related to nonsense-mediated mRNA decay (NMD) – the major machinery evolved to protect against harmful products of nonsense mutations. This is a posttranscriptional translation-dependent surveillance mechanism that prevents the synthesis of proteins carrying PTCs. NMD has been shown to

Table 3 Clinical trials in CF (class I mutations)

	Type of administration	Type of study	Results	References
Gentamicin	Nose drops	Pilot	Significant improvement of NPD	Kerem et al. (1997)
		Double-blind, placebo-controlled	Significant improvements of NPD and chloride secretion compared with placebo. Positive immunofluorescent staining in the treatment group	Hermann (2007)
			Specific for patients with class I mutations with no effect in the control group of patients homozygous for the p.F508del mutation	
			Vast majority of patients with PTCs expressed at least one copy of the p.W1282X CFTR mutation	
	Intravenous	Pilot	Significant improvement of NPD	Howard et al. (1996)
		Pilot	Significant improvement of NPD in CF patients carrying the p.Y122X mutation	Bedwell et al. (1997)
Ataluren	Oral	Phase II clinical trial	Two consecutive 28-day cycles, each of 14 days of treatment followed by 14 days of washout.	Sermet-Gaudelus et al. (2007)
			Significant improvement of NPD	
			Modest but statistically significant improvements in lung function and bodyweight	
		Open-label extension study	NPD improvements were reported over time in both the higher- and lower-dose treatment groups including 4 patients who did not respond to ataluren in the 2-week study (see above)	Linde et al. (2007)
			Modest improvements in pulmonary function and a significant reduction in quantitative cough assessment	
		Phase II clinical trial	Significant improvement in NPD and nasal epithelial CFTR protein by immunofluorescence	Welch et al. (2007)
		Phase III clinical trial	Disappointing results, no difference in FEV_1 between the ataluren and placebo groups	Du et al. (2008)
			In patients not receiving chronic inhaled tobramycin: The mean pulmonary exacerbation rate was 40% lower in the ataluren arm	

(continued)

Table 3 (continued)

	Type of administration	Type of study	Results	References
			Similar difference in mean relative change from baseline in % predicted FEV_1 at week 48 was 5.7% favoring ataluren with a mean change from baseline of −0.7% in the ataluren arm, and 6.4% in the placebo arm was observed	
			In patients who received chronic inhaled tobramycin: No significant difference in mean relative change from baseline in % predicted FEV_1 at week 48 between ataluren and placebo	
			In patients who received other antibiotics (e.g., colistin and aztreonam): No modification in the treatment effect of ataluren	

degrade transcripts carrying disease-causing nonsense or frameshift mutations. It is the efficiency of NMD which affects the level of transcripts carrying PTCs, which govern the response to read-through treatment. Response to gentamicin was found only in patients with a higher level of transcripts (Linde et al. 2007). Downregulation of NMD in cells carrying the p.W1282X mutation increased the level of CFTR nonsense transcripts and enhanced the CFTR Cl^- channel activity in response to gentamicin. This may have a critical clinical correlation in the read-through of PTCs in various diseases. However, the inconvenience of parenteral administration and the potential for serious toxic effects preclude long-term systemic use of gentamicin for suppression of nonsense mutations.

Another PTC suppressor ataluren was developed through an extensive high-throughput screening program using a luciferase-based system (Welch et al. 2007). The molecule is a 1,2,4 oxadiazole benzoic acid and is reported to interact with mammalian ribosomes in a manner distinct from aminoglycosides. Ataluren does not have antibiotic activity and is orally bioavailable. Studies in myocytes isolated from the *mdx* mouse defined target doses and exposures to rescue dystrophin function. After treatment with ataluren, full-length dystrophin was localized in skeletal and cardiac tissue. In the p.G542X-hCFTR mouse oral and intraperitoneal administration led to detectable full-length CFTR localization at the apical cell membrane of intestinal glandular cells by immunofluorescent staining together with improved Cl^- conductance as assayed by transepithelial ion transport (Du et al. 2008). Correction of CFTR Cl^- transport was incomplete. Less than 30% of the short-circuit current that was observed in wild-type mice occurred in the

treated CF mice. This suggests that potential clinical benefit would only need partial restoration of protein function. Phase 2 clinical trials show clear improvement of NPDs (Kerem et al. 2008; Sermet-Gaudelus et al. 2010; Wilschanski et al. 2011); however, a phase 3 clinical trial showed no difference in FEV_1 between the ataluren and placebo group (Kerem et al. 2014) (Table 3). Although the primary outcome was not achieved, the findings are encouraging since it demonstrates a positive effect of disease-modifying therapy using a corrector of CFTR. A confirmatory phase 3 efficacy and safety trial of ataluren in CF patients not receiving chronic inhaled tobramycin (ACT CF trial) is ongoing (Kerem et al. 2014).

5.1.4 Class II Mutations

Class II mutations include the most common mutation, p.F508del. High-throughput screening assays have been used to screen drug libraries, and some compounds called correctors have shown promise in improving CFTR processing (Pedemonte et al. 2005; Varga et al. 2008). One drug, VX-809 (lumacaftor), an orally bioavailable p.F508del corrector, showed moderate improvement in lung function in a phase 2 trial in adults homozygous for p.F508del. This breakthrough may be enhanced by using these compounds with a potentiator for class II mutations like VX-809, the logic being that the CFTR will be trafficked to the apical surface of the cell and then activated by a potentiator.

The results of a combination therapy have recently been published in the *New England Journal of Medicine* (Wainwright et al. 2015). 1108 patients with CF who were homozygous p.F508del were randomized to lumacaftor, which corrects cellular misprocessing to increase the amount of functional mutated CFTR, with ivacaftor, which increases opening of the channel protein, or to placebo. Results at 24 weeks showed that patients treated with the combination therapy had a mean improvement of 2.6–4.0% in FEV_1, when compared with participants randomized to placebo ($P < 0.001$).

The rate of pulmonary exacerbations in the two trials was 30–39% lower among the patients given the combination treatment. This combination therapy has now been approved for CF patients homozygous for p.F508del.

5.1.5 Class III Mutations

Class III mutations require drugs that activate the protein, which is inactive but properly targeted. These compounds are termed potentiators. VX-770 or ivacaftor is a very promising potentiator and is the first FDA-approved drug for patients carrying the p.G551D *CFTR* mutation. It was discovered by high-throughput screening. In vitro studies of the effect of VX-770 on CFTR-mediated Cl^- secretion were performed on both recombinant cell lines and primary cultures of human bronchial epithelial cells. Van Goor et al. demonstrated that VX-770 increases Cl^- transport by increasing the open probability of the CFTR channel and increases

apical fluid height and ciliary beat frequency (Van Goor et al. 2009). In an important clinical study, orally administered ivacaftor improved CFTR function in patients carrying the p.G55D mutation as measured by sweat Cl^- concentration and NPD measurements. A phase 3 trial demonstrated significant improvements in FEV_1 from baseline, average weight gain, concentration in sweat Cl^-, and reductions in pulmonary exacerbations (Ramsey et al. 2011). Ivacaftor has shown similar results in patients carrying other class III mutations (De Boeck et al. 2014).

5.1.6 Class IV and V Mutations

Class IV defects may be susceptible to augmentation of channel function. Possible compounds of potential treatments are flavonoid, compounds like genistein which acts directly on the channel to increase open probability.

Class V mutations often include splicing mutations which produce a variable phenotype. The disease expression is inversely related to the level of correctly spliced transcripts. The effect of overexpression of splicing factors on the level of correctly spliced CFTR transcripts was studied. It has been shown that increasing the level of correctly spliced RNA activated the CFTR channel and restored function. Class IV and V mutations may respond to potentiators like ivacaftor and these studies are ongoing.

These new developments provide hope that a treatment strategy could be applied to the basic defect rather than downstream manifestations of the disease.

5.2 Possible Treatments in Pancreatitis

The protective role of CFTR against alcohol-induced pancreatitis and pancreatic damage has been demonstrated in the *Cftr* knockout mouse (Maléth et al. 2015). As a corollary, compelling evidence indicates that CF-causing mutations are associated with an increased prevalence of pancreatitis (De Boeck et al. 2005). Furthermore, the loss-of-function CFTR phenotype, caused by ethanol, palmitic acid, and palmitoleic acid, at least partly, can be attributed to attenuated biogenesis, accelerated PM turnover, and channel inhibition of CFTR (Maléth et al. 2015). Thus, restoring the functional cell surface expression of CFTR may partly alleviate the ethanol-induced exocrine pancreas damage.

While the precise molecular mechanism of CFTR downregulation has not been fully elucidated, based on sustained cytosolic $[Ca^{2+}]$ elevation and mitochondrial Ca^{2+}-overload in ethanol- or palmitoleic acid-exposed cells (Maléth et al. 2015), it is plausible to assume that the folding capacity of molecular chaperone network, confined to the ER and cytosol, is reduced. Depletion of cytosolic [ATP] due to mitochondrial dysfunction upon cellular Ca^{2+}-overload could also contribute to impaired CFTR domain assembly. These events, jointly, elicit the conformational destabilization of newly synthesized CFTR, which leads to the channel accelerated

metabolic turnover at the ER and PM (Maléth et al. 2015). Accordingly, we envision multifaceted therapeutic approaches to alleviate the exocrine pancreas damage in pancreatitis.

Cytosolic Ca^{2+}-overload and the associated mitochondrial dysfunction during ethanol-induced pancreatitis could be attenuated by inhibiting the PM Ca^{2+} entry channels (such as the store operated Ca^{2+} channel Orai1 (Gerasimenko et al. 2013)). Intriguingly, inhibiting PM Ca^{2+}-channels also upregulates several molecular chaperones, which could be an added advantage in stabilizing CFTR. This treatment had some success in rescuing mutant β-glucocerebrosidase in Gaucher patient-derived fibroblasts (Mu et al. 2008). The cytosolic folding capacity of the chaperone systems could be increased also by inducing the unfolded protein response (UPR) or inhibiting the histone deacetylase 7 (HDAC7). Inhibiting the ER-associated degradation and PM mistargeting may help to increase the number of functional channels at the cell surface (Balch et al. 2011). Finally, the recently FDA-approved pharmacological chaperone, lumacaftor (VX-809) (Van Goor et al. 2011), that partially corrects the p.F508del CFTR folding/processing defect and the CFTR gating activator ivacaftor (VX-770) (Van Goor et al. 2009) can be considered as potential therapeutics. While both drugs are assumed to directly bind to mutant and wild-type CFTR, lumacaftor has been shown to stabilize the NBD1/MSDs interface in CFTR variants both in the ER and the PM (Ren et al. 2013; Okiyoneda et al. 2013). In contrast, ivacaftor can stimulate the gating of several missense mutations with impaired channel activation upon PKA-mediated phosphorylation (Van Goor et al. 2014). Thus, lumacaftor and ivacaftor may have the potential to revert the conformational and functional defects, respectively, of the ethanol-induced dysfunctional CFTR, a scenario that has to be evaluated experimentally in the future.

Acknowledgments Our research was supported by the Hungarian Scientific Research Fund (PD115974 (JM), K116634 (PH), NF101116 (ZR)); the Momentum Grant of the Hungarian Academy of Sciences (LP2014-10/2014); the Cystic Fibrosis Canada (GL), NIH R01-DK75302 (GL); and the Canadian Institute of Health Research (GL), the NIH DK096327 (AU), NIH DK097820 (AU), NIH DK096518 (JE), Cystic Fibrosis Trust (UK) INOVCF- SRC003 (MAG), National Pancreas Foundation Grant (AU), NIH/NIDCR intramural grant DE000735 (SM), NIH R01-DK058088 (MST), and GL is a recipient of a Canada Research Chair.

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Rev Physiol Biochem Pharmacol (2016) 170: 67–100
DOI: 10.1007/112_2015_5003
© Springer International Publishing Switzerland 2016
Published online: 21 January 2016

Signature and Pathophysiology of Non-canonical Pores in Voltage-Dependent Cation Channels

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Abstract Opening and closing of voltage-gated cation channels allows the regulated flow of cations such as Na^+, K^+, and Ca^{2+} across cell membranes, which steers essential physiological processes including shaping of action potentials and triggering Ca^{2+}-dependent processes. Classical textbooks describe the voltage-gated cation channels as membrane proteins with a single, central aqueous pore. In recent years, however, evidence has accumulated for the existence of additional ion permeation pathways in this group of cation channels, distinct from the central pore, which here we collectively name non-canonical pores. Whereas the first non-canonical pores were unveiled only after making specific point mutations in the voltage-sensor region of voltage-gated Na^+ and K^+ channels, recent evidence indicates that they may also be functional in non-mutated channels. Moreover, several channelopathies have been linked to mutations that cause the appearance of a non-canonical ion permeation pathway as a new pathological mechanism. This review provides an integrated overview of the biophysical properties of non-canonical pores described in voltage-dependent cation channels (K_V, Na_V,

The authors declare no conflict of interest.

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Ca_v, H_v1, and TRPM3) and of the (patho)physiological impact of opening of such pores.

Keywords Alternative ionic pathway • Ca_v • H_V1 and TRPM3 • K_V • Na_V • Voltage-dependent cation channels

Contents

1 Introduction

Living cells are delimited by the lipid bilayer membrane, which separates an internal environment from a drastically different external medium. Whereas the total ionic concentration is similar on both sides of the bilayer, the concentration of specific ion species (e.g., sodium ion (Na^+)) is different. The electrodiffusion of the ions down their electrochemical gradient generates a charge separation across the membrane, which is named the membrane potential. The membrane potential translates into an intense electric field, which acts on charges within the membrane field.

Voltage-dependent cation channels comprise a broad number of different channel families, all sharing the property of voltage-dependent regulation. In general, cation channels regulated by voltage contain a voltage-sensing module that enables them to detect and react to changes in voltages. This module is called the voltage-sensing domain (VSD) and is intensively studied in voltage-gated potassium (K_V)

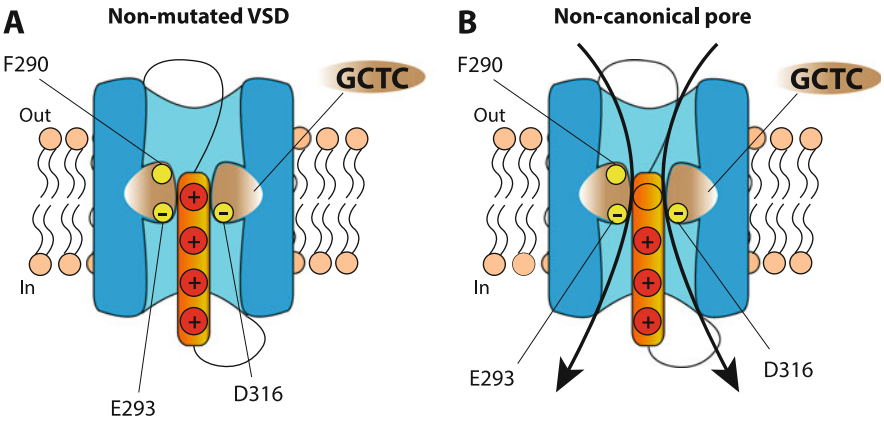

Fig. 1 Cartoon of a non-canonical pore in the Shaker K^+ channel. Schematic illustration of a cut-open view on the voltage-sensing domain (VSD) with the S4 voltage sensor. Gating charges R1–R4 in S4 are indicated as *plus signs* in *red*. In the center of the VSD, shaping the path of the S4 arginines is the gating charge transfer center (GCTC) with the amino acids F290, E293, and D316. The GCTC builds a hydrophobic plug and thereby separates the external and internal water crevices. (**a**) If arginines are located at all the positions R1–R4, no non-canonical pore current can occur. (**b**) If the arginine at position R1 is exchanged for a smaller, uncharged amino acid, the hydrophobic plug is interrupted. This leads to the connection of the external and internal water crevices and allows the flow of ions through the resulting pore. This ion flux occurs in the hyperpolarized state of the voltage sensor, when the substituted amino acid is positioned in the center of the GCTC

and sodium channels (Na_V) (Bezanilla 2000; Gandhi and Isacoff 2002; Horn 2002). Voltage-gated cation channels generally consist of two modules, the VSD formed by transmembrane segments S1–S4 and the pore domain (PD) formed by transmembrane segments S5 and S6 and their interconnecting pore loop (Gandhi and Isacoff 2002; Catterall 2010; Bezanilla 2000; Stock et al. 2013). The S4 of the VSD is the actual voltage sensor as it carries a number of positive charges like arginines (R) and lysines (K), normally at every third amino acid position (Fig. 1). The residues in between these positive charges are classically of hydrophobic nature (Catterall 2010; Bezanilla 2000). Upon depolarization of the cell membrane, the positive S4 charges are moved over the membrane electric field within the VSD (Bezanilla 2002). This charge transport is accomplished due to the movement of S4, which leads to a conformational change of the channel protein, pulling, among others, the S4–S5 linker and thereby opening the central pore and allowing ion flux over the cell membrane (Horn 2002; Catterall 2010). As the movement of the positively charged residues in S4 leads to the gating of the channel, these residues are also often named "gating charges" (Fig. 1a) (Bezanilla 2002). The movement of gating charges can be measured as a small transient current that is called "gating current."

In addition to the current through the central pore, some voltage-dependent cation channels possess alternative, non-canonical pore currents that share unique properties. The existence of this alternative ion permeation pathway was first

discovered in the Shaker K^+ channel, where substitution of arginine residues in the fourth transmembrane domain (S4) by histidines caused a proton wire through the VSD, and a substitution of an arginine for even smaller, uncharged amino acids caused a metal-ion-specific cation pore within the VSD. It was proposed that the ions pass through a pathway that is normally occupied by the gating charges of S4 (Fig. 1). Therefore, this pathway was called the "gating pore" and the occurring current "gating pore current." Also the expression "omega current" was introduced, which should differentiate the pathway from the standard ion pathway through the pore domain of ion channels, also called the "alpha pore" (Starace and Bezanilla 2004; Tombola et al. 2005). In the meantime, the number of studies reporting alternative ionic currents through the VSD in voltage-dependent cation channels increased significantly. However, it is not clear whether all reported ionic currents through the VSDs are occurring through the pathway that is normally occupied by arginines. In addition, alternative ionic currents are described in naturally occurring channels that do not possess arginines at the standard positions in S4 and therefore must possess a different gating mechanism. Here, we propose a different terminology for the alternative pores, namely, "non-canonical pores."

In this review, we aim to provide a historical overview of the identification of non-canonical pores in different voltage-dependent cation channels. Further, we will illuminate the biophysical characteristics and discuss possible activation mechanisms of the reported non-canonical pores. Additionally, we will illustrate the role of non-canonical pores in physiological as well as pathophysiological conditions.

2 Structure and Function of the Voltage-Sensing Domain

2.1 A Focused Electric Field Within the VSD

The voltage-sensing domains have an important property in focusing the membrane electric field on a small voltage-sensitive area of S4 (Starace et al. 1997; Starace and Bezanilla 2004; Ahern and Horn 2005). This could be shown in experiments using histidine-scanning mutagenesis, where the residues R1–R4 in the voltage sensor of the Shaker K^+ channel were individually exchanged for histidines. Measuring gating currents in different internal and external pH conditions indicated that all residues are accessible to the external solution at depolarized potentials and to the internal solution at hyperpolarized potentials, resulting either in proton transport or in proton conduction. Therefore, narrow hydrophilic water crevices must surround the hydrophobic environment of the protein and center the membrane electric field on a small area of the voltage sensor (Starace and Bezanilla 2001, 2004; Starace et al. 1997). This specific structure of the voltage sensor allows transport of many charges over the entire membrane electric field by only small conformational movements of S4 (Starace and Bezanilla 2004; Starace et al. 1997; Bezanilla 2002).

2.2 The Role of S1–S3 of the VSD

The voltage-sensing domain is formed by transmembrane segments S1–S4 where the S4 segment is the actual voltage sensor as it carries a number of positive charges (Fig. 2b). However, the other segments of the VSD (S1–S3) play an equally important role in the regulation and movement of the VSD. Various studies have identified conserved amino acids on S1–S3 that are involved in the voltage-sensing process (Papazian et al. 1995; Tao et al. 2010; Pless et al. 2011; Lacroix et al. 2014; Tombola et al. 2005). It was shown that certain residues in S2 and S3 (E283, E293 and D316) are important for the stabilization of S4. A model was proposed in which a positive residue in S4 (K374) of the Shaker K^+ channel electrostatically interacts with two negative charges in S2 and S3 (E293 and D316) in the resting state, while R377 in S4 is stabilized by similar negative residues (E293 and D316) in the activated state of the channel. Moreover, a weak interaction was proposed for K374 and R377 with E283 in S2 (Papazian et al. 1995). Later studies indicated that substitution of arginine gating charge residues for smaller amino acids create a leak current through the voltage-sensing domain. This could be explained by the disruption of a hydrophobic area within the VSD, leading to the connection of the internal and external water crevices and allowing an ion flux (Starace and Bezanilla 2001, 2004; Tombola et al. 2005). Later, other groups also reported the importance of amino acids located in S1–S3 for the stabilization of S4. It was postulated by Tao et al. that F290, E293, and D316 configure an occluded cation binding site, the so-called gating charge transfer center (GCTC), which is a hydrophobic region in the VSD where residues of S1–S3 interact with the gating charge residues in S4 and thereby separate the internal and external water crevices. The GCTC catalyzes the transfer of gating charges over the membrane electric field in case of voltage-sensor movement (Tao et al. 2010). It was proposed that a ring at position 290 is required to enable proper voltage-sensor movement. Additionally, the possibility of cation–π interactions between the aromatic ring of F290 and the gating charges in S4 was further investigated but could not be confirmed (Tao et al. 2010). In contrast, a correlation was shown between the electronegative surface potential of the aromatic ring at position 290 in the Shaker K^+ channel and shifts in the G–V curve of the gating charge currents (Pless et al. 2011). On the other hand, Pless et al. contradicted the proposed electrostatic charge–charge interactions of residues E293 and D316 with gating charges of S4 and proposed a potential role of these residues in the formation of the water crevices surrounding the GCTC (Pless et al. 2011).

A recent study highlighted the importance of the physicochemical properties of certain amino acid side chains in S1–S3 for specific functional features of the voltage sensor. This was shown by a correlation study in which changes in amino acid side chain properties were linked with changes in biophysical properties of the mutant channel (voltage dependence of charge movement and gating kinetics). Importantly, five residues (S240, I287, F290, F244, and I237) were identified that require specialized physicochemical properties (size or hydrophobicity) to enable

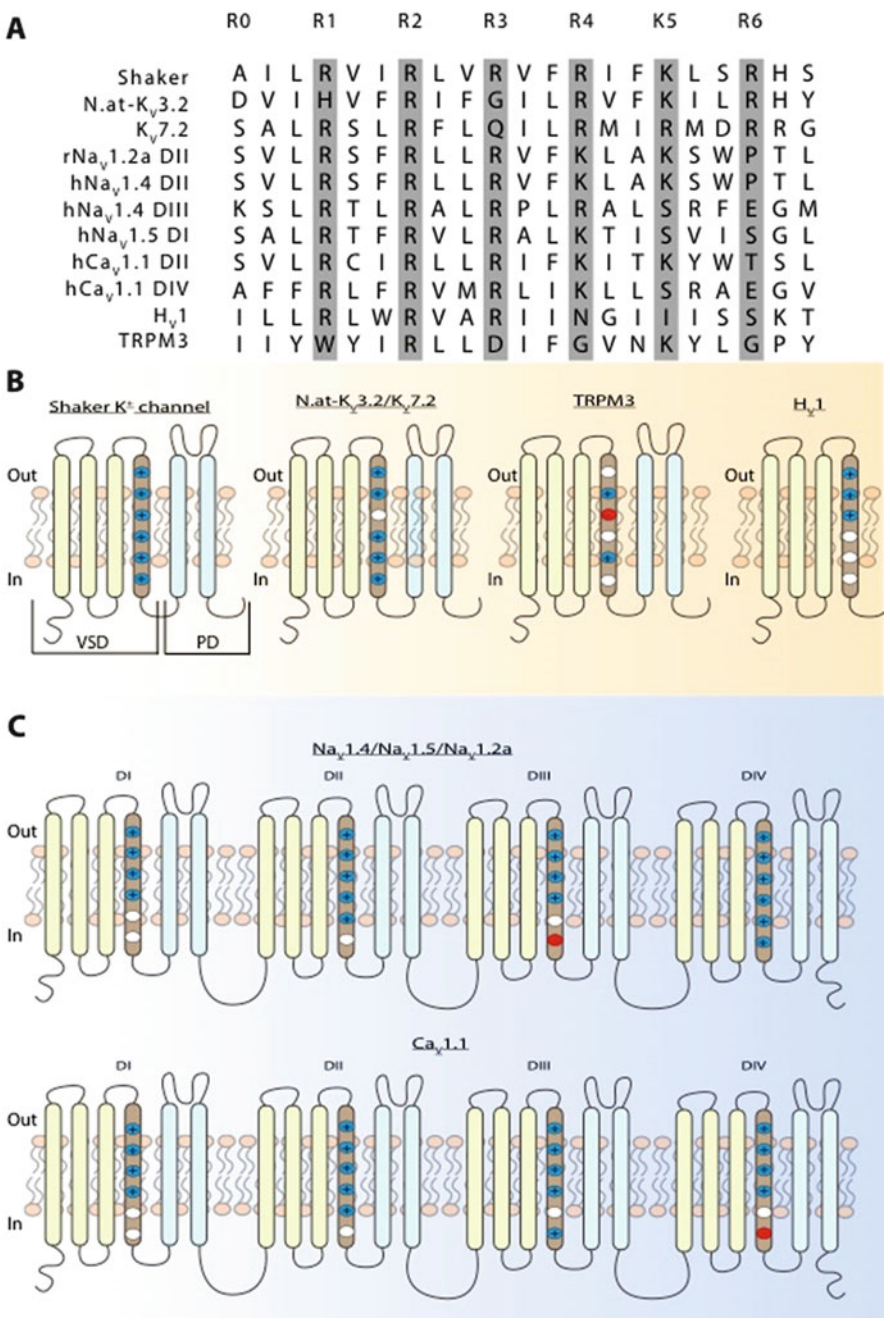

Fig. 2 Voltage-dependent cation channels with reported non-canonical pores. (**a**) Alignments of the S4 regions of voltage-dependent cation channels with reported non-canonical pores. Here only the wild-type sequences are shown. Note that only N.at-K_V3.2, H_V1, and TRPM3 show non-canonical pore currents through the wild-type channel. (**b**) All channels listed in (**a**) that belong to the group of multi-subunit ion channels. These channels build multimers to create a channel protein. (**c**) All channels listed in (**a**) that belong to the single-subunit ion channels. They

proper voltage-sensor movement (Lacroix et al. 2014). To summarize, many different studies implicated the importance of residues within the S1–S3 region for the proper functioning of the voltage sensor by either stabilizing the S4 or participating in the formation of the water crevices that surround the GCTC and thereby influencing the centered electric field that is necessary for proper voltage sensing. Nevertheless, since the knowledge about the S1–S3 region of the VSD is still in its infancy, additional experiments are required to unravel the importance of the interplay between residues of S1–S3 and S4 in the voltage-sensing process.

2.3 *Models of Voltage-Sensor Movement*

In the past, two major models were proposed to describe the movement of the S4 segment by changes in voltage. A first model is called the *sliding helix* or *helical screw* model, in which the S4 arginines are located within a transmembrane helical environment. The charged residues in S4 build ion pairs with negatively charged residues in S1–S3 (Catterall 1986, 2010; Horn 2002; Bezanilla 2002; Gandhi and Isacoff 2002). Upon depolarization, the positive gating charges are pulled outward following a spiral path stabilized by the negatively charged residues, which allows S4 to move along a low-energy pathway during gating charge movement (Catterall 2010). In this model, gating charges are insulated from the lipid bilayer by a polar protein environment. An alternative model that has been proposed was the *paddle model*, where S4 is lying at the outside of the VSD having the arginine residues located in the lipid environment of the membrane. Upon depolarization, the S4 segment voltage-sensor paddle flips up and transports gating charges over the membrane at the protein–lipid interface (Jiang et al. 2003). This model emerged due to an X-ray crystal structure from the microbial potassium channel K_VAP. Nevertheless, the obtained crystal structure might not represent the natural constitution of the VSD, as it was created by coupling Fab fragments to the voltage sensors with the idea to hold the potentially highly flexible voltage sensors attached to the channel (Jiang et al. 2003). Moreover, additional evidence was provided by experimental results supporting the *helical screw* model, rather than the *paddle model* (Pathak et al. 2007; Tombola et al. 2005, 2007; Sokolov et al. 2005; Gamal El-Din et al. 2010, 2014; Khalili-Araghi et al. 2012; Gosselin-Badaroudine et al. 2012a). It is in the course of intense research efforts to distinguish between the helical screw and paddle models that non-canonical pores in voltage-dependent cation channels first observed, and their discovery represented strong evidence in favor for the former model. However, further research is required to obtain a

Fig. 2 (continued) consist of four domains (equivalent to subunits) that shape together a functional channel. (**a**, **b**) The residues at positions R1–R6 in the voltage sensors are indicated as *plus* and *blue-colored* for positively charged, as *minus* and *red-colored* for negatively charged, and without *sign* and *white-colored* for neutrally charged

complete picture of the actual voltage-sensor movement. One should keep in mind that movement of S4 might also differ in several VSDs from various proteins depending on the structure of the specific VSD and the electrostatic interactions between different residues and so may probably not be generalized.

3 Voltage-Dependent Cation Channels Containing a Non-canonical Pore

The following part provides an overview of non-canonical pores described in different classes of voltage-dependent cation channels.

3.1 Voltage-Gated K^+ Channels

Voltage-gated K^+ channels (K_v) are selectively permeable to K^+ ions and comprise a large family including 12 subfamilies (K_V1–K_V12) (Shah and Aizenman 2014). The activities of these proteins are regulated by changes in membrane potential and are critical for maintaining or restoring the resting membrane potential in both excitable and non-excitable cells (Miller 2000). Voltage-gated potassium channels consist of four subunits each possessing a VSD (S1–S4) and a PD (S5–S6) (Fig. 2b) (Miller 2000; Shah and Aizenman 2014). Furthermore, K_V α subunits can bind to regulatory β subunits or other K_V channel-interacting proteins that modify channel properties (Shah and Aizenman 2014). The S4 segments of K_V channels are highly positively charged and are key segments in voltage sensing (Fig. 2a). The first evidence of a non-canonical pore current was reported in a mutant channel of the Shaker K^+ channel where a proton-specific current through the voltage sensor was observed when the first (R1) or last S4 arginine (R4) was substituted with histidine (Starace and Bezanilla 2001, 2004). In addition, Tombola et al. reported a metal-ion-specific current through the voltage-sensing domain of the Shaker K^+ channel, when R1 was mutated to even smaller, uncharged amino acids (Tombola et al. 2005).

Later, a naturally occurring gating pore current was described in a member of the K_V3 family, N.at-$K_V3.2$ from a flatworm (Klassen et al. 2008). N.at-$K_V3.2$ differs from other K_V channels in that the first and third basic residue positions within S4 are not the traditional arginine or lysine, but instead histidine (H325) and glycine (G331) (Fig. 2a, b). Typically, the channel showed a low selectivity to a number of cations and displayed an unusual weak inward rectifier phenotype. Reversal mutations of H325 and G331 to positive charges (R or K) changed the phenotype back to a typical K^+-selective delayed rectifier.

Recently, non-canonical pore currents were linked to mutations in R4 of $K_V7.2$ channels, causing benign familial neonatal seizures (BFNS) and peripheral nerve

hyperexcitability (PNH) (Fig. 2a) (Miceli et al. 2012) (explained in more detail in Sect. 5 of this review).

3.2 *Voltage-Gated Na^+ Channels*

Voltage-gated sodium channels (Na_{Vs}) are large proteins consisting of a pore-forming α subunit, associated with one or two β1-4 auxiliary subunits (Catterall et al. 2005a). The α subunit encompasses 24 transmembrane segments. In particular, it is arranged into four homologous but not identical channel domains (DI-DIV), each made up of six transmembrane segments S1–S6 (Fig. 2c). Each domain plays the role of a subunit-like entity, so that the channel is arranged by the complex organization of these four domains around the pore. The four domains are linked by three different intracellular loops of unequal lengths. The pore is formed by the S5–S6 segments of each domain and is surrounded by the S1–S4 segments responsible for the voltage sensing (Fig. 2c) (Catterall 2010; Catterall et al. 2005a). The S4 segments of Na_V channels are closely homologous to the S4 domain of K_V channels, including the cluster of positive charges involved in voltage sensing (Fig. 2a). The first non-canonical pore currents in voltage-gated sodium channels were reported in the brain sodium channel $Na_V1.2a$, where paired arginine mutations in DII induced gating pore currents (Sokolov et al. 2005). Interestingly, mutations of the outermost gating charges in DIIS4 of $Na_V1.2a$ induced an inward gating pore current at negative potentials, while mutation of the innermost arginines induced an outward gating pore current at positive potentials (Fig. 2c) (Sokolov et al. 2005). These observations strongly support the hypothesis that an ion gap is created when the mutated residues in S4 are positioned in the narrowest part of the pore through which S4 moves (Sokolov et al. 2005). Non-canonical pore currents are also described in a disease-linked $Na_V1.4$ mutant (Sokolov et al. 2007). Mutations of S4 arginines in $Na_V1.4$ channels result in a hyperpolarization-activated gating pore leak current, causing hypokalemic periodic paralysis (HypoPP) in patients (explained in more detail in Sect. 5). Evidence for the existence of a non-canonical pore current in $Na_V1.4$ channels was provided by experiments in which tetrodotoxin was used to block the canonical central pore of wild-type and mutant $Na_V1.4$ channels. The results of this study showed a prominent nonlinear current component in mutant channels at hyperpolarized potentials (−50 mV to −140 mV), which was absent in wild-type $Na_V1.4$. Interestingly, these gating pore currents were observed with single gating charge mutations (R663H and R666G/H), in contrast to the former study in the brain sodium channel $Na_V1.2a$ (Sokolov et al. 2005, 2007). Later, more HypoPP mutations in $Na_V1.4$ were identified and associated with the introduction of an alternative ion permeation pathway (Struyk and Cannon 2007; Struyk et al. 2008; Francis et al. 2011; Wu et al. 2011; Mi et al. 2014; Groome et al. 2014). A later study identified a critical residue in the $Na_V1.4$ channel, causing normokalemic periodic paralysis (NormoPP) (see Sect. 5) in patients when mutated (R675G/Q/W) (Vicart et al. 2004). Interestingly, mutation

of this critical residue in $Na_V1.4$ also induced non-canonical pore currents (Sokolov et al. 2008).

In addition, evidence for non-canonical pores in Na_V channels was provided in the heart sodium channel $Na_V1.5$ (Gosselin-Badaroudine et al. 2012b; Moreau et al. 2015b). Insertion of a single point mutation in the DI–S4 segment of the heart sodium channel $Na_V1.5$ (R219H) causes mixed arrhythmias and dilated cardiomyopathy in patients (see Sect. 5), resulting from a proton leak or a proton wire through a non-canonical pore at hyperpolarized potentials (Gosselin-Badaroudine et al. 2012b).

3.3 Voltage-Gated Ca^{2+} Channels

Voltage-gated calcium (Ca_V) channels, comprising ten members, conduct Ca^{2+} ions into cells in response to depolarization. Like sodium channels, Ca_Vs have a large, pore-forming subunit (designated α1) that is composed of four homologous domains (DI–DIV), and each domain comprises again a VSD (S1–S4) and a PD (S5–S6) (Fig. 2c) (Catterall et al. 2005b). The S4 segments of Ca_V channels contain at least four positive charges, like most voltage-dependent channels (Fig. 2a). However, calcium channels have a different set of auxiliary subunits, including Ca_V β, γ and α2δ subunits (Catterall et al. 2005b). The first evidence for non-canonical pore currents in voltage-gated Ca^{2+} channels was given in a study describing a $Ca_V1.1$ mutant channel (Wu et al. 2012a). The R528H mutation in the outermost arginine of the S4 segment of domain II revealed an inward current at hyperpolarized potentials in $Ca_V1.1$, like homologous mutations in the skeletal muscle Na_V channel $Na_V1.4$ (see above), causing HypoPP (Wu et al. 2012a). A later study succeeded in expressing the NormoPP causing $Ca_V1.1$ channel mutant R1242G, localized to the third positive charge of the domain IV voltage sensor, in a heterologous expression system. The electrophysiological measurements of the R1242G mutant channel indicated depolarization-activated gating pore currents and hyperpolarization-activated gating pore currents after a long depolarizing pre-pulse (Fan et al. 2013). This result was consistent with the location of the mutation at position R3 in S4DIV.

3.4 Voltage-Gated Proton Channel

The voltage-gated proton channel (H_V1) is a proton channel that plays a vital role in the fast proton translocation, thereby controlling a wide range of physiological functions, including the phagocytic respiratory burst, sperm motility, apoptosis, and metastatic cancer (Li et al. 2015). The H_V1 channel is a VSD protein that lacks a discernible pore domain (S5–S6) (Fig. 2b). Both voltage activation and proton conduction are carried out by a VSD with strong similarity to canonical VSDs in

voltage-dependent cation channels (Ramsey et al. 2006; Sasaki et al. 2006). Functional H_V1 is expressed as a dimer in biological membranes, with each monomer containing a separate proton pathway (Tombola et al. 2008, 2009; Lee et al. 2008; Koch et al. 2008). The S4 segment of H_V1 has multiple basic residues, which are characteristic of the VSDs of voltage-gated cation channels and voltage-sensing phosphatases (Fig. 2a). Electrophysiological studies indicate that S4 moves and functions as a voltage sensor in the H_V channels (Gonzalez et al. 2010, 2013; Kulleperuma et al. 2013), which moves upward relative to other helices upon activation as it does in K_V and Na_V channels. In H_V channels, S4 has only three positively charged residues (Fig. 2a) and comparison of S4 of H_V1 and Shaker K^+ channels aligns asparagine 214 (N214) of H_V1 at position R4 of Shaker channels. Mutating N214 to a cysteine makes the proton pore susceptible for a block with MTS reagents. Additionally, mutating N214 in H_V1 to an arginine completely abolishes the proton current. These two results, plus the fact that the proton pore of H_V1 opens at positive voltages, when the voltage sensor is in a depolarized position, support the idea that movement of the voltage sensor upon depolarization positions N214 at the narrowest part of the VSD and therewith opens a proton pore (Tombola et al. 2008, 2009). This implies the existence of a naturally occurring non-canonical pore within the H_V1 proton channel. Several models for molecular mechanisms of gating and permeation have been proposed (Gonzalez et al. 2010). Recently, Asp 112 located in S1 was identified as a critical component of the selectivity filter of H_V1, crucial to both proton selectivity and charge selectivity (Musset et al. 2011; Berger and Isacoff 2011). These results are different from the proton currents described in K_V and Na_V channels, where histidine shuttles protons through the VSDs with Arg to His mutations.

3.5 Transient Receptor Potential Melastatin 3

The transient receptor potential melastatin 3 channel (TRPM3) is a member of the melastatin subfamily of transient receptor potential (TRP) channels. TRP channels contain six transmembrane spanning domains with a voltage-sensing domain (S1–S4) and a pore-forming reentrant loop between the fifth (S5) and the sixth (S6) transmembrane domain (Fig. 2b) (Wu et al. 2010). Similar to the structure of K_V channels, functional TRP channels are tetramers, where four subunits together build the channel protein (Hoenderop et al. 2003). However, the number of basic amino acids in S4 of TRP channels is generally low, and in many TRP channels, voltage dependence is weak or nonexistent. It has been suggested that most structural elements within TRP channels and K_V channels are not sufficiently related to allow for the creation of hybrid channels and that the transmembrane regions of TRP channels have more constraining packing interactions than have been observed in K_V channels (Kalia and Swartz 2013). TRPM3 can be activated by chemical ligands, such as the neurosteroid pregnenolone sulfate (PS) and the synthetic compound nifedipine, as well as by heat and by depolarization (Wagner

et al. 2008; Vriens et al. 2011). It was generally accepted that in TRP channels physical and chemical activating stimuli lead to gating of a single, central cation-conducting pore formed by S5–S6 and the interconnecting pore loop (Wu et al. 2010). For TRPM3, stimulation by heat or chemical compounds like PS and nifedipine opens the classical central pore and induces outwardly rectifying currents in TRPM3 expressing cells. The central pore is highly permeable for Ca^{2+} and Mg^{2+} (Oberwinkler et al. 2005) and can be blocked by the nonspecific channel blocker La^{3+} (Grimm et al. 2003; Held et al. 2015; Vriens et al. 2014). At −150 mV, single-channel currents were measured with an average amplitude of −7.1 pA and an estimated single-channel conductance of ~50 pS (Vriens et al. 2014). In a search for TRPM3 modulators, clotrimazole (Clt), a drug used to treat yeast infections, caused PS-activated TRPM3 currents to acquire an inwardly rectifying component at negative voltages, resulting in a biphasic conductance–voltage relationship. Evidence was presented that the inward current might reflect the permeation of cations through the opening of a non-canonical pore. Typically, this alternative ion permeation pathway showed (i) a strong inward rectification, (ii) a low permeability to Ca^{2+} and Mg^{2+}, (iii) a single-channel conductance of ~13 pS, (iv) resistance to Ca^{2+}-dependent desensitization, and (v) a low sensitivity to the open pore blocker La^{3+} (Vriens et al. 2014). The S4 region of TRPM3 contains less positive charges than voltage-dependent Na^+, K^+, or Ca^{2+} channels, with only one positively charged arginine residue (Fig. 2a). This could explain the presence of a naturally occurring non-canonical current and the presence of an inward current. Indeed, introduction of an arginine at the position corresponding to R1 of the Shaker K^+ channel of the S4 region (W982R) specifically disrupts the Clt-PS-induced inward current (Vriens et al. 2014). The data indicate that TRPM3 represents one of the first examples of a naturally occurring voltage-dependent cation channel with two distinct pores, a classical central pore and a non-canonical pore. Recently, a novel small-molecule TRPM3 agonist, CIM0216, was identified, whose potency and apparent affinity greatly exceeds that of the endogenous TRPM3 agonist PS. Remarkably, application of CIM0216 caused the opening of both the canonical calcium-conducting pore and the non-canonical pore of TRPM3 (Held et al. 2015). The presence of an alternative pore in TRPM3 might seem like a biophysical oddity observed only under very contrived conditions. Nevertheless, evidence was provided that it occurs in vivo and can contribute to the genesis of pain signals and in the release of insulin in pancreatic β cells (Held et al. 2015; Vriens et al. 2014). However, Clt and CIM0216 are clearly not the endogenous agonists of this alternative pore. Possibly an inflammatory mediator released during irritation or an endogenous activator released after high blood glucose levels has the same pore-opening property, if existing at all?

4 Biophysical Characteristics of Non-canonical Pores

The next section provides an overview of permeability properties and pore block of non-canonical pores.

4.1 Ionic Selectivity

In general, ionic currents through non-canonical pores are mediated by cations. When *N*-methyl-D-glucamine ($NMDG^+$) was substituted for Na^+ in the extracellular solution, most of the voltage-dependent cation channels lacked an inward current component, what could indicate that the inward current is principally carried by exclusively cations (Starace and Bezanilla 2004; Starace et al. 1997; Tombola et al. 2005, 2007, 2008; Sokolov et al. 2005, 2007, 2010; Francis et al. 2011; Struyk and Cannon 2007; Struyk et al. 2008; Klassen et al. 2008; Groome et al. 2014; Held et al. 2015; Vriens et al. 2014; Moreau et al. 2015b; Berger and Isacoff 2011). The exclusion of anions can be explained by a collection of negatively charged residues in the GCTC, where two negative charges repulse one another (Khalili-Araghi et al. 2012). However, $NMDG^+$ permeability through the non-canonical pore is reported in $Na_V1.4$ mutant channels and H_V1 channels (Sokolov et al. 2007; Francis et al. 2011; Struyk et al. 2008; Struyk and Cannon 2007). Still, it is possible that these currents recorded in $NMDG^+$ conditions might occur due to proton permeation over the non-canonical pore (Struyk and Cannon 2007; Struyk et al. 2008).

Based on the relative permeability of cations with increasing dimensions, the diameter of non-canonical pores at their narrowest point has been estimated to be around ~3.5 Å in $K_V1.2$ channel (Khalili-Araghi et al. 2012) and <6.8 Å in all voltage-gated cation channels that showed impermeability to $NMDG^+$ when Na^+ was replaced, including TRPM3 (Vriens et al. 2014).

Most of the non-canonical pores showed an ionic selectivity sequence $Cs^+>K^+>Na^+>Li^+$, which corresponds to an Eisenmann I or II selectivity sequence, suggesting that the non-canonical pores have a weak cation binding site (Tombola et al. 2005; Sokolov et al. 2007; Struyk et al. 2008; Francis et al. 2011; Vriens et al. 2014). Therefore, larger cations are favored to permeate the pore, as they have a lower dehydration energy (Hille 2001). In general, the Eisenmann I or II sequence selectivity of the non-canonical pore is different from the sequence selectivity of the central canonical pore, which is predominantly selective for a specific cation in K_V, Na_V, and Ca_V channels. Interestingly, whereas wild-type voltage-gated proton channels are highly specific for protons, neutralization of a single Asp (D112) residue in H_V1 converts a proton channel into a predominantly anion selective channel. This makes aspartate 112 as a crucial component in the selectivity filter of H_V1 (Musset et al. 2011).

A remarkable group of non-canonical pores is formed by proton-conducting channels, including mutant channels with arginine to histidine substitution and naïve H_V1 channels. This group is not able to conduct metal ions or larger compounds due to their

Table 1 Selectivity sequences reported for different non-canonical pores

Channel	Selectivity	Publication
Shaker K^+ channel	$Gua^+ > Cs^+ > K^+ > Li^+$	Tombola et al. (2005)
$rNa_V1.2a$	$K^+ > Cs^+ = Li^+ > Na^+ > TMA^+ = TEA^+$	Sokolov et al. (2005)
$rNa_V1.4$ R666G	$Cs^+ = K^+ > Na^+ = Li^+ > TEA^+ = NMDG^+$	Sokolov et al. (2007)
	$K^+ > Na^+$	Struyk et al. (2008)
	$Gua^+ > Ethylgua^+ > Na^+$	Sokolov et al. (2010)
R666H	$Gua^+ > H^+$	Sokolov et al. (2010)
R1125Q	$K^+ > Na^+ >> NMDG^+$	Francis et al. (2011)
R1128H	$Gua^+ > Li^+ = Na^+ = K^+$	Groome et al. (2014)
R1128C	$Gua^+ > Li^+ = Na^+ = K^+$	Groome et al. (2014)
$hNa_V1.5$ R222Q	$Cs^+ > K^+ > Na^+$	Moreau et al. (2015b)
R225W	$Cs^+ > K^+ > Na^+$	Moreau et al. (2015b)
N.at-$K_V3.2$	$K^+ > Cs^+ > Gua^+ = Li^+ > Na^+ = Ba^{2+}$	Klassen et al. (2008)
H_V1 D112S-R211S	$Gua^+ > Li^+ > Na^+ = K^+ = Cs^+ > H^+$	Berger and Isacoff (2011)
TRPM3	$Gua^+ > Na^+ > Ca^{2+}$	Vriens et al. (2014)
	$Gua^+ > Na^+$	Held et al. (2015)

restriction in size. Anyhow, two studies have reported permeation of ions as large as K^+, Li^+, Na^+, and Gua^+ in case of an arginine to histidine mutation in $Na_V1.4$ channels (Sokolov et al. 2010; Groome et al. 2014). An overview of the ionic selectivities of non-canonical pores identified in different cation channels is provided in Table 1.

Another characteristic of several non-canonical pores is the higher permeability for guanidinium (Gua^+) compared to metal ions (Tombola et al. 2005; Sokolov et al. 2010; Groome et al. 2014; Held et al. 2015; Vriens et al. 2014; Berger and Isacoff 2011). These observations are in line with the proposition that alternative currents occur through a narrow constriction area that is normally occluded by arginine residues (Tombola et al. 2005). In channels with non-canonical pores where the arginine is replaced by a smaller amino acid, the arising pore can perfectly store a guanidine side group (Tombola et al. 2005). The naturally occurring non-canonical pores of H_V1 and N.at-$K_V3.2$ form the exception on this characteristic. N.at-$K_V3.2$ shows a lower permeability for Gua^+ compared to K^+ and Cs^+ (Klassen et al. 2008), while the H_V1 channel is blocked by Gua^+ (Tombola et al. 2008; Berger and Isacoff 2011).

In addition, non-canonical pores generally have a very low Ca^{2+} and Mg^{2+} permeability (Tombola et al. 2005; Sokolov et al. 2007, 2010; Held et al. 2015; Vriens et al. 2014; Berger and Isacoff 2011; Francis et al. 2011). For the non-canonical pore of TRPM3, it was described that the permeability of Ca^{2+} was very low compared to the permeability of Na^+, which is in strong contrast with the high Ca^{2+} permeability of the canonical pore of TRPM3 (P_{Ca2+}/P_{Cs+} ~10) (Wagner et al. 2010) (Held et al. 2015; Vriens et al. 2014).

Altogether, the limited similarities between the properties of the non-canonical pore of the different voltage-gated cation channels illustrate that non-canonical pores are clearly not all the same and that their properties depend on the VSD, in particular the location of the non-canonical pore and the nature of the amino acid in the narrow constriction area of the alternative pore.

4.2 Blockers

The fact that selective blockers of the canonical pore, including pore-specific toxins, did not influence the current via the non-canonical pore, was an important element in elucidating the origin of alternative ion permeation pathways (Sokolov et al. 2005; Tombola et al. 2005; Klassen et al. 2008). Oppositely, at this moment blockers that inhibit the non-canonical pore of voltage-gated channels without affecting the canonical pore are scarce and their specificity is limited Table 2.

A first group of blockers of non-canonical currents are bivalent cations such as Zn^{2+}, Ca^{2+}, Mg^{2+}, Sr^{2+}, Ni^{2+}, and Ba^{2+} (Tombola et al. 2005; Sokolov et al. 2007, 2010; Held et al. 2015; Vriens et al. 2014; Berger and Isacoff 2011; Francis et al. 2011). Additionally, trivalent (Gd^{3+}, Yb^{3+}, La^{3+}, Lu^{3+}, Y^{3+}, Tl^{3+}) and quadrivalent cations (Hf^{4+}) are also described to block non-canonical pore currents (Sokolov et al. 2010; Held et al. 2015; Vriens et al. 2014). However, the ability of bi- and trivalent cations to block non-canonical pores of $Na_V1.4$ mutant channels (R663H, R666G, R1125Q) and $Na_V1.5$ mutant channel (R219H) was not observed in some studies (Struyk and Cannon 2007; Struyk et al. 2008; Francis et al. 2011; Gosselin-Badaroudine et al. 2012b).

In Shaker K^+ channels and $Na_V1.4$ mutant channels, guanidine derivatives, like ethylguanidinium (~50% block at 10 mM) and 1-(2,4-xylyl)guanidine carbonate (~50% block at 5 mM), are described as blockers of the non-canonical pore

Table 2 Blockers reported for different non-canonical pores

Channel	Block	No block	Publication
Shaker K^+ channel	$Ethylgua^+$, Mg^{2+}		Tombola et al. (2005)
r$Na_V1.4$ R663H		Ni^{2+}, Cd^{2+}, Li^+, Ba^{2+}	Struyk and Cannon (2007)
R666G	Zn^{2+}, Ba^{2+}, Ca^{2+}		Sokolov et al. (2007)
		Zn^{2+}, Ba^{2+}, Ca^{2+}	Struyk et al. (2008)
	1-(2,4-xylyl)-gua^+, Ba^{2+}, Zn^{2+}, Gd^{3+}, Yb^{3+}, La^{3+}, Lu^{3+}, Y^{3+}, Tl^{3+}, Hf^{4+}		Sokolov et al. (2010)
R1125Q	Ni^{2+}, Zn^{2+}	Ca^{2+}, Ba^{2+}	Francis et al. (2011)
h$Na_V1.5$ R219H		Ni^{2+}, Cd^{2+}, Zn^{2+}, La^{3+}, $Ethylgua^+$	Gosselin-Badaroudine et al. (2012b)
N.at-$K_V3.2$	4-AP		Klassen et al. (2008)
H_V1	Gua^+		Tombola et al. (2008)
	Gua^+, Zn^{2+}		Berger and Isacoff (2011)
TRPM3	Mg^{2+}, Ba^{2+}, Sr^{2+}, Ca^{2+}		Vriens et al. (2014)
	Mg^{2+}, Ca^{2+}		Held et al. (2015)

(Tombola et al. 2005; Sokolov et al. 2010). Like guanidine, these guanidine derivatives may enter the non-canonical pore, but due to their larger size, they may not be able to permeate and thus block the pathway. Interestingly, the proton channel H_V1 is blocked by Gua^+ (~85% block at 10 mM and $pH_i = 6.0$, $pH_o = 7.5$; ~35% at 10 mM and $pH_i = pH_o = 6.0$), possibly due to the limited size of the H_V1 pore size (Tombola et al. 2008; Berger and Isacoff 2011). Further support for this idea was given by Berger and Isacoff showing that the block by Gua^+ was altered when mutations at position R3 and D112 were introduced, allowing Gua^+ as well as metal ions to permeate through the gating pore (Berger and Isacoff 2011). Since guanidine derivatives selectively affect the non-canonical pore, these compounds are proposed as potentially interesting compounds for treatment of pathophysiological diseases caused by non-canonical pore leaks.

Finally, 4-AP was shown to block the gating pore currents in the naturally occurring non-canonical pore of N.at-Kv3.2 (~60% block at 10 mM) (Klassen et al. 2008).

The identification of blockers that selectively inhibit the non-canonical pore of specific channels without affecting the canonical pore may not only have therapeutic potential, as specific treatment for diseases linked to the opening of non-canonical pores (see Sect. 5), but would also represent powerful tools for further biophysical characterization of non-canonical pores and VSDs.

4.3 Conductance

Ionic currents through the non-canonical ion pathway occur through a relatively narrow pore within the VSD, and gating strongly depends on the membrane potential when the voltage sensor occupies a specific position (Tombola et al. 2005). Consequently, the ionic flow through the non-canonical pore of voltage-dependent cation channels is in general a relatively small "leak" current, occurring at potentials where most central pores are in a closed conformation. In the Shaker K^+ channel mutant E283D/R1C, it was determined that at −200 mV the amplitude of the non-canonical current was around 6% of the central pore currents at +60 mV (Tombola et al. 2005). A similar small conductance for the non-canonical pore at −140 mV of around 1% of the central pore currents at −10 mV was described for $Na_V1.4$ channels (Sokolov et al. 2007, 2008, 2010; Struyk and Cannon 2007; Struyk et al. 2008). Given the diversity of the gating pores investigated and the methods used to carry out the investigations, no consensus seems to have emerged concerning the conductance of gating pores. To compare the conductance values obtained from different studies, Moreau et al. have converted all the values into Siemens (Moreau et al. 2014). The calculated conductances span in a wide range of 0.17–1,060 fS for non-canonical pores created with single mutations and up to 3,400 fS for non-canonical pores created with multiple mutations (Moreau et al. 2014). In the cation channels containing a naturally occurring non-canonical pore, a conductance of around 13 pS at −150 mV was

described in HEK293 cells expressing TRPM3 and 38–140 fS in eosinophils expressing in H_V1 (Vriens et al. 2014; Cherny et al. 2003). A more detailed overview of the conductance values of the different cation channels and mutations is given by Moreau et al. (2014).

In general, the conductances of the non-canonical pores are lower than that of typical canonical pores. The large variety in conductance levels can be explained by variations in the size or shape of surrounding water crevices, as well as in the physicochemical properties of the residues within the VSD.

5 Potential Mechanisms Underlying Opening of the Non-canonical Pore

Several distinct mechanisms may contribute to the gating of the non-canonical pore.

5.1 Voltage-Gated Na^+, K^+, and Ca^{2+} Channels

The voltage sensitivity of the voltage-dependent cation channels is provided by the interaction of highly conserved charged residues. The S4 segment contains a series of at least three positively charged residues, while the S1, S2, and S3 segments contain organized structures of highly conserved negatively charged amino acids that appear to be involved in the stabilization of S4 in different conformational states, as discussed earlier (Papazian et al. 1995; Tao et al. 2010; Pless et al. 2011). At the center of each VSD is the gating charge transfer center (GCTC) that separates the intra- and extracellular water crevices, which gives rise to a hydrophobic septum that modulates the kinetics of the S4 segment. The larger the septum, the higher the free energy barrier is, meaning that charged amino acids such as arginine and lysine need more energy to move through the septum. During membrane depolarization, the S4 segment moves toward the extracellular medium and causes a conformational change that results in the opening of the canonical pore (Yang and Horn 1995; Yang et al. 1996). Mutagenesis studies have illustrated that neutralizing the positively charged amino acids of S4 interacting with the GCTC at a given state creates a non-canonical pore (Starace and Bezanilla 2004; Tombola et al. 2007; Gamal El-Din et al. 2010, 2014; Gosselin-Badaroudine et al. 2012a). When the charges of the GCTC are not counterbalanced by the charges of S4, the region becomes hydrophilic. The change from hydrophobic to hydrophilic implies that the water crevices are no longer separated, which allows ions to permeate through the newly formed non-canonical pore (Fig. 3a).

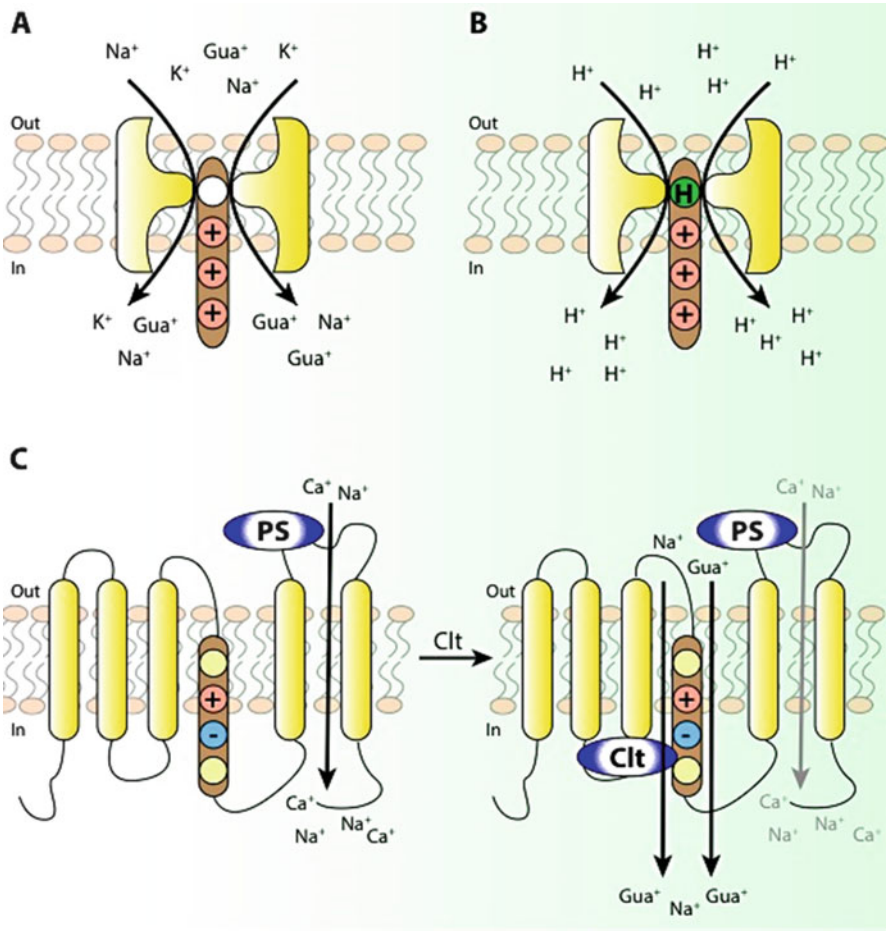

Fig. 3 Opening mechanisms of non-canonical pores. (**a**) An ionic flux through a non-canonical pore can occur due to a pore formation in the VSD by substitution of a small, uncharged amino acid at the position that is normally occupied by an arginine in S4. (**b**) A proton flux can occur due to a proton wire that is created by a histidine residue at the interface of the external and internal water crevices. (**c**) A non-canonical pore current can occur by conformational changes induced in the VSD due to ligand binding

5.2 Naturally Occurring Non-canonical Pores

5.2.1 H_V1 Channel

The gating mechanism of H_V1 channels appears to involve at least two major channel rearrangements, as revealed following the fluorescence changes of a probe attached to S4 (Gonzalez et al. 2010). These experiments showed that the fluorescence is biphasic with two fluorescence components both during depolarization and during repolarization (Qiu et al. 2013). During depolarization, the first fluorescence component is a

decrease associated with voltage-sensor movement from the resting to the activated state preceding pore opening. The second fluorescence component is an increase thought to reflect a conformational change from the activated to the open state of the pore (Qiu et al. 2013). Therefore, during H_V1 gating, concerted motions of the S1 and S4 segments take place, with S4 preceding the movement of S1 that concomitantly opens the channel (Mony et al. 2015). Using site-specific fluorescent labels to monitor changes in the environment near the external ends of both S1 and S4 helices in H_V1, it was shown that the kinetics of fluorescence signals from a probe on top of S4 is rapid with respect to activation of the proton conductance and also displays a voltage dependence that precedes pore opening. These observations are similar to those from fluorescent labels in S4 of Shaker and Na_V channels (Chanda and Bezanilla 2002; Mannuzzu et al. 1996; Cha and Bezanilla 1997). In contrast, the fluorescent label at the top of S1 monitors a relatively slower change in local environment than that reported by the label on S4. Both the slower kinetics and the voltage dependence of the fluorescence signals from the probe on S1 correlate with opening of the proton pore (Mony et al. 2015). Notably, D112 in the middle of S1 was found to be critical for proton selectivity (Berger and Isacoff 2011; Musset et al. 2011). The S4 helix moves upon depolarization with an exponential time course preceding H^+ current detection. The S1 segment in H_V1 channels contains three negatively charged residues (D160, E167 and D171), which may move inward at positive voltages and therefore could carry some of the total gating charge (Mony et al. 2015). Altogether, two distinct, but interdependent, rearrangements of S1 and S4 take place during H_V1 gating. However, the gating charges of H_V1 appear to be mostly contained in the arginines of S4, indicating that S1 motion is a consequence of the voltage-dependent rearrangement of S4.

However, several considerations must be kept in mind when discussing different movements of S4. Activation of the VSDs in voltage-activated ion channels necessarily involves transitions through multiple states from deep resting to intermediate to fully activated conformations (Bezanilla 2008). Although classical models associate such states with various positions of S4 as it moves outward during activation, it is intriguing to consider the possibility that some of these steps might instead reflect movements in other helices.

5.2.2 TRPM3

The induction of a non-canonical pore current after application of a ligand has been described in TRPM3.

Stimulation of the channel by the endogenous agonist PS results in the opening of a canonical pore. Interestingly, combined stimulation with PS and clotrimazole (Clt) leads to the activation of two distinct permeation pathways: the classical pore and a non-classical ion permeation pore (Vriens et al. 2014). However, it should be noted that in the absence of PS, no current increase was observed in TRPM3 cells after stimulation with Clt. Remarkably, co-application of Clt with other TRPM3-activating stimuli such as nifedipine or heat did not result in the opening of a non-canonical pore. A possible mechanism could be that PS binding on TRPM3

affects the hydrophobicity of a putative GCTC (Fig. 3c). This PS-induced change in hydrophobicity could allow S4 to move slightly deeper in the VSD during membrane hyperpolarization. The modification in S4 movement of TRPM3 is further stabilized by interaction with clotrimazole or creates an interaction side for clotrimazole on TRPM3.

However, further investigation is required to identify a possible interaction side for ligands of the non-canonical pore on TRPM3.

6 Pathophysiology of Non-canonical Pores

The impact of non-canonical pores is illustrated by the many disease-causing mutations in channelopathies linked to the existence of non-canonical currents. Currently, over 45 missense mutations have been identified in various channelopathies located at S4 arginines in Na_V, Ca_V, and K_V channels (Cannon 2010). In the following part, we will introduce different pathologies caused by mutations with reported non-canonical pore currents. An overview is given in Fig. 4 and Table 3.

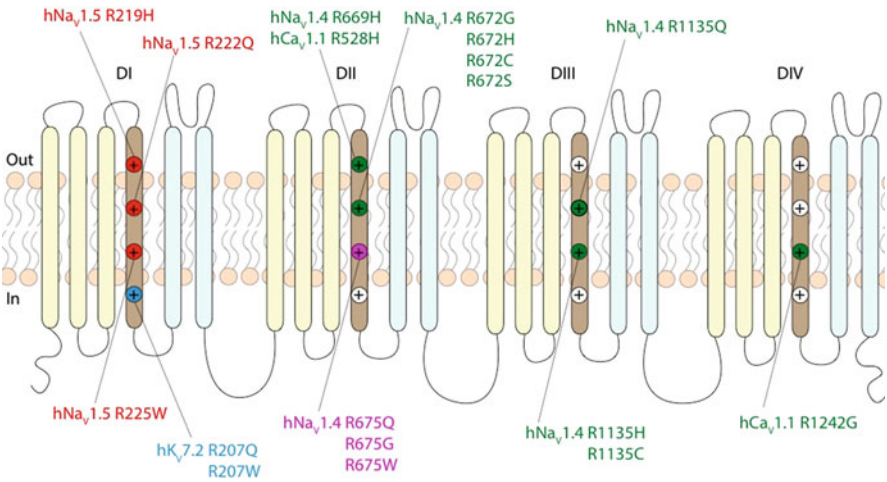

Fig. 4 Illustration of all reported disease-causing mutations leading to non-canonical pores. The locations of the mutations within the channel are indicated. The *color code* links the mutation to a specific disease: *red* dilated cardiomyopathy and cardiac arrhythmias, *blue* neuronal hyperexcitability, *magenta* normokalemic periodic paralyses, and *green* hypokalemic periodic paralyses. Note that the illustration of the channel does not properly represent the structure of the hK$_V$7.2 channel. hK$_V$7.2 forms a four-subunit channel and not a single-subunit channel with four connected domains as shown in the illustration

Table 3 Overviewing disease-causing mutations showing non-canonical pore currents

Channel	Location	Mutation	Disease
$hNa_V1.4$	DIIR1	R669H	HypoPP
$hNa_V1.4$	DIIR2	R672G/H/C/S	HypoPP
$hNa_V1.4$	DIIR3	R675Q/G/W	NormoPP
$hNa_V1.4$	DIIIR2	R1132Q	HypoPP
$hNa_V1.4$	DIIIR3	R1135H/C	HypoPP
$hCa_V1.1$	DIIR1	R528H	HypoPP
$hCa_V1.1$	DIVR3	R1242G	HypoPP
$hNa_V1.5$	DIR1	R219H	Mixed arrhythmias and DCM
$hNa_V1.5$	DIR2	R222Q	Mixed arrhythmias and DCM
$hNa_V1.5$	DIR3	R225W	Mixed arrhythmias and DCM
$hK_V7.2$	R4	R207Q/W	BFNS and PNH

6.1 Periodic Paralysis

Periodic paralyses are skeletal muscle disorders caused by mutations in sodium, potassium, and calcium channel genes. The disease has been characterized by periodic muscle weakness in relation to variations of the serum potassium levels (Platt and Griggs 2009; Raja Rayan and Hanna 2010). Depending on the serum potassium levels, the diseases can be categorized as hyperkalemic periodic paralysis (HyperPP) (high serum K; >5.5 mEq/L), HypoPP (low serum K; <3.5 mEq/L)), and NormoPP (normal serum K; between 3.5 and 5.5 mEq/L) (Korgaonkar et al. 2010; Joshi et al. 2010). Recently, HypoPP and NormoPP were linked to mutations of the voltage sensor of skeletal muscle cation channels that result in non-canonical currents through the VSD (Sokolov et al. 2007, 2008, 2010; Struyk and Cannon 2007; Struyk et al. 2008; Francis et al. 2011; Groome et al. 2014; Wu et al. 2011, 2012a; Mi et al. 2014; Fan et al. 2013).

HypoPP is a rare autosomal-dominant disorder characterized by recurrent attacks of muscle weakness together with low serum potassium levels that are triggered by stimuli like rest after an exercise, carbohydrate-rich food, or exposure to cold. Regularly, HypoPP patients wake up in a paralyzed state and generally the attacks last several hours to days. At older age it can develop to persistent muscle weakness. The paralysis episodes can be explained by a change in resting membrane potential (from −85 mV to −60 mV) under hypokalemic conditions, which will lead to muscle fiber inexcitability (Platt and Griggs 2009; Jurkat-Rott et al. 2009, 2012; Matthews and Hanna 2010; Rudel et al. 1984). Missense mutations in the alpha subunits of the skeletal muscle sodium and calcium channels, $Na_V1.4$ and $Ca_V1.1$, have been found to cause HypoPP. Six out of seven missense mutations of $Ca_V1.1$ (CACNA1S) causing HypoPP1 are located at the position of an arginine in S4 in the VSD (Fig. 4). The $Ca_V1.1$ mutations account for around 60% of the HypoPP cases, while around 20% of the cases are caused by mutations in the $Na_V1.4$ (SCN4A) channel (HypoPP2). All existing HypoPP $Na_V1.4$

mutations are located at arginine residues in S4 (Platt and Griggs 2009; Cannon 2010; Raja Rayan and Hanna 2010; Wu et al. 2012a; Jurkat-Rott et al. 2012; Matthews and Hanna 2010). In general, non-canonical pore currents are described as the common mechanism leading to the muscular pathology (HypoPP1 and HypoPP2) (Sokolov et al. 2007; Struyk and Cannon 2007). This was the conclusion after investigating the specific location of all different missense mutations (S4 segments) in the two genes encoding $Ca_V1.1$ and $Na_V1.4$. An overview of the different disease-causing mutations is given in Table 3. In general, two different types of mutations have been identified: a first group consists of mutations causing the formation of a non-canonical cationic current (Sokolov et al. 2007) and a second group of mutations are mutations inducing a proton-specific leak current (Struyk and Cannon 2007). The existence of a non-canonical cationic current was first shown by Sokolov et al. indicating that a HypoPP causing mutation on the S4 segment of $Na_V1.4$ (R666G) unveils non-canonical currents (Sokolov et al. 2007). Later, Struyk and colleagues revealed a proton-selective non-canonical current in the HypoPP mutant R669H (Struyk and Cannon 2007), followed by additional studies implicating non-canonical pores in HypoPP mutations (Struyk et al. 2008; Francis et al. 2011; Wu et al. 2011, 2012a; Groome et al. 2014; Mi et al. 2014). Interestingly, Wu et al. introduced the HypoPP causing $Na_V1.4$ channel mutation (R669H) in a knockin mouse model, which enabled them to study its effects on the disease level as well as on a biophysical level. These mice exhibited a clear HypoPP phenotype and measurements in muscle fibers of the knockin mice revealed the existence of a hyperpolarization-activated non-canonical current (Wu et al. 2011). A similar technique was used to insert a $Ca_V1.1$ channel mutation (R528H) resulting in a HypoPP phenotype (Wu et al. 2012a). K^+-sensitive normokalemic periodic paralysis was proposed to be an intermediate form of periodic paralyses, where patients have periodic muscle weakness, but maintain normal serum potassium levels during attacks (3.5–5.5 mEq/L) (Sokolov et al. 2008; Platt and Griggs 2009; Joshi et al. 2010). However, it was reported that some NormoPP diagnosed patients contain typical HyperPP mutations or showed Hypo- or HyperPP phenotypes, suggesting that it is just a phenotypic variant of Hypo- or HyperPP (Chinnery et al. 2002; Vicart et al. 2004). Sokolov et al. were the first to describe non-canonical pore currents in NormoPP causing mutations of the $Na_V1.4$ channel. In this study, the existence of non-canonical currents was indicated occurring at depolarized potentials or in the slow-inactivated state after long depolarization in R669Q/G/W of the rat $Na_V1.4$ channel (Sokolov et al. 2008). Although the contribution of non-canonical pore currents to sodium influx during an action potential is minor, they do have a large effect during resting phases. A large amount of fibers stay in the slow-inactivated state at rest. In addition, the number of channels in the slow-inactivated state will further increase after trains of action potentials during forceful contractions (during exercise). In these conditions sodium would be primarily entering the cell, leading to a sodium overload and an impairment of action potential generation, resulting in the pathology of NormoPP (Sokolov et al. 2008). After that, another study described a NormoPP mutation of $Ca_V1.1$ channels

(R1242G) and linked it to non-canonical currents occurring through the VSD (Fan et al. 2013).

The downstream molecular mechanisms connected to non-canonical pores in Hypo- and NormoPP are not fully understood yet. Nevertheless, different theories were suggested. It was proposed that non-canonical pores lead to an elevation in resting membrane potential (V_{Rest}) by the non-canonical cationic currents. The increase in V_{Rest} would result in a reduced excitability of muscle fibers (Sokolov et al. 2007; Moreau et al. 2014). Missense mutations leading to a non-canonical proton current were proposed to induce acidosis, which can impair myoplasmic pH homeostasis and changes in V_{Rest} (Struyk and Cannon 2007). Moreover, acidification could lead to excitation–contraction uncoupling by reduction of the troponin C affinity to Ca^{2+}. Further, acidification can disrupt connections of gap junctions through block of connexins (Moreau et al. 2014). It was suggested that an overload of Na^+, in case of metal ion conducting non-canonical pores, might block the Na^+/H^+ exchanger or induce a reverse mode, leading also to acidosis (Moreau et al. 2014, 2015b). Against this, some groups put forward that all non-canonical pore mutations, even the proton-specific ones, might lead to an overload of sodium in the cell, resulting in a depolarization of the resting membrane potential (Sokolov et al. 2007, 2008; Struyk and Cannon 2007; Gosselin-Badaroudine et al. 2014). This occurs either direct via a metal-ion-selective non-canonical current over the membrane (Na^+ influx) or indirect via an increased activity of the Na^+/H^+ exchanger induced by a proton flux over the non-canonical pore (Struyk and Cannon 2007; Sokolov et al. 2008; Gosselin-Badaroudine et al. 2012b). The sodium overload can have diverse effects. First, it augments the activity of the Na^+/K^+ pump, which might explain, for example, the persistent, modest hypokalemia phenotype in case of HypoPP patients (Struyk et al. 2008). Second, sodium overload might induce mitochondrial production of reactive oxygen species (ROS), leading to cellular remodeling, excitation–contraction uncoupling, and electrical disturbances (Moreau et al. 2014). Third, it can induce increased activity of the Na^+/Ca^{2+} exchanger in reverse direction leading to a Ca^{2+} overload (Moreau et al. 2015b; Gosselin-Badaroudine et al. 2012b, 2014). Increased Ca^{2+} can induce excitation–contraction uncoupling due to decreasing troponin C affinity for Ca^{2+} as well as uncoupling of gap junctions via connexin block (Gosselin-Badaroudine et al. 2012b; Moreau et al. 2015b). In short, an ionic homeostasis imbalance, induced by either a proton or a sodium leak through non-canonical pores, can influence many downstream cellular processes by changes in ionic transporter activities and thereby causing the phenotypes observed in Hypo- and NormoPP. Another theory proposes that the increased membrane potential due to non-canonical currents prevents the ability of muscle cells to withstand reductions in serum potassium levels (Struyk et al. 2008; Cannon 2010; Jurkat-Rott et al. 2012; Moreau et al. 2015b). This ability is normally guaranteed through the K_{IR} potassium channel, which is important to maintain the resting membrane potential. K_{IR} works against a small depolarizing leak conductance over the membrane. If extracellular potassium drops, the K_{IR} activity will decrease. At very low potassium concentrations under 1 mM, I_{KIR} becomes too small to compensate I_{Leak}. This situation will

induce an abrupt jump of the resting membrane potential to more depolarized values (from around −90 mV to −65 mV) due to the nonlinear I–V characteristics of I_{KIR}. A non-canonical pore current would increase I_{Leak} even more and therefore shift the depolarization threshold against physiological potassium concentrations (Struyk et al. 2008; Cannon 2010; Jurkat-Rott et al. 2012). This would be a unifying explanation for the effects of proton as well as sodium conducting non-canonical pores, resulting in the same disease phenotype in HypoPP.

6.2 Mixed Arrhythmias and Dilated Cardiomyopathy

Cardiac diseases are often caused by mutations in the SCN5A gene encoding the cardiac voltage-gated sodium channel $Na_V1.5$. Dilated cardiomyopathy (DCM) results in dilated cardiac chambers and a reduced systolic function, inducing mixed arrhythmias and heart failure (Gosselin-Badaroudine et al. 2012b, 2014). DCM causing mutations are either located in the intracellular loops or in the VSD of $Na_V1.5$ (Fig. 4). All intracellular loop mutations induce a persistent sodium current (Gosselin-Badaroudine et al. 2014). Fittingly, a $Na_V1.5$ mutation at position R219H in S4, inducing cardiac conduction disorder and DCM, was linked to the presence of a non-canonical ionic current (Gosselin-Badaroudine et al. 2012b). Similarly, other $Na_V1.5$ mutations, R222Q and R225W, causing complex arrhythmias and dilated cardiomyopathy, are linked to the presence of non-canonical cationic currents at depolarized potentials or in the slow-inactivated state of the channel (Moreau et al. 2015b).

At the moment no common disease-causing mechanism for DCM is described, as clinical phenotypes cannot be solely explained by changes in the electrical properties of the sodium channel. A possible explanation could be the induction of an ionic homeostasis imbalance through persistent non-canonical pore currents. These non-canonical currents will result in an unstable resting membrane potential and premature ventricular depolarization as well as dysfunctions in heart rhythm or cardiac contractile functions (Gosselin-Badaroudine et al. 2012b, 2014; Moreau et al. 2015a).

6.3 Neuronal Hyperexcitability

Benign familial neonatal seizure (BFNS) is an autosomal-dominant form of epilepsy in newborns. BFNS is characterized by recurrent seizures in newborn babies. The seizures begin around day 3 after birth and usually self-resolve within 1–4 months. The seizures can involve one side (focal seizures) or both sides of the brain (generalized seizures). Typically, seizures are the characteristic symptom of BFNS, and prognosis is generally good (10% seizure recurrence) with usually normal developmental outcome. The risk of seizures during adulthood is around 15%

(Miceli et al. 2012; Jurkat-Rott et al. 2012). BFNS is caused by mutations in two genes, *KCNQ2* and *KCNQ3*, encoding for $K_V7.2$ and $K_V7.3$ potassium channels, respectively. Mutations in *KCNQ2* are more frequent than mutations in *KCNQ3*. The voltage-dependent potassium channels $K_V7.2$ or $K_V7.3$ are functionally expressed in neurons of the brain, where they coassemble to produce the so-called M-current. This current is involved in the control mechanism of the membrane potential around the action potential threshold and prevents neurons to continually transmit signals to other cells. Mutations in one of these channel genes result in a reduced or altered M-current, which give rise to peripheral nerve hyperexcitability (PNH). Seizures develop when neurons in the brain are abnormally excited, which is characterized by muscle overactivity and will escalate in muscle cramps (Jurkat-Rott et al. 2012). Most of the BFNS causing mutations in $K_V7.2$ are located in the C-terminal domain or in the VSD, and some have been implicated in the existence of non-canonical pores, when expressed in the background of analogous $K_V7.4$ channels (Fig. 4) (Miceli et al. 2012). The pathological mechanism of PNH could be explained by the presence of non-canonical pore currents, which could result in a persistent depolarization of the membrane potential. This increase in V_{Rest} might facilitate action potential firing, thereby causing neuronal hyperexcitability (Miceli et al. 2012).

6.4 Naturally Occurring Non-canonical Pore Currents

In clear contrast to the classical voltage-dependent cation channels, H_V1 lacks a pore domain and thus permeation necessarily occurs through the voltage-sensing domain. The main role of voltage-gated proton channels is to extrude protons from the intracellular milieu when, mediated by different cellular processes, the H^+ concentration increases. The H_V1 channel is involved in a wide variety of specialized physiological processes such as chondrocyte alkalization after hypotonic shock (Sanchez et al. 2006), fertilization in human sperm (Lishko et al. 2010), acid secretion in airways (Fischer et al. 2002), and in the facilitation of B-cell activation during immune response (Capasso et al. 2010). The best characterized function of proton channels is the electron balance mediated by the H^+ flux during the phagocytic respiratory burst. H_V1 channels appear to be key for optimization of NADPH oxidase activity when the pH in the phagosomal lumen and cytoplasm decreases (El Chemaly et al. 2010). Altered function of the proton channel entails several physiological dysfunctions. For example, H_V1 channels have been implicated in the enhancement of brain damage after an ischemic stroke. Mice lacking H_V1 are protected from NOX-mediated neuronal death and brain damage 24 h after stroke (Wu et al. 2012b).

Evidence was provided for the presence of a naturally occurring non-canonical pore in wild-type TRPM3 channels. Activation of a non-canonical pore current can contribute to the genesis of pain signals. This is substantiated by the observation that intraplantar injection of mice with Clt alone does not produce nocifensive

responses but does potentiate responses to injection of PS in a TRPM3-dependent manner (Vriens et al. 2014). Moreover, the nocifensive behavior to CIM0216 was remarkably more exhaustive compared with intraplantar injection of a sixfold higher dose of PS (Held et al. 2015). These observations provide evidence that activation of a non-canonical pore in TRPM3 exacerbates nociceptive behavior. This can be explained by the large inward current, which under physiological conditions is carried mainly by Na^+. In excitable cells, such as sensory neurons, robust Na^+ influx may facilitate membrane depolarization and concomitant Ca^{2+} influx via voltage-gated Ca^{2+} channels. High expression of TRPM3 is also described in the brain with the most prominent expression in epithelial cells of the choroid plexus (Oberwinkler et al. 2005; Hasselblatt et al. 2009). The choroid plexus is responsible for the formation and regulation of the cerebrospinal fluid. Possible TRPM3 might be involved in the regulation of the cerebrospinal fluid and dysregulation of TRPM3 gating may result in the formation of choroid plexus cysts or in the formation of choroid plexus papillomas (Hasselblatt et al. 2009). In mouse brain, TRPM3 transcripts could also be detected in several other brain regions like the cerebellum, cerebrum, brain stem, hippocampus, forebrain, and hypothalamus (Lee et al. 2003; Kunert-Keil et al. 2006; Hoffmann et al. 2010; Oberwinkler et al. 2005). Although the physiological role of TRPM3 in these brain regions is not known, it can be envisaged that activation of the non-canonical pore in TRPM3 might have neurological and psychiatric consequences.

7 Conclusions and Perspectives

There is extensive evidence for the existence of non-canonical pores in some mutant voltage-gated K^+, Na^+, and Ca^{2+} channels (Starace and Bezanilla 2001; Tombola et al. 2005; Sokolov et al. 2007; Wu et al. 2012a). Additionally, wild-type TRPM3 is described to have a naturally occurring non-canonical pore that can be opened with the right combination of agonists (Held et al. 2015; Vriens et al. 2014). Furthermore, a non-canonical pore is also defined for the H_V1 proton channel, although the channel is unique in that it contains the pore and gate within its voltage-sensing domain (Ramsey et al. 2006; Tombola et al. 2008). The segment of the channel comprising the non-canonical pore ordinarily functions as the passageway for the charged residues of the voltage-sensor (S4) region, but mutations of these charged residues in voltage-gated K^+, Na^+, and Ca^{2+} channels can open the way for positively charged ions.

The impact of non-canonical pore currents has been shown by the different disease-causing mutations. To date, different phenotypes, like periodic paralysis, arrhythmias with dilated cardiomyopathy, and peripheral nerve hyperexcitability, have been clearly associated with the appearance of non-canonical pore currents (Sokolov et al. 2007; Gosselin-Badaroudine et al. 2012b; Miceli et al. 2012). These pathologies are directly caused by mutations in Na_V, Ca_V of K_V channels. Additionally, the discovery that a non-canonical pore of TRPM3 contributes to the

genesis of pain signals make non-canonical pores an attractive target for new analgesics (Held et al. 2015; Vriens et al. 2014). The better comprehension of non-canonical pores might offer insights in an increasing number of disease phenotypes caused by channels with non-canonical pores and might reveal other pathologies caused by the appearance of non-canonical pore currents.

At the moment there is a strong need for the development of blockers that selectively inhibit the non-canonical pore of specific channels without affecting the canonical pore. Unfortunately, none of the reported non-canonical pore blockers is clinically useful. However, identification of a selective blocker of the non-canonical pore would be very potentially helpful for patients, since they only benefit from symptomatic treatment targeting the downstream consequences of the mutation (paralysis or arrhythmias). In the future, we can look forward to the development of non-canonical pore blockers to complement the central pore blockers already available for many voltage-gated ion channels. However, maintaining the gating properties and the canonical pore functions of channels is a major challenge for developing therapies to specifically treat non-canonical pore current-induced pathologies.

Currently, only a few crystal structures of voltage-gated ion channels have been reported (Jiang et al. 2003; Long et al. 2005, 2007; McCusker et al. 2012; Payandeh et al. 2011; Zhang et al. 2012), but no structural information is available of the mutant channels containing the non-canonical pore. Recently, a 3.45-Å resolution crystal structure of a chimeric construct of the mouse proton channel mH_V-cc, containing parts of mH_V, C*i*VSD (VSD of voltage sensitive phosphatase from *Ciona intestinalis*, C*i*VSD), and a fragment of the GCN4 coiled coil, has been reported, providing the first glimpses of a proton channel structure, including its close agreement with a consensus VSD scaffold and the relative positions of functionally critical residues. However, due to the unorthodox nature of the chimeric construct, details on the individual role of the gating charges, plus the mechanisms of proton permeation and cooperative gating, remain to be established (Takeshita et al. 2014). However, structural information related to TRPM3 may be within reach given the recent successes in determination of the structure of TRPV1 and TRPA1 using cryoEM (Liao et al. 2013; Paulsen et al. 2015). These findings could provide a platform for understanding the general principles of voltage sensing and gating of non-canonical pores.

Furthermore, the knowledge related to the biophysical properties of non-canonical pores is very incomplete. The difficulties to find a consensus may be caused by the fact that the described non-canonical pores are occurring in various channels that possess differences in their VSDs. Unifying biophysical properties might therefore not exist, and instead, every non-canonical pore has to be treated as a pore with unique properties. Additional investigations are required to unravel open questions like the dynamics and interactions of other segments of the VSD involved in opening of the non-canonical pore and the number of S4 segments involved in the appearance of a non-canonical current. Identifying the mechanisms by which specific ligands open the non-canonical pore in TRPM3 or other naïve

channels is a challenge for the future. Unaddressed questions remain the location and/or existence of a possible binding site in ligand-gated non-canonical pores.

TRPM3 represents one of the first examples of a naturally occurring channel with what appears to be a non-canonical pore. The question will raise whether other non-canonical pores in other ion channels that contain uncharged S4 regions exist and what will be the physiological impact of opening of the non-canonical pore? Moreover, what will be the endogenous activator, if any, for the non-canonical pore and when is it released?

Acknowledgments We thank all members of the Laboratory of Experimental Gynecology and the members of the Laboratory of Ion Channel Research for helpful discussions. This work was supported by grants from the Belgian Federal Government (IUAP P7/13 to T.V.), the Research Foundation-Flanders (G.0565.07 and G.0825.11 to T.V. & J.V. and G.084515N to J.V.), and the Research Council of the KU Leuven (PF-TRPLe to T.V.). K.H. is funded by the FWO Belgium.

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Rev Physiol Biochem Pharmacol (2016) 170: 101–128
DOI: 10.1007/112_2015_5004
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Published online: 8 January 2016

Cardiac Response to Oxidative Stress Induced by Mitochondrial Dysfunction

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Abstract The heart works without resting, requiring enormous amounts of energy to continuously pump blood throughout the body. Because of its considerable energy requirements, the heart is vulnerable to oxidative stress caused by the generation of endogenous reactive oxygen species (ROS). Therefore, the heart has effective regulatory and adaptive mechanisms to protect against oxidative stress. Inherited or acquired mitochondrial respiratory chain dysfunction disrupts energy metabolism and causes excessive ROS production and oxidative stress. The physiological cardiac response to oxidative stress can strengthen the heart, but pathological cardiac responses or altered regulatory mechanisms can cause heart disease. Therefore, mitochondria-targeted antioxidants have been tested and some are used clinically. In this review, we briefly discuss the role of mitochondrial DNA mutations, mitochondrial dysfunction, and ROS generation in the development of heart disease and recent developments in mitochondria-targeted antioxidants for the treatment of heart disease.

Keywords Heart disease • Mitochondrial dysfunction • Mitochondrial medicine • Mitochondrial reactive oxygen species • Oxidative stress

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Conflict of interest: None.

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1 Introduction

Approximately 30 kg adenosine triphosphate (ATP) is generated and used by the human heart each day (Dorn 2013). Because the heart muscle has ATP reserves for only 20–40 strokes, it requires a highly efficient energy production system to enable continuous pumping of the blood throughout the body. Mitochondrial oxidative phosphorylation is capable of producing >30 ATP molecules per glucose molecule, providing the heart with >95% of the required ATP. To meet the energy demands of the heart, mitochondria comprise more than 30% of its mass (Page and McCallister 1973). Oxidative phosphorylation also produces various reactive oxygen species (ROS) and reactive nitrogen species (RNS), including superoxide radical anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot} + OH^-$), and peroxynitrite ($ONOO^-$) (Sovari et al. 2012). At physiological concentrations, ROS and RNS function as molecular messengers to modulate biological activities such as cell growth, anti-inflammatory responses, cell differentiation, and hormone synthesis. However, when produced in excess, ROS rapidly oxidize biomolecules (e.g., DNA, proteins, lipids), resulting in cellular dysfunction and cell death (Fig. 1). Therefore, effective systems for producing and clearing ROS are necessary for cell survival. For this purpose, various endogenous antioxidants including manganese or copper and zinc superoxide dismutase (MnSOD or Cu/ZnSOD), catalase, glutathione peroxidase, and peroxiredoxin existed in mitochondria or cell. An imbalance between ROS production and clearance leads to oxidative stress, which can cause a wide range of cardiovascular diseases including hypertension (de Champlain et al. 2004), coronary artery disease (Vichova and Motovska 2013), hypertrophy (Takimoto and Kass 2007), cardiomyopathy, and heart failure (Seddon et al. 2007). In this context, it is important to understand the role of ROS in both

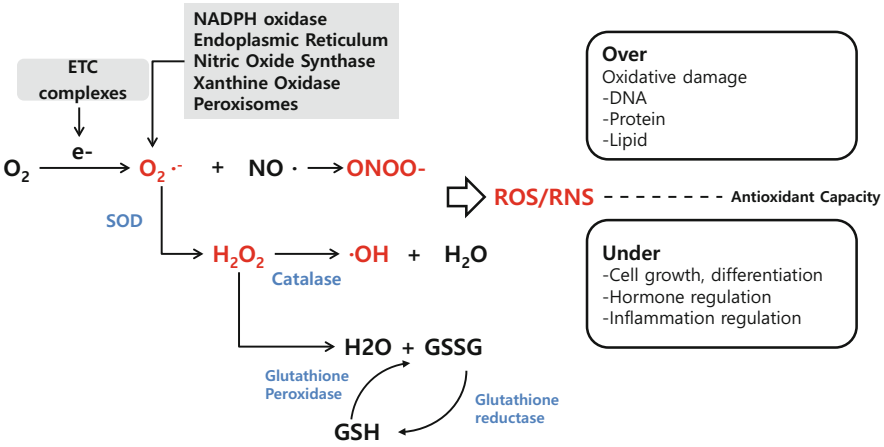

Fig. 1 Generation, clearance, and role of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Sources of intracellular ROS generation are depicted in *gray boxes*; ROS/RNS are shown in *red* and antioxidants in *blue*. ROS/RNS function as signaling molecules that regulate physiological functions (*lower box*). Oxidative damage occurs when ROS/RNS production exceeds the cell's antioxidant capacity (*upper box*). *ETC* electron transport chain, *SOD* superoxide dismutase, *GSSG* oxidized glutathione, GSH, reduced glutathione

normal physiological processes and disease states and the cardiac response to ROS in ischemia/reperfusion injury, diabetes, hypertrophy, and endothelial shear stress.

2 Oxidative Stress and Mitochondrial Dysfunction

The cause-and-effect relationship between mitochondrial dysfunction and oxidative stress has not been completely elucidated. Oxidative stress induces mitochondrial dysfunction and apoptosis. As a result, the damaged mitochondrial electron transfer chain (ETC) complexes produce even more ROS, further increasing oxidative stress in the mitochondrion itself, as well as other subcellular organelles such as the endoplasmic reticulum (ER), sarcoplasmic reticulum, nucleus, and plasma membrane. The primary sources of mitochondrial ROS production are complex I and complex III; however, recent studies have implicated complexes II and IV in $O_2^{\bullet -}$ production in disease conditions (Chen and Zweier 2014). Specific cellular mechanisms underlying mitochondrial ROS generation are well explained in the excellent review by Chen and Zweier (2014).

A unique feature of mitochondria is that these organelles contain their own DNA. Human mitochondrial DNA (mtDNA) is a circular, covalently closed, double-stranded DNA molecule that contains 37 genes encoding 13 ETC component proteins, 2 ribosomal RNAs, and 22 transfer RNAs (Anderson et al. 1982). Recently, a fourteenth mitochondrial-derived peptide (humanin) was identified in

three different groups (Guo et al. 2003; Hashimoto et al. 2001; Ikonen et al. 2003). Humanin is encoded in the mitochondrial genome by the 16S ribosomal RNA gene. It has neuroprotective and cytoprotective roles and IGFBP-3 binding ability, which is involved in Alzheimer's disease, apoptosis regulation, and IGF-1 signaling (Guo et al. 2003; Hashimoto et al. 2001; Ikonen et al. 2003). Further detailed information of humanin is well described in the review of Cohen group (Yen et al. 2013).

Since mtDNA is located near the ETC, which is the major site of ROS generation, it is easily damaged, resulting in mutations or degradation (Shokolenko et al. 2009). Previously, it was thought that mtDNA lacks a DNA repair system, increasing its susceptibility to oxidative stress (Ames et al. 1995; Cadenas and Davies 2000). However, recent studies have described a mitochondrial DNA repair system similar to that of nuclear DNA, including base excision repair, single- and double-strand break repair, and mismatch repair (for review see (Alexeyev et al. 2013; Berneburg et al. 2006)). Mutations in mtDNA have been associated with a number of conditions including seizures, ataxia, cortical blindness, dystonia, diabetes, cardiomyopathy, hearing loss, kidney failure, and various cancers (DiMauro and Schon 2001; Lu et al. 2009; Wallace 1997). These findings suggest that mtDNA mutations impair mitochondrial function, thereby causing tissue-specific dysfunction or disease. This hypothesis has been supported by studies of mitochondrial dysfunction in mtDNA mutant cell lines (Ishikawa et al. 2008) and animal models (Ahlqvist et al. 2012; Dai et al. 2010; Hashizume et al. 2012; Hiona et al. 2010; Kolesar et al. 2014; Kujoth et al. 2005; Logan et al. 2014; Lu et al. 2009; Mito et al. 2013; Safdar et al. 2011; Trifunovic et al. 2004; Vermulst et al. 2008; Yamada et al. 2012).

Several research groups have generated mitochondrial DNA polymerase gamma mutant ($Polg^{D257A}$) mice (Kujoth et al. 2005; Trifunovic et al. 2004). These mice show a high frequency of mtDNA mutations in multiple tissues and a premature aging phenotype, with decreased oxidative phosphorylation and increased oxidative stress (Kujoth et al. 2005; Logan et al. 2014; Trifunovic et al. 2004; Trifunovic et al. 2005; Vermulst et al. 2008). The $Polg^{D257A}$ mice also exhibit sarcopenia, muscle weakness, cardiac hypertrophy, and dilatation, which are associated with significant defects in ETC complex I, III, and IV assembly (Dai et al. 2010; Hiona et al. 2010; Kolesar et al. 2014; Yamada et al. 2012). Similarly, a recently generated specific mtDNA mutation in the gene encoding NADH dehydrogenase subunit 6 (*ND6*) resulted in deficient complex I activity and ROS overproduction in a mouse tumor cell line, enhancing its metastatic potential (Ishikawa et al. 2008). Mice with the *ND6* G13997A mutation also showed deficient complex I activity and excessive ROS production in addition to lactic acidosis, diabetes, multiple tissue defects, and an elevated risk of lymphoma (Hashizume et al. 2012). Mutations in genes encoding proteins involved in mtDNA replication (e.g., mitochondrial transcription factor A (Wang et al. 1999), mitochondrial helicase TWINKLE (Milenkovic et al. 2013)) or the regulation of oxidative phosphorylation (e.g., adenine nucleotide transporter 1 (Narula et al. 2011)) result in the depletion of mtDNA, subsequent mitochondrial dysfunction, and cardiac diseases including hypertrophy, dilated cardiomyopathy, and conduction blocks (Kujoth et al. 2007).

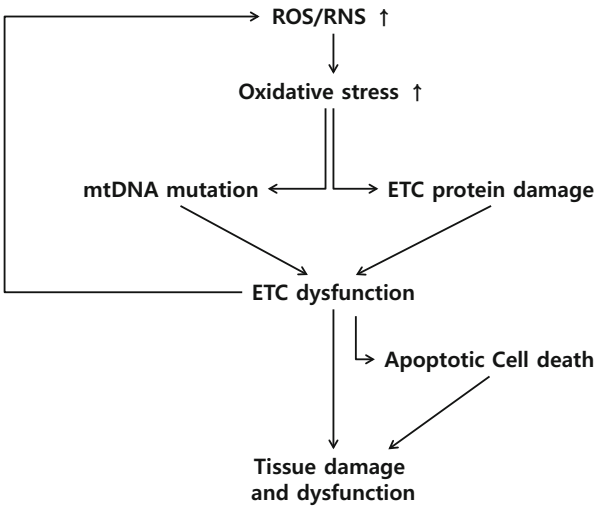

Fig. 2 Oxidative stress-induced mtDNA mutations result in mitochondrial dysfunction and tissue damage. Production of ROS/RNS that exceeds the cell's antioxidant capacity increases intracellular oxidative stress. The resulting damage to ETC proteins and mtDNA results in impaired oxidative phosphorylation and ROS overproduction, inducing apoptosis and causing tissue damage. *ETC* electron transport chain, *RNS* reactive nitrogen species, *ROS* reactive oxygen species

Conversely, overexpression of TWINKLE attenuates cardiac fibrosis and heart failure in mice with pressure overload hypertrophy (Tanaka et al. 2013). These results support the idea that oxidative stress increases mtDNA mutations and damages ETC proteins, thereby impairing oxidative phosphorylation and ultimately leading to cell death and tissue failure (Fig. 2).

3 Mitochondrial Quality Control System: Mitophagy and ROS

In addition to endogenous antioxidants, mitophagy acts as a mitochondrial quality control system to protect the cell. Mitophagy involves the autophagosomal degradation of abnormal mitochondria containing damaged components or producing excessive ROS. Pathological heart conditions including I/R injury, diabetic cardiomyopathy, and cardiac hypertrophy cause oxidative damage to cardiac mitochondria, leading to ROS overproduction, activation of inflammatory signals, and local tissue injury via NF-kappa B and NOD-like receptor family 3 (NLRP3) signaling (Gottlieb et al. 2011). In addition, mtDNA released from damaged mitochondria activates the NLRP3 inflammasome. By decreasing the number of damaged mitochondria, mitophagy decreases ROS production and inflammation to prevent further cardiac damage. Conversely, impairment of autophagy increases the number of

damaged mitochondria in cardiomyocytes, exacerbating inflammation, oxidative stress, and heart damage (Gottlieb et al. 2011). Autophagy is induced by starvation through AMPK signaling, and ROS play an important role in autophagy and mitophagy (Chen et al. 2009; Gottlieb and Carreira 2010; Korolchuk et al. 2010; Scherz-Shouval et al. 2007). A recent study by Scherz-Shouval demonstrated that starvation stimulates the formation of hydroperoxide, which is essential for autophagy, whereas antioxidant treatment prevents autophagosome formation and protein degradation (Scherz-Shouval et al. 2007). Excessive mitophagy can impair cardiac function; therefore, mitophagy must be carefully regulated to maintain normal heart function (Tang et al. 2015).

4 Non-mitochondrial Cytosolic ROS Sources

In addition to mitochondria, sources of ROS generation include NADPH oxidase (Nox) family (Griendling et al. 2000), ER (Dickinson and Chang 2011; Gross et al. 2006), nitric oxide synthase (NOS) (Landmesser et al. 2003; Umar and van der Laarse 2010; Zhang et al. 2012), xanthine oxidase (Kelley et al. 2010), and peroxisomes (Antonenkov et al. 2010), depending on the tissue and cell type. These intracellular ROS sources are closely linked, and the cumulative ROS levels modulate heart function under physiological and pathological conditions.

4.1 Cardiac NADPH Oxidase

As one of the major cellular sources of ROS, cardiac Nox plays an important role in a wide range of physiological and pathological processes including hypoxic adaptation, hypertrophy, apoptosis, and heart failure (Brandes et al. 2010). In particular, the isoforms Nox2 and Nox4 appear to have major roles within the myocardium, with Nox2 producing superoxide and Nox4 generating only hydrogen peroxide (Zhang et al. 2012). Nox2- and Nox4-derived $O_2 \cdot^-$ and H_2O_2 are involved in the growth response of vascular smooth muscle cells, cardiac cells, and fibroblasts; JNK/p38 MAPK and Akt signaling; and the expression of cardiovascular-related genes involved in hypertrophy and development of atherosclerosis and hypertension (Griendling et al. 2000).

4.2 Endoplasmic Reticulum: Ero1p and Nox4

The ER also produces ROS through Ero1p, an enzyme that transfers electrons from thiol substrates to molecular oxygen (Gross et al. 2006). In addition, Nox4 generates H_2O_2 from $O_2 \cdot^-$ in the ER by two-electron reduction (Chen et al. 2008). ROS

production and oxidative stress are closely related to ER stress and the unfolded protein response, which regulates intracellular signaling transduction and cell death (Santos et al. 2009). A recent study suggested that under ER stress, ROS production is increased by the Nox family of enzymes, which may contribute to the development of hypertension and other cardiovascular diseases (Santos et al. 2014). In cardiomyocytes, Nox4 mediates autophagy in response to energy stress by stimulating the protein kinase RNA-activated-like ER kinase signaling pathway (Sciarretta et al. 2013).

4.3 Nitric Oxide Synthases

Nitric oxide synthases produce NO, a highly reactive signaling molecule, through oxidative conversion of L-arginine to L-citrulline. In the heart, neuronal NOS and endothelial NOS constitutively produce NO in distinct subcellular locations, whereas inducible NOS is upregulated under certain pathological conditions such as I/R injury (Umar and van der Laarse 2010). Tetrahydrobiopterin is an essential cofactor for NO production by all three NOS isoforms. In the absence of tetrahydrobiopterin, NOS functions in an uncoupled state, producing ROS instead of NO. The lower NO bioavailability and increased oxidative stress in the heart lead to pathological cardiac remodeling (hypertrophy, fibrosis) and heart failure (Landmesser et al. 2003; Umar and van der Laarse 2010; Zhang et al. 2012).

4.4 Xanthine Oxidase

Another major source of ROS production in the heart is xanthine oxidase, which is converted from xanthine dehydrogenase by the oxidation of sulfhydryl residues or by limited proteolysis. Xanthine oxidase produces both $O_2^{\bullet -}$ and H_2O_2 through the oxidative hydroxylation of purine substrates. Under inflammatory conditions, xanthine oxidase levels are increased, resulting in excess ROS formation and oxidative damage in the cardiovasculature. Accordingly, xanthine oxidase inhibition attenuates oxidative damage in heart disease (Kelley et al. 2010; Kumar et al. 2011; Zhang et al. 2012).

4.5 Peroxisomes

Peroxisomes are multifunctional organelles that play an important role in maintaining oxidative balance. Peroxisomes degrade various biomolecules through alpha- and beta-oxidation, alone or in cooperation with mitochondria, producing H_2O_2 as a metabolic by-product (Antonenkov et al. 2010). The H_2O_2 is normally

broken down into water and oxygen by catalase or peroxidases; however, impairment of the antioxidant system allows ROS accumulation and subsequent damage to proteins, lipids, DNA, and organelles, resulting in neurodegenerative disease, type 2 diabetes, and cardiovascular disease (Fransen et al. 2012; Terlecky et al. 2012).

5 Cardiac Response to Oxidative Stress

Diseases caused by mtDNA mutations can be categorized as inherited or acquired mitochondrial disorders, depending on when the mtDNA mutation occurred. Approximately 40 different congenital mitochondrial diseases have been identified; they result in symptoms by 10 years of age and are associated with multiple tissue defects. The prevalence of mitochondrial mutations or disease is 4.7 in 100,000 in children and 11.5 in 100,000 for all ages (Schaefer et al. 2004). Inheriting a large mtDNA deletion or mutation results in one of the mitochondrial myopathies, which are functional defects of the mitochondrial respiratory chain, primarily affecting complexes I, III, and IV (Holt et al. 1988). Specific congenital conditions caused by mtDNA mutations include Kearns–Sayre syndrome, chronic progressive external ophthalmoplegia, mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes, myoclonic epilepsy with ragged-red fibers, neurogenic weakness with ataxia and retinitis pigmentosa, and Leigh syndrome (DiMauro and Davidzon 2005). These inherited diseases are associated with a wide range of heart dysfunctions including left ventricular hypertrophy, cardiac fibrosis, systo-diastolic dysfunction, and impaired conduction (Anan et al. 1995; Fayssoil 2009; Galetta et al. 2014; Thorburn and Rahman 1993), demonstrating the significant relationship between mtDNA mutations and heart disease in humans.

The prevalence of acquired mitochondrial dysfunction and related diseases (e.g., type 2 diabetes, Parkinson's disease, Alzheimer disease, cardiovascular disease) is considerably higher than that of inherited mitochondrial diseases. In acquired mitochondrial diseases, aging and oxidative stress lead to the accumulation of mtDNA mutations that impair oxidative phosphorylation. In turn, the damaged ETC further increases oxidative stress and mtDNA mutations, resulting in a vicious circle. Because the heart is the organ most dependent on mitochondrial oxidative phosphorylation, mitochondrial dysfunction often manifests as cardiomyopathy. In animal models, mtDNA mutations/deletions in the heart commonly cause defects in mitochondrial oxidative phosphorylation and severe cardiomyopathies, including dilated cardiac dysfunction (Zhang et al. 2000), cardiac hypertrophy (Esposito et al. 1999; Graham et al. 1997), and atrioventricular heart conduction blocks (Li et al. 2000b; Wang et al. 1999). These findings suggest that the integrity of mtDNA and mitochondrial oxidative phosphorylation are essential for normal cardiac function.

Age-dependent mitochondrial dysfunction and cardiomyopathy are closely associated with an imbalance between mitochondrial ROS production and

detoxification (Ames et al. 1995; Bratic and Trifunovic 2010; Cui et al. 2012; Dai et al. 2012). In the aging heart, increased ROS production is accompanied by decreased complex I and IV activity and state 3 respiration. This ETC impairment directly increases mitochondrial ROS generation (Dai and Rabinovitch 2009). However, the effect of age on antioxidant capacity or enzyme activity remains unclear (Rikans and Hornbrook 1997). It is generally accepted that antioxidant capacity increases through an adaptive response to aging-associated oxidative stress, but this increased capacity is not sufficient to prevent overwhelming oxidative stress and mitochondrial damage (Wei and Lee 2002). Thus, increased ROS generation in the aging heart induces cardiac abnormalities such as ventricular hypertrophy (Lakatta 2003), fibrosis, and diastolic dysfunction (Khouri et al. 2004). These age-related cardiac abnormalities are significantly attenuated in mice overexpressing mitochondrial catalase, providing further evidence for the role of elevated oxidative stress in mitochondrial dysfunction-induced heart disease (Dai and Rabinovitch 2009).

5.1 Ischemia/Reperfusion Injury

Loss of blood or coronary artery blockage can reduce the supply of blood to the cardiac myocardium. The resulting lack of oxygen and glucose (i.e., ischemia) eventually causes irreversible cardiac cell death, myocardial infarction, and cardiac dysfunction. Cardiomyocyte cell death is the result of depolarization of mitochondrial membrane potential, decreased oxidative phosphorylation and ATP generation, and increased ROS generation (Kim et al. 2011). Restoration of the blood supply (i.e., reperfusion) by thrombolytic therapy or percutaneous coronary intervention can reduce cardiac damage. However, the process of reperfusion can also induce or exacerbate cardiomyocyte death in a process known as ischemia/reperfusion (I/R) injury (Hausenloy and Yellon 2013). During the first few minutes of reperfusion, ROS overproduction and Ca^{2+} overload open the mitochondrial permeability transition pore, leading to cardiomyocyte apoptosis (Hausenloy et al. 2003). Ischemia/reperfusion strongly increases ROS generation in various mitochondrial sites including the Krebs cycle (e.g., aconitase) and electron transfer chain complexes I, II, III, and IV via site-specific mechanisms (Chen and Zweier 2014). Detailed information on the targets, roles, and mechanisms of mitochondrial ROS generation during I/R injury were well documented in the recent review by Chen and Zweier (2014).

In 1986, Murry et al. demonstrated that brief (<5 min) and repeated (four times) ischemic episodes before prolonged ischemia significantly attenuate myocardial infarction in dogs (Murry et al. 1986). This ischemic preconditioning is an endogenous cardioprotective response against I/R injury mediated through the activation of G-protein-coupled receptors, ATP-dependent potassium channel (K_{ATP}), and various protein kinases including protein kinase C, tyrosine kinase, and the mitogen-activated protein kinase (MAPK) family (Das and Das 2008) or

inactivation of proapoptotic p53 signaling (Mocanu and Yellon 2003). Ischemic preconditioning also inhibits opening of the mitochondrial permeability transition pore by preserving mitochondrial membrane potential and activity of NADH dehydrogenase and cytochrome c oxidase and by reducing ROS production (Halestrap et al. 2007). Despite its powerful cardioprotective effect, ischemic preconditioning is difficult to apply in patients with acute coronary disease. In 2003, Zhao et al. reported that brief ischemic episodes during early reperfusion (i.e., ischemic postconditioning) attenuated myocardial infarction in dogs, similar to the effects of ischemic preconditioning (Zhao et al. 2003). Furthermore, preconditioning and postconditioning can also protect remote regions and distant organs (i.e., remote ischemic pre- or postconditioning) (Kerendi et al. 2005; Przyklenk et al. 1993). These findings opened the possibility of clinical applications (Bousselmi et al. 2014; Hausenloy and Yellon 2009). For example, ischemic preconditioning has been used in open heart and coronary bypass surgery to preserve cardiac function and reduce tissue damage (Lu et al. 1997; Szmagala et al. 1998). Regarding ischemic postconditioning, three clinical trials in children and adults with cardiovascular disease have reported positive results (Luo et al. 2007; Luo et al. 2008a; Luo et al. 2008b). The discovery of signaling pathways underlying ischemic conditioning has provided novel pharmacological targets for the development of pharmacological agents including adenosine, GLP-1, atrial natriuretic peptide, and cyclosporine A (Hausenloy and Yellon 2009).

Bursts of ROS during reperfusion impair cellular defense mechanisms against oxidative stress. In the first stage, hydrophilic antioxidants (e.g., ascorbate and glutathione disulfide) are readily oxidized by increased ROS; further oxidative stress diminishes lipophilic antioxidants (e.g., vitamin E and ubiquinol-9) (Haramaki et al. 1998). Bursts of ROS also oxidize thiol groups and lipids, leading to membrane damage and necrosis. Severe oxidative stress inhibits the activity of mitochondrial superoxide dismutase (SOD). Results of a clinical study showed that increased oxidative stress during I/R injury is associated with transient left ventricular dysfunction or stunning (Ferrari et al. 2004). Because increased oxidative stress during I/R is a major cause of myocardial infarction, the ability of antioxidants to protect against I/R-induced cardiac damage has been tested (Marczin et al. 2003). Several studies have shown that supplementation with vitamin C, vitamin E, or the glutathione (GSH) precursor *N*-acetylcysteine limits oxidative stress and enhances cardiac function after I/R in animals and patients (Dingchao et al. 1994; Ferrari et al. 1991; Mickle et al. 1991).

Cytosolic Cu/ZnSOD and mitochondrial MnSOD are both antioxidant enzymes that convert superoxide to H_2O_2. However, the cardioprotective effect of MnSOD after I/R is significantly higher than that of Cu/ZnSOD because of its location (Asimakis et al. 2002; Jones et al. 2003). These findings suggest a site-specific role for ROS and indicate that antioxidant intervention in I/R injury should target mitochondrial ROS (Marczin et al. 2003).

5.2 Cardiac Hypertrophy and Fibrosis

Cardiac hypertrophy (CH) is a morphologic adaptation to work overload and is associated with an abnormal response to beta-adrenergic stimulation. Oxidative stress is considered a major cause of CH, which strongly increases the risk of heart failure, cardiac arrhythmia, and sudden cardiac death (Maulik and Kumar 2012). In a well-designed study by Dai et al. (2011), overexpression of mitochondria-targeted catalase, but not cytosolic catalase, was shown to protect against CH, fibrosis, and mitochondrial damage in mouse models of cardiomyopathy. Overexpression of mitochondria-targeted catalase prevented the accumulation of mitochondrial protein carbonyls, DNA deletions, increased autophagy, and activation of MAP kinase extracellular signal-regulated kinase1/2 in the heart. These findings demonstrated that mitochondrial ROS are not just involved in cellular damage but have important roles in cell signaling.

Fibrosis is caused by pathological remodeling of the extracellular matrix (ECM) mediated by matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) (Spinale 2007). Cardiac ECM provides physical connections and enables signal transduction among cardiomyocytes, cardiac fibroblasts, and blood vessels within the myocardium. Cardiac ECM undergoes remodeling in response to diverse stimuli in pathological cardiac conditions, such as myocardial infarction and overload and dilated cardiomyopathy (Spinale 2007). The major components of ECM include collagen types I and III, IV, V, and VI and fibronectin, laminin, elastin, fibrillin, proteoglycans, and glycoproteins. These ECM proteins are produced primarily by cardiac fibroblasts (Fan et al. 2012). Cardiac fibroblasts also regulate ECM homeostasis through the production of MMPs and TIMPs, which degrade ECM and inhibit ECM degradation, respectively (Spinale 2007). Because MMP activation and overexpression are common in heart disease, the inhibition of MMP expression/activity has been investigated to attenuate maladaptive cardiac remodeling. Selective MMP inhibitors such as PG11680 have been shown to prevent myocardial remodeling after myocardial infarction (Hudson et al. 2006), and inhibition of the renin–angiotensin–aldosterone pathway decreases MMP levels and cardiac remodeling (Li et al. 2000a; Sakata et al. 2004). Together with various cytokines, ROS-mediated oxidative stress is a potential activator of MMPs in the heart (Grieve and Shah 2003). ROS activates MMPs by upregulating MMP expression (Nian et al. 2004; Siwik et al. 2001; Wainwright 2004) and through posttranslational modification and activation of pro-MMPs (Fu et al. 2001; Fu et al. 2004; Yoon et al. 2002). Antioxidants have been shown to significantly reduce MMP activity, diabetes-induced cardiac dysfunction, and hypertension-induced cardiac hypertrophy (Bilginoglu et al. 2009; Rizzi et al. 2013). These findings demonstrate the important role of ROS in ECM remodeling in patients with heart disease.

5.3 Diabetic Cardiomyopathy

Diabetes is a metabolic disease caused by abnormal energy metabolism in multiple organs including the pancreas, heart, liver, and skeletal muscle. Although the primary cause of diabetes is unknown, mitochondrial dysfunction may be a major contributor to insulin resistance in major organs (including skeletal muscle and liver) and defects in insulin secretion by pancreatic beta cells (Lowell and Shulman 2005). In particular, abnormal mitochondrial fatty acid oxidation is thought to increase the accumulation of intracellular fatty acyl coenzyme As and diacylglycerol, which activate signaling pathways inhibiting insulin-stimulated glucose transport activity. This hypothesis was supported by a clinical study demonstrating that elderly individuals with severe insulin resistance in skeletal muscle had higher triglyceride levels in muscle and liver and decreased mitochondrial oxidative phosphorylation activity (Petersen et al. 2003). In addition, the insulin-resistant subjects had fewer mitochondria in muscle cells and decreased expression of nuclear-encoded genes that regulate mitochondrial biogenesis, such as peroxisome proliferator-activated receptor gamma coactivator 1alpha and 1beta (PGC-1alpha and PGC-1beta) (St-Pierre et al. 2003; Wu et al. 1999).

The pancreas produces and releases insulin in response to blood glucose levels. In obesity, insulin deficiency is caused by beta cell mass that is insufficient to meet metabolic demands due to the inadequate proliferation or apoptosis of beta cells. Because apoptosis is regulated primarily by mitochondria, mitochondrial dysfunction is a major cause of beta cell loss. Mitochondrial dysfunction also reduces ATP levels in beta cells, which inhibits the opening of K_{ATP} channels and prevents membrane depolarization, a critical signal for the opening of voltage-gated calcium channels required for insulin secretion (Maechler and Wollheim 2001). Thus, mitochondrial dysfunction contributes to both insulin deficiency and insulin resistance in the development of diabetes.

Cardiovascular disease is the most common complication and primary cause of death in patients with diabetes mellitus. Diabetes significantly increases the risk of heart disease and vulnerability to pressure overload or ischemia. Diabetic cardiomyopathy (DCM) is ventricular dysfunction occurring in diabetic patients who do not have severe coronary artery disease or hypertension (Bell 2003; Bugger and Abel 2010). Left ventricular hypertrophy and systolic/diastolic dysfunctions are often observed in diabetes patients, along with hyperglycemia, hyperlipidemia, increased activation of protein kinase C and the renin–angiotensin system, and aldosterone-induced fibrosis (Boudina and Abel 2010; Hayat et al. 2004). Potential mechanisms underlying the development of DCM include disruptions in intracellular ion homeostasis and energy metabolism, the polyol pathway, and enhanced oxidative stress (Wold et al. 2005). In addition, mitochondrial dysfunction has been suggested as a major contributor to the development of DCM in various animal and human studies (Bugger and Abel 2010). Studies in animal models have revealed impaired state 3 mitochondrial oxygen consumption, decreased activity of respiratory chain complexes, and defects in mitochondrial ultrastructure and proliferation

in the heart (Boudina et al. 2005; Duncan et al. 2007; Kuo et al. 1983). Similarly, patients with type 2 diabetes show abnormal ATP generation, fatty acid utilization, and oxidative phosphorylation in cardiac mitochondria (Anderson et al. 2009; Peterson et al. 2004; Scheuermann-Freestone et al. 2003).

Besides mitochondrial dysfunction, factors that increase oxidative stress in diabetes include increased fatty acid oxidation, polyol pathway flux, advanced glycation end products, and activation of protein kinase C-dependent NADPH oxidase (Watanabe et al. 2010; Wold et al. 2005). As in other cardiomyopathies, increased ROS leads to mitochondrial dysfunction, cardiac cell death, increased fibrosis, and contractile dysfunction in DCM; however, these effects can be attenuated by the overexpression of MnSOD, catalase, or metallothionein (Cai et al. 2006; Ye et al. 2003; Ye et al. 2004). Interestingly, increased mitochondrial ROS generation reduces cardiac efficiency by upregulating the expression and activity of mitochondrial uncoupling proteins in DCM (Boudina et al. 2005; Echtay et al. 2002; Murray et al. 2005). Proper coupling of oxygen consumption to ATP generation is essential for cardiac contraction/relaxation. Overexpression or activation of uncoupling protein 3 promotes proton leak across the mitochondrial membrane, decreasing ATP generation and increasing oxygen consumption. This is known as cardiac inefficiency and is a major cause of cardiac contractile dysfunction in DCM (Bugger and Abel 2010). Taken together, these findings demonstrate the multiple roles of oxidative stress in the development of DCM.

5.4 Benefits of Reactive Oxygen Species During Exercise

Regular exercise has beneficial effects on the cardiovascular system, significantly decreasing the risk of cardiovascular disease. However, skeletal muscles generate ROS during exercise, which increases oxidative stress. The health consequences of exercise-induced oxidative stress remain unclear (Powers and Jackson 2008). The first direct evidence for exercise-induced ROS production and subsequent tissue damage was provided by Davies et al. (1982). This was followed by studies demonstrating that vitamin E supplementation reduces exercise-induced damage in skeletal and cardiac muscles (Jackson et al. 1985; Kumar et al. 1992). However, recent studies have shown that exercise-induced ROS exert beneficial effects (Gomez-Cabrera et al. 2005; Gomez-Cabrera et al. 2008; Kang et al. 2009; Meilhac et al. 2001; Ristow et al. 2009). Skeletal and cardiac muscles show increased antioxidant capacity after moderate oxidative stress due to acute or chronic exercise, which strengthens cellular defense mechanisms against severe oxidative stress due to I/R injury and age-related cardiac dysfunction (Bowles et al. 1992; Gomez-Cabrera et al. 2008; Kwak et al. 2006; Starnes et al. 2007). In addition, ROS signaling appears to be essential for exercise-induced enhancement of PGC1-alpha-mediated mitochondria biogenesis (Kang et al. 2009), MAPK–nuclear factor kappa B signaling (Gomez-Cabrera et al. 2005), insulin sensitivity (Ristow et al. 2009), and prevention of atherosclerosis (Meilhac et al. 2001). Thus, although

high levels of oxidative stress can damage cellular components, low-to-moderate levels of oxidative stress regulate gene expression, cell signaling pathways, and skeletal muscle force production (Powers and Jackson 2008).

5.5 *Pathophysiological Role of Mitochondrial ROS in Endothelial Cells*

Coronary blood flow is a key modulator of cardiac function. In the coronary artery, mitochondrial H_2O_2 acts as a vasodilator to increase the activity of the large-conductance (119 pS) $Ca2^+$- and voltage-activated K^+ (BKCa) channel (Barlow and White 1998). The H_2O_2 is produced in the endothelium by shear stress and is therefore considered an endothelium-derived hyperpolarizing factor (Chen and Zweier 2014). Mitochondrial-derived ROS (mtROS) also activates endothelial NOS through AMPK signaling, which modulates vascular relaxation (Quintero et al. 2006). Another mitochondria-mediated vasoregulation component, mitochondrial membrane potential depolarization, regulates vascular tone by activating nitric oxide synthase (Katakam et al. 2013). In isolated rat cerebral arteries, membrane potential depolarization was induced by activating the mitochondrial ATP-sensitive potassium channel, demonstrating its key role in vascular tone modulation through ROS-dependent or ROS-independent mechanisms. These findings indicate the importance of mtROS in vascular endothelium.

However, overproduction of mtROS in endothelial cells, smooth muscle cells, and macrophages is a major cause of atherosclerosis. ROS induces oxidative modification of phospholipids, resulting in increased transport of oxidized low-density lipoprotein into the artery wall, damaging endothelial cells, and eventually causing atherosclerosis (Madamanchi et al. 2005). Oxidative stress-mediated vascular dysfunction is frequently observed in patients with diabetes mellitus (Mackenzie et al. 2013). Although antioxidant treatment of atherosclerosis in humans has not been successful to date (Lonn et al. 2005), in vitro studies and experiments in animal models support the therapeutic potential of antioxidant therapy in atherosclerosis and metabolic disease (Mackenzie et al. 2013; Mercer et al. 2012).

6 Cardioprotective Effects of Mitochondria-Targeted Antioxidants

Various therapies for cardiomyopathy and ischemic heart disease target mitochondrial dysfunction (Walters et al. 2012). These include inhibitors of mitochondrial permeability transition pore opening, activators of mitochondrial K_{ATP} channel and respiratory chain complexes, AMPK signaling modulators, and mitochondrial

Table 1 Mitochondria-specific antioxidant agents

Agent	Remark	Clinical status	Reference
MitoQ	Coenzyme Q10 derivative	Phase II (NASH)	Jauslin et al. (2003)
MitoE	Vitamin E derivative	Not yet tested	Jauslin et al. (2003)
MitoPBN	Nitrone radical trap alpha-phenyl-tert-butylnitrone	Not yet tested	Maples et al. (2004)
MitoPeroxidase (ebselen analog)	Increases mitochondrial glutathione activity	Not yet tested	Filipovska et al. (2005)
MitoGSH	Increases mitochondrial glutathione activity	Not yet tested	Sheu et al. (2006)
MitoNAC	Increases mitochondrial glutathione activity	Not yet tested	Sheu et al. (2006)
SS31 (Bendavia)	Peptide antioxidants targeted to the inner mitochondrial membrane	Phase II (AMI)	Szeto (2006)
SS02		Preclinical	Szeto (2006)
Edaravone	Used for brain and cardiac I/R injury	In use (stroke, Japan) phase IV (AMI)	Higashi et al. (2006)
NecroX	ROS/RNS scavenger, mitochondria Ca^{2+} uniporter blocker	Phase II (STEMI)	Kim et al. (2010), Thu et al. (2012)
Phenolic antioxidant prodrugs	Mitochondria beta-oxidation-mediated drug delivery	Not yet tested	Roser et al. (2010)

I/R ischemia/reperfusion, *RNS* reactive nitrogen species, *ROS* reactive oxygen species, *STEMI* ST-segment elevation in myocardial infarction, *AMI* acute myocardial infarction, *NASH* nonalcoholic steatohepatitis

antioxidants (Armstrong 2007; Szewczyk and Wojtczak 2002; Toogood 2008; Walters et al. 2012). The primary goal of antioxidant treatments, whether mitochondria-targeting or non-mitochondria-targeting, is to decrease excessive ROS and oxidative stress in order to prevent functional loss of intracellular organelles and the cell itself. The primary reason for developing mitochondria-targeting antioxidants is the biological importance of the mitochondrion, which is the control center for energy metabolism, apoptosis, Ca^{2+} homeostasis, and cell signaling (Sheu et al. 2006). Large-scale clinical studies including the Heart Outcomes Prevention Evaluation (HOPE) study (Yusuf et al. 2000) and the Heart Protection Study (HPS) (MRC/BHF 1999) have demonstrated the ineffectiveness of conventional antioxidant therapies in patients, perhaps because these antioxidants are not efficiently taken up by mitochondria (Murphy and Smith 2007). To solve problem, a number of mitochondria-targeted antioxidants have been developed. Accumulated evidence shows that mitochondria-specific antioxidants are more effective than nonspecific antioxidants in their mitochondria protective role (Sheu et al. 2006; Smith and Murphy 2011). Here, we briefly describe a number of mitochondria-targeted antioxidants with their current clinical status (Table 1).

MitoQ and MitoE are derived from coenzyme Q10 and vitamin E, respectively. The antioxidant effects of these compounds are 100- to 350-fold more potent than their untargeted analogs (idebenone and Trolox), preventing cell death from endogenous oxidative stress in cultured fibroblasts of patients with Friedreich ataxia (Jauslin et al. 2003). The safety and effectiveness of MitoQ were demonstrated in a phase II clinical trial (Smith and Murphy 2010). MitoPBN, a mitochondria-targeted nitrone radical trap alpha-phenyl-tert-butylnitrone, provides neuroprotection against ischemic stroke by blocking oxidative stress-induced lipid peroxidation (Maples et al. 2004). MitoGSH is a choline ester of GSH, a nonprotein thiol that serves as an endogenous antioxidant. Although mitochondrial GSH comprises only 15% of total cellular GSH, MitoGSH provides cytoprotective effects (Sheu et al. 2006). Similarly, a mitochondria-targeted analog of ebselen (MitoPeroxidase) (Filipovska et al. 2005) and a choline ester of *N*-acetylcysteine (MitoNAC) were developed to increase GSH activity in mitochondria and decrease oxidative stress-induced mitochondrial depolarization and apoptosis (Sheu et al. 2006). The Szeto–Schiller peptides (SS02 and SS31) represent a novel class of cell-permeable antioxidants that target the inner mitochondrial membrane (Szeto 2006). These peptide antioxidants scavenge mitochondrial ROS and inhibit mitochondrial permeability transition, thereby suppressing oxidative stress-induced apoptosis and necrosis in isolated mitochondria, cell cultures, and ischemic tissue (Cho et al. 2007; Szeto 2006). Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a strong free radical scavenger developed by Mitsubishi-Tokyo Pharmaceuticals Inc. Because of its strong mitochondrial antioxidant effect, edaravone is widely used in patients with brain and cardiac I/R injury and may be useful for improving endothelial function in patients with cardiovascular disease (Higashi et al. 2006). NecroX compounds, developed by LG Life Science Ltd., have been shown to protect cultured cardiac cells and heart mitochondria from cardiotoxic agents (tertiary butyl hydroperoxide, sodium nitroprusside, and doxorubicin), hypoxic injury, and I/R injury (Kim et al. 2010; Lee et al. 2014; Thu et al. 2012). Our recent study demonstrated that NecroX-5 inhibits the mitochondria Ca^{2+} uniporter during hypoxia/reoxygenation to prevent mitochondrial Ca^{2+} overload-induced apoptosis (Thu et al. 2012). Finally, biotransformation of phenolic antioxidant prodrugs by the mitochondrial beta-oxidation pathway protects against I/R injury in isolated cardiomyocytes (Roser et al. 2010).

7 Conclusions and Perspectives

Accumulated evidence clearly demonstrates that ROS-induced oxidative stress in mitochondria plays an important role in the development of heart disease (Fig. 3). For that reason, mitochondria-targeted therapies represent a promising clinical strategy for the treatment of heart disease. However, despite promising results in animals (Mercer et al. 2012), the effects of antioxidant treatment in patients with cardiovascular disease have been inconsistent (Lonn et al. 2005). This discrepancy

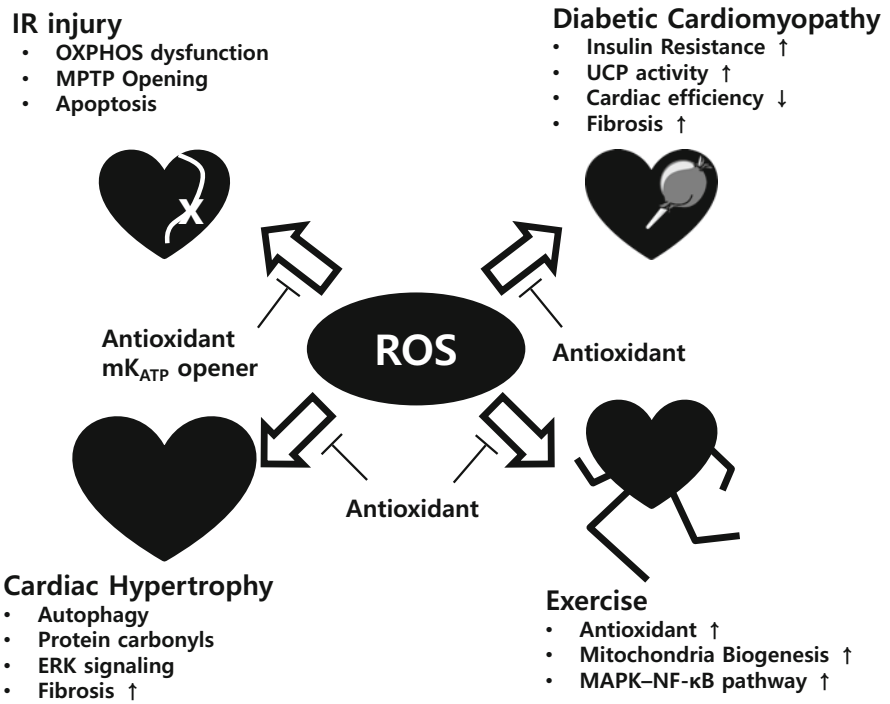

Fig. 3 Pathophysiological response of heart to reactive oxygen species (ROS). ROS mediates cardiac responses to exercise and various pathophysiological stimuli such as I/R injury, cardiac hypertrophy, and diabetic cardiomyopathy. A number of antioxidant treatments attenuate these cardiac responses, providing evidence for the mediatory role of ROS. Non-antioxidant treatments are also effective in specific conditions. For example, in I/R injury, a mitochondrial K_{ATP} channel opener also decreases oxidative stress and apoptosis in the heart. *ERK* extracellular signal-regulated kinase, *I/R* ischemia/reperfusion, *mK_{ATP}* mitochondrial ATP-dependent potassium channel, *MAPK* mitogen-activated protein kinase, *MPTP* mitochondrial permeability transition pore, *NF-kappa B* nuclear factor kappa B, *OXPHOS* oxidative phosphorylation, *UCP* uncoupling protein

may be due to the timing of treatment (before disease onset vs. after onset), antioxidant bioavailability, effects of other treatments (e.g., aspirin, angiotensin receptor blockers, statins), and, in particular, the ability to target the mitochondria (Firuzi et al. 2011; Mitra et al. 2011). An efficient antioxidant delivery system may therefore be needed to restore the function of damaged mitochondria. The ideal therapeutic agent would be selectively taken up by mitochondria within the target organs, where it can prevent oxidative damage and be recycled back to the active antioxidant form. In addition, it should be a pharmaceutically tractable and stable small molecule with acceptable oral bioavailability (Murphy and Smith 2007; Smith and Murphy 2011).

Acknowledgments This study was supported by a grant from the Priority Research Centers Program and Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (2010–0020224, 2012R1A2A1A03007595, 2012R1A1A2005240, and 2011–0028925), Republic of Korea.

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Rev Physiol Biochem Pharmacol (2016) 170: 129–130
DOI: 10.1007/112_2016_1
© Springer International Publishing Switzerland 2016
Published online: 16 March 2016

Erratum to: Intrauterine Growth Retardation (IUGR) as a Novel Condition of Insulin-Like Growth Factor-1 (IGF-1) Deficiency

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Erratum to: Rev Physiol Biochem Pharmacol
DOI: 10.1007/112_2015_5001

The spelling of the author I. Castilla de Cortázar was incorrect. The name should read as I. Castilla-Cortázar.

The online version of the original chapter can be found under
DOI: 10.1007/112_2015_5001

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