Advances in Biochemistry in Health and Disease

C.C. Kartha
Surya Ramachandran
Radhakrishna M. Pillai *Editors*

Mechanisms of Vascular Defects in Diabetes Mellitus

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Preface

Diabetes is a major public health and economic problem worldwide. Alarmingly, both the prevalence and incidence of the disease continue to escalate globally, even in low- and middle-income countries. The major cause of both morbidity and mortality in patients with diabetes is vascular complications of the disease. Patients with diabetes are at a higher risk of vascular disease in all the vessel walls of the human body.

In *Mechanisms of Vascular Defects in Diabetes Mellitus*, we bring together a panel of experts in the field of vascular biology and diabetology from different parts of the world to integrate the current understanding of the pathogenesis and pathophysiology of vascular diseases in diabetes mellitus.

This book has 24 chapters divided into six sections. These consider the global burden, pathogenesis, molecular mechanisms, hemostatic factors, metabolic factors, and pharmacological therapies. In Chap. 1, Viswanathan Mohan and Rajendra Pradeepa present the current global burden of diabetes and its vascular complications. Dwaipayan Bharadwaj and Anjali Singh in the next chapter survey the approaches to identify genetic risk factors for diabetes and discuss the results of the genetic studies in type 2 diabetes, identification of genetic factors for coronary artery disease, as well as epigenetics of complications in patients with diabetes.

Diabetes causes vascular remodeling through multiple pathways. How diabetes causes these changes is not well understood. Srikanth Vallurupalli and Jawahar L. Mehta in Chap. 3 review the mechanisms of vascular remodeling in diabetes mellitus. In the chapter that follows, Devendra Agrawal and colleagues critically analyze the effect of hyperglycemia in the pathogenesis of the atherosclerotic plaque and its rupture.

The trigger for initiation and then progression of vascular disease is injury to the vascular endothelium. S. Chandel, R. Tiwari, and Madhulika Dixit summarize in Chap. 5 the present knowledge on the molecular mechanisms that contribute to endothelial dysfunction in diabetes. Uma Siakia and Suvradeep Mitra in the following chapter describe the morphological changes in the vascular smooth muscle cells and the basis for vascular smooth muscle cell proliferation in the diabetic milieu and their significance. Surya Ramachandran, M.R. Pillai, and C.C. Kartha delineate in

Chap. 7 the role of monocyte-associated cytokines in early atherogenesis and vascular disease in diabetes. They also comment on the current approaches to repress atherosclerosis by modulating cytokine action. In Chap. 8, Adriana Georgescu and colleagues highlight the significance, mechanisms, and therapeutic implications of dysfunction of endothelial progenitor cells in diabetes and its vascular complications. Saumik Biswas and Subrata Chakrabarti in the subsequent chapter provide insights into the cellular and molecular mechanisms, including epigenetic alterations that are associated with the development and progression of diabetic retinopathy. In Chap. 10, Anita Mahadevan and S. K. Shankar detail the pathological features of diabetic neuropathy and discuss metabolic, inflammatory, and ischemic mechanisms involved in its pathogenesis. They also analyze various clinical manifestations and their pathologic bases and treatment prospects.

Section 3 on molecular mechanisms begins with a report by Ana Cristina Simões e Silva, R. N. Ferreira, and A. S. Miranda on clinical and experimental evidences for (i) the role of angiotensin-converting enzyme-2–angiotensin-(1–7)–Mas axis in glycemic control, diabetic nephropathy, and cardiovascular complications in diabetes and (ii) Mas receptor agonists as possible therapeutic targets. In the following chapter, Camille M. Balarini draws attention to how adipokines influence the development of vascular disease in diabetes. In Chap. 13, Madhu B. Anand-Srivastava dwells upon the changes in heterodimeric G proteins and associated signal transduction systems which regulate vascular function in hyperglycemic conditions and their role in vascular remodeling in diabetes.

Recent evidences suggest that deacetylation/acetylation of histones contributes to vascular dysfunction. Ashok Srivastava and Paulina Pietruczk in Chap. 14 evaluate the role of histone deacetylases in the regulation of vasoactive peptides and growth factor genes and in the pathogenesis of vascular disease. In Chap. 15, Sumi Surendran and C.C. Kartha review the dysregulation of ncRNAs associated with vascular complications in diabetes and their likely role for use as biomarkers and therapeutic targets.

Section 4 deals with hemostatic factors. In the first chapter of this section, Kanjaksha Ghosh delineates the pathways through which diabetes induces a hypercoagulable and thrombophilic environment. In Chap. 17, Etheresia Pretorius scrutinizes how diabetes and associated inflammation affect the coagulation system and how this in turn contributes to aberrant clot lysis and impaired vascular function. They also narrate new methods to monitor the signs of both hypercoagulability and hypofibrinolysis in diabetes. Gundu H. R. Rao in Chap. 18 provides an overview of the physiological function of platelets and its relation to vascular dysfunction and how the altered platelet function in diabetes contributes to cardiovascular complications of diabetes.

In Sect. 5, there are three articles on metabolic factors. Accumulation of advanced glycation end products (AGEs) is implicated in the development of insulin resistance and in the pathogenesis of complications in diabetes. Mahesh Kulkarni and colleagues discuss the mechanisms of formation of AGEs, their degradation, and their role in inflammatory signaling as well as vascular complications of diabetes. They also remark on both chemical and natural product inhibitors of AGEs for

prevention of complications of diabetes. Krishnan Venkitaraman and colleagues in Chap. 20 focus on the derangements in lipid and lipoprotein metabolism in diabetes and the contribution of lipotoxicity to the progression of atherosclerosis and vascular complications. The next chapter by Pankaj Chaturvedi is a description of the role of homocysteine in the pathogenesis of vascular disease.

The final section in this volume contains three articles on therapeutic targets in the mechanisms that lead to vascular complications of diabetes. Hina Nizami and Sanjay K. Banerjee provide an extensive analysis of a large number of drugs that are used to prevent or treat endothelial dysfunction in diabetes and the mechanisms of their action. They also consider nutritional therapies and probiotics which have promise as remedies. Zahra Bahadoran, Parvin Mirmiran, and Asghar Gahsemi in Chap. 23 assess the potential of inorganic nitrate and nitrite as a supplement to treat vascular dysfunction and hypertension in patients with diabetes.

Myeloperoxide-mediated oxidants are considered to play a significant role in inflammatory response. As atherosclerosis is an inflammatory process, myeloperoxide could be a target to attenuate atherosclerotic process. In the concluding chapter, Sampath Parthasarathy and colleagues debate on the development of small organic molecules, organometallic scaffolds, and aptamers as myeloperoxide inhibitor.

In summary, this text provides a comprehensive update on the current knowledge pertaining to cellular and molecular mechanisms for the pathogenesis of various forms of vascular complications linked to diabetes and also on known and potential targets for therapeutic intervention to mitigate macrovascular and microvascular diseases in patients with diabetes.

We thank all the contributing authors and the staff at Springer for their support in the compilation and production of this compendium. We are also grateful to Professor Naranjan S. Dhalla, series editor for *Advances in Biochemistry in Health and Disease*, for inviting us to plan and assemble this volume.

Thiruvananthapuram, Kerala, India

C.C. Kartha
Surya Ramachandran
Radhakrishna M. Pillai

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Part I
Introduction

Chapter 1
The Global Burden of Diabetes and Its Vascular Complications

Viswanathan Mohan and Rajendra Pradeepa

Abstract Diabetes, one of the most common metabolic disorders, is no longer a disease of affluent nations. The prevalence rates of diabetes are rising steeply in low and middle-income nations that have rapidly improved their economy and adopted a 'westernized' life style. The worldwide explosion of diabetes also increases the propensity for developing both micro and macro vascular complications and this result in a huge burden due to mobility and mortality in addition to increasing the costs of therapy. As both micro and macrovascular complications share common pathophysiological mechanisms, several studies have shown a strong association between the various vascular complications. Thus, screening for all diabetic complications simultaneously is recommended. If the burden due to diabetes and its complications is to be reduced, there is need for a multi-prolonged strategy involving early diagnosis of diabetes, screening for its complications and offering optimal therapy at all levels of care. Luckily, effective interventions are available, making such efforts justifiable.

Keywords Diabetes • Disease burden • Epidemiology • Cardiovascular diseases • Vascular complications • Microvascular complications • Macrovascular complications

1.1 Introduction

The alarming increase in prevalence and incidence of diabetes has made it a major global public health, and economic, problem. Diabetes is no longer a disease of affluent developed nations, as the prevalence of diabetes is steadily increasing

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globally [1, 2]. Indeed, nearly two thirds of the world's population with diabetes currently lives in low- and middle-income regions [2]. Nations that have rapidly improved their economy and adopted a westernized life style have seen the steepest rises in diabetes rates [3]. While type 1 diabetes and other types account for 5–10% of all cases of diabetes, type 2 diabetes remains by far, the most common form of diabetes. Type 2 diabetes which was earlier considered as a mild disorder of the aged, has now a changed profile and now affects young adults and even children in whom the disease course also appears to be more aggressive [4].

Several developing countries have experienced rapid transitions in social structures, economics, nutrition and lifestyles and these have largely contributed to the global diabetes epidemic. While some risk factors eg. ageing and genetic makeup are non-modifiable, there are also several modifiable risk factors including physical inactivity, unhealthy diet, obesity, tobacco use, excess alcohol use, lack of sleep, and depression which if controlled can decrease the incidence of diabetes [5]. Unfortunately, the greatest impact of these behavioural risk factors are observed in developing countries, which probably reflects the underlying socioeconomic determinants such as poverty, illiteracy, social inequality and poor health infrastructure. Moreover, these countries are still grappling with the unfinished agenda of communicable diseases, thereby their focus on non-communicable disease like diabetes still quite limited.

Unfortunately the worldwide explosion of diabetes also parallely increases the propensity for developing micro and macrovascular complications [6]. The vascular complications of diabetes can have a devastating effect on quality of life and can increase the mobility and mortality. Vascular alterations associated with diabetes include anatomic, structural, and functional changes leading to multiorgan dysfunction [7–9]. Complications of diabetes can be broadly divided into small vessel (microvascular) and large vessel (macrovascular) disease and these are seen in both type 1 and type 2 diabetes. This chapter will deal with the current global burden of diabetes (which is predominantly type 2 diabetes) and its complications.

1.2 Global Burden of Diabetes

Globally, the number of people with diabetes started to perceptibly rise in the 1990s and from the year 2000 onwards, there has been an explosion in the number of people with diabetes [10–13]. The global burden of diabetes has been estimated by various groups. McCarty et al. [10] estimated the global burden of diabetes using data from population-based epidemiological studies and reported that 110 million people had diabetes in 1994 and predicted that it would double to 239 million by 2010. King et al. [11] estimated the global burden at 135 million in 1995, and projected that the number would reach 299 million by the year 2025. In 1997, the global burden of diabetes was estimated to be 124 million people and the projected

number was 221 million people by the year 2010 [12]. However recent studies have shown that these numbers were all gross under estimates as the numbers of people with diabetes worldwide rose from 194 million in the year 2003 [14] to 415 million in 2015 [15]. According to the IDF, there would be 642 million people with diabetes by the year 2040, with three-quarters of these people be occurring in low- to middle-income countries [15].

Of the top ten countries in terms of the number of individuals with diabetes, listed by IDF in 2015, eight are developing countries: China, India, Brazil, the Russian Federation, Mexico, Indonesia, Egypt and Bangladesh [15]. The highest number of people with diabetes in the world currently is in China (109.6 million) where these numbers are expected to increase to 150.7 million by 2040. The corresponding numbers for India are 69.2 million and 123.5 million respectively. An overview of the burden of diabetes (absolute numbers and prevalence percentage) in the seven IDF Regions – Africa (AFR), Europe (EUR), Middle East and North Africa (MENA), North America and Caribbean (NAC), South and Central America (SACA), South-East Asia (SEA) and Western Pacific (WP) over the past 15 years is presented in Fig. 1.1 [14–20]. The figure shows that the largest increases will take place in the regions dominated by developing nations. Undoubtedly the increase in absolute numbers of people with diabetes is primarily driven by the larger population sizes in these regions. However, the rates at which diabetes is increasing is also higher in the developing nations most probably due to rapid epidemiological and a nutritional transition. This is substantiated by the Global Burden of Disease Study, which noted that the absolute growth in number of people with diabetes was partially explained by population growth and aging in the world's largest countries (e.g., India and China) [21]. Indeed Asians both resident and migrant comprise about ~4 billion out of the world's 6.95 billion inhabitants [22].

A recently published pooled analysis of 751 population-based studies with 4·4 million participants from 146 countries [3] reported that global age-standardised prevalence of diabetes increased from 4.3% in 1980 to 9.0% in 2014 in men, and from 5.0 to 7.9% in women respectively. This group also reported that the number of adults with diabetes in the world increased from 108 million in 1980, to 422 million in 2014. This study, for the first time, tries to explain the causes of these increases and concludes that 28.5% of the increase in diabetes was due to the rise in prevalence, 39.7% due to population growth and ageing, and 31.8% due to interaction of these two factors.

There have been few national studies on prevalence of diabetes in India. The Indian Council of Medical Research-INdia DIABetes (ICMR-INDIAB) study is being conducted in all states and union territories of India, in a phased manner [23]. In 2011, the prevalence of diabetes in four regions of the country was reported and it was found to be 10.4% in Tamil Nadu, 8.4% in Maharashtra, 5.3% in Jharkhand, and 13.6% in Chandigarh. [23].

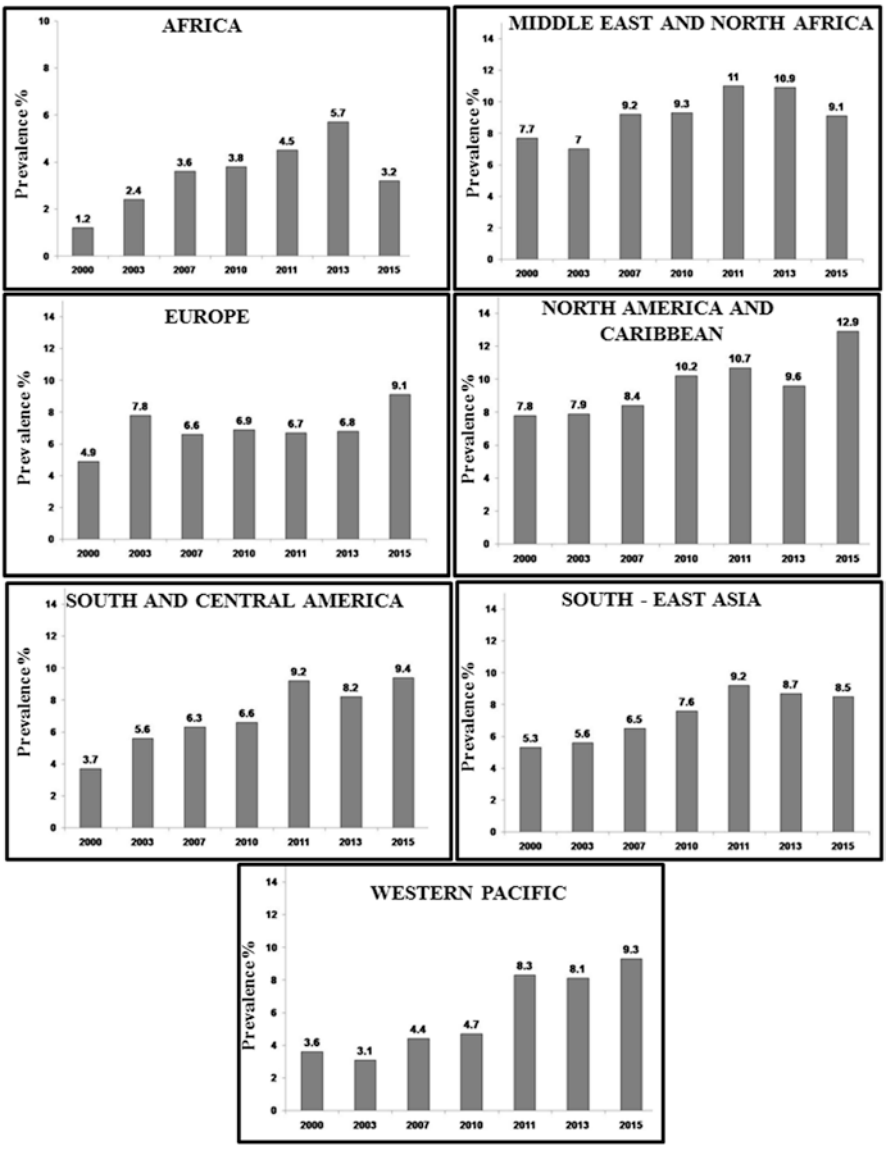

Fig. 1.1 Prevalence of diabetes in IDF regions (2000–2015) Refs. [14–20]

Unfortunately a large percentage of people with diabetes still remain undiagnosed – 46.5% according to the recent IDF atlas report [15]. The rate of undiagnosed diabetes is higher in developing countries due to less developed health care systems. Unfortunately, many people with undiagnosed diabetes already have complications associated with diabetes [24]. Moreover, undiagnosed diabetes can substantially increase the risk of developing complications in the future.

1.3 Vascular Complications

Microvascular complications affect the body's most intricately vascularized organs, in particular, the retina (diabetic retinopathy-DR), kidney (diabetic nephropathy) and the peripheral nerves (diabetic neuropathy). The macrovascular complications encompass disorders of large blood vessels which affect the heart (cardiovascular disease), brain (cerebrovascular disease) and the peripheral arteries (peripheral vascular disease) [9]. The prevalence of both micro and macrovascular complications are related to the type and duration, of diabetes. Vascular complications are usually progressive. For example nephropathy, retinopathy and neuropathy, begin insidiously, but over time, may result in severe damage to the organs and this contributes significantly to morbidity and mortality. The increasing epidemic of type 2 diabetes in low and middle income nations thus portend that unless effective strategies are taken up to control diabetes effectively, there could be huge increases in the burden due to these complications [15].

Globally, several epidemiological studies have been conducted to assess the prevalence (and incidence) of diabetic complications. However, due to lack of standardized methods used to assess the complications associated with diabetes, comparison between different populations is difficult. The rest of this renew with deal with the published literature on the various diabetes related complications.

1.3.1 Microvascular Complications

1.3.1.1 Diabetic Retinopathy (DR)

DR is considered as the most specific complication of diabetes. It is clinically defined by the presence of visible opthalmoscopic retinal microvascular lesions in individuals with diabetes, which results from a combination of systemic and ocular abnormalities. Prevalence of DR among type 2 diabetic subjects has been reported to range from 7.6% in Brazil to 50.3% in USA (Table 1.1) [25–52]. The landmark studies which assessed the prevalence of DR, include the WESDR (USA) [25], the Barbados Eye Study (West Indies) [26], Beaver Dam Eye Study (USA) [34], Blue Mountains Eye Study (Australia) [31], Liverpool Diabetic Eye Study (UK) [32], Los Angeles Latino Eye study (USA) [29], Chennai Urban Rural Epidemiology study (CURES) Eye Study (India) [39], Multi-Ethnic Study of Atherosclerosis (MESA,USA) [40] and the Singapore Malay Eye Study [41] among others.

DR is increasingly recognized as one of the most important causes for visual impairment and blindness. A recent meta-analysis conducted from 1990 to 2010 to estimate the prevalence and number of persons visually impaired specifically by DR reported that globally the number of persons with visual impairment due to DR is rising. DR represents an increasing proportion of all cause blindness/moderate and severe vision impairment (MSVI). Age-standardized prevalence of DR-related

Table 1.1 Prevalence rates of diabetic retinopathy in different populations Refs. [25–52]

Author (year)	Place	Participants with diabetes (n)	Age (years)	Prevalence of retinopathy (%)
Klein et al. (1984) [25]	Southern Wisconsin, USA	1313	≥40	50.3
Haffner et al. (1988) [26]	San Antonio, Texas	351	≥40–74	44.3
Hamman et al. (1989) [27]	Colorado,USA	360	≥40–74	35.3
Chen et al. (1992) [28]	Taiwan, China	527	≥40	35.0
Klein et al. (1992) [29]	Wisconsin, USA	410	43–86	35.1
Nagi et al. (1997) [30]	Wakefield, UK	991	≥15	37.8
Mitchell et al. (1998) [31]	Blue Mountain, Australia	252	≥50	29.0
Broadbent et al. (1999) [32]	Liverpool, UK	395	13–92	33.6
Leske et al. (1999) [33]	Barbados, West Indies	615	≥40	28.8
McKay et al. (2000) [34]	Melbourne, Australia	233	≥40	27.5
West et al. (2001) [35]	Arizona, USA	899	≥40	44.3
Tapp et al. (2003) [36]	Australia	703	≥25	13.7
Varma et al. (2004) [37]	Los Angeles, USA	1,217	≥40	46.9
Hanley et al. (2005) [38]	Ontario, Canada	133	14–79	23.3
Rema et al. (2005) [39]	Chennai, India	1,715	≥20	17.6
Wong et al. (2006) [40]	USA	778	45–85	33.2
Wong et al. (2008) [41]	Singapore	757	40–79	35.0
Xie et al. (2008) [42]	China	362	≥45	27.9
Raman et al. (2009) [43]	Chennai, India	1,414	>40	18.0
Zhang et al. (2010) [44]	USA	1,006	≥40	28.5
Chiang et al. (2011) [45]	Singapore	401	>24	25.4
Jee et al. (2013) [46]	Korea	1,678	>40	15.8
Papali'i- Curtin et al. (2013) [47]	Northland, New Zealand	5,647	9–97	19.0
Schellini et al. (2014) [48]	Sao Paulo, Brazil	407	≥30	7.62
Win Tin et al. (2014) [49]	Pacific Island countries	459	≥35	47.1
Dutra Medeiros et al. (2015) [50]	Portugal	52,739	≥45	16.3
Papakonstantinou et al. (2015) [51]	Iran	529	40–80	29.6
Thomas et al. (2015) [52]	UK	91,393	>30	32.4

blindness/MSVI was higher in sub-Saharan Africa and South Asia. One out of 39 blind people was due to DR, and 1 out of 52 visually impaired people was due to DR [53].

A pooled analysis using data from 35 population-based studies around the world reported that the prevalence rates of DR, proliferative DR, diabetic macular edema, and vision-threatening DR among individuals with diabetes were 34.6 %, 7.0 %, 6.8 %, and 10.2 %, respectively. In absolute numbers, there were approximately 93 million people with DR, 17 million with proliferative DR, 21 million with diabetic macular edema, and 28 million with VTDR worldwide in the year 2011 [54]. However, there are some limitations as the data pooled are from studies performed at different time points, using different methodologies and the population characteristics may differ widely. Zheng et al. [55] extrapolated these findings to the global number of individuals with diabetes and estimated that the number of people with DR will grow from 126.6 million in 2011 to 191.0 million by 2030, and the number of people with vision-threatening DR will increase from 37.3 million to 56.3 million.

1.3.1.2 Diabetic Nephropathy

Diabetic nephropathy (DN) has a number of functional and structural abnormalities. Functional changes include initial renal hyperfiltration / hyperperfusion with subsequent development of microalbuminuria which is defined as urinary excretion of albumin in the range of 30–300 mg/day. Microalbuminuria is not only an important predictor of risk of developing overt DN, but also a marker of endothelial dysfunction. Progression from microalbuminuria to overt nephropathy occurs in 20–40% within a 10-year period with approximately 20% of these patients progressing to end-stage renal disease (ESRD) [56]. Clinically, DN is generally characterized by a progressive increase in proteinuria and decline in glomerular filtration rate, hypertension, and a high risk of cardiovascular morbidity and mortality both from renal failure as well as from cardiovascular disease.

Earlier studies have shown that individuals with type 1 diabetes were at higher risk of DN, compared with those with type 2 diabetes [57]. Recent, studies have shown that diabetic nephropathy is probably more frequent in type 2 than in type 1 diabetes [58, 59]. However, the prevalence/incidence of DN and the rates of its progression are less clear in type 2 compared with type 1 diabetes, mainly due to the more insidious nature of onset of diabetes in type 2 diabetes which makes accurate estimation of the duration of the disorder difficult to determine. According to Ritz [60], the reasons for the increase in the prevalence of nephropathy in type 2 diabetes are increasing prevalence, ageing population and improved survival. The United Kingdom Prospective Diabetes Study (UKPDS), reported that after a median 15 years of follow-up, 38% of participants developed microalbuminuria, while reduced GFR occurred in 29% of participants [57]. In the Pima Indian population type 2 diabetes cohort [61], the cumulative incidence of macroproteinuria was reported to be 50% at 20 years' duration.

In a systematic review conducted among individuals with self reported diabetes, the incidence rates of end-stage renal disease (ESRD) due to all causes ranged from

Table 1.2 Prevalence of micro and macroalbuminuria in people with diabetes in different population based studies Refs. [49, 67–76]

Author, year	Place	No: of diabetic subjects	Prevalence of microalbuminuria (%)	Prevalence of macroalbuminuria (%)
Gatling et al. 1988 [67]	Poole, UK	450	–	7.0
Neil et al. 1993 [68]	Oxford, UK	246	15.0	4.0
Klein et al. 1993 [69]	Wisconsin, USA	798	25.9	16.0
Wirta et al. 1995 [70]	Finland	188	29.0[a]	4.0[a]
			27.0[b]	7.0[b]
Collins et al. 1995 [71]	Western Samoa	162	22.0[a]	3.9[a]
			17.2[b]	6.3[b]
Bruno et al. 1996 [72]	Italy	1574	32.1	17.6
Atkins et al. 2004 [73]	Australia	832	24.9[a]	4.6[a]
			15.9[b]	1.3[b]
Unnikrishnan et al. 2007 [74]	Chennai, India	1716	26.9	2.2
Pedro et al. 2010 [75]	North-East of Spain	8187	17.8	6.7
Lee et al. 2014 [76]	Korea	971	19.3	5.5
Win et al. 2014 [49]	Nauru	100	71.0	–
Win et al. 2014 [49]	Solomon Islands	160	36.0	–
Win et al. 2014 [49]	Vanuatu	199	51.0	–

[a]*NDD* New detected diabetes
[b]*KD* Known diabetes

132.0 to 167.0 per 100,000 person-years, whereas the incidence rates of ESRD due to diabetic nephropathy varied from 38.4 to 804.0 per 100,000 person-years [62]. In most cross sectional studies of type 2 diabetes populations, the prevalence of kidney disease at any point in time is estimated to 30–50% [63].

The Third National Health and Nutrition Examination Survey conducted among the US population reported that prevalence of microalbuminuria was 28.8% in persons with previously diagnosed diabetes [64]. Earlier studies on migrant Asian Indians had suggested a high prevalence of microalbuminuria and kidney disease compared to the white European population [65, 66]. Table 1.2 provides the prevalence of micro and macroalbuminuria in type 2 diabetes in different population based studies [49, 67–76]. The prevalence ranges from 15% in the UK to 71% in the Nauru population. The CURES study from India reported that the overall prevalence of overt nephropathy was 2.2% while that of microalbuminuria was 26.9% [74].

1.3.1.3 Diabetic Neuropathy

Neuropathy, is a descriptive term meaning a demonstrable disorder of peripheral nerves, either clinically evident or sub clinical, that occurs in the setting of diabetes mellitus without other obvious causes for peripheral neuropathy. Diabetic neuropathy is not a single entity, but a group of disorders classified by the affected organ [77]. The neuropathic disorders include manifestations in the somatic and/or autonomic parts of the peripheral nervous system. It affects nearly 50% of all diabetic subjects and is considered to be the main cause for morbidity. The most common among the neuropathies are chronic sensorimotor distal symmetric polyneuropathy and autonomic neuropathy [77]. Severity and duration of diabetes play an important role in the extent of the functional and anatomical abnormalities of diabetic neuropathy. It is estimated that at the time of diagnosis, neuropathy is present in 10% of diabetic patients and over 50% of patients may develop neuropathy after 25-year duration of the disease [78, 79].

Table 1.3 shows the wide variation in prevalence rates of diabetic neuropathy in population based surveys [80–93]. In the Rochester Diabetic Neuropathy Study [81] conducted in late 1980s, 66% of the type 1 and 59% of the type 2 diabetic individuals had some form of neuropathy. The prevalence of peripheral neuropathy was reported to be 11.5% in the National Health and Nutrition Examination Survey [94]. The Diabcare Africa project, which was conducted across six sub-Saharan African countries reported that 48% of the study population had neuropathy [95]. These differences could be because of different diagnostic criteria used in assessing neuropathy.

Neuropathy significantly increases the risk for amputation among patients with diabetes [96]. A review of global variability in incidence of lower extremity amputations reported that in the population with diabetes, the incidence of all forms of lower extremity amputation ranged from 46.1 to 9600 per 10^5 while major amputation ranged from 5.6 to 600 per 10^5 [97].

1.3.2 Macrovascular Complications

1.3.2.1 Cardiovascular Disease

Cardiovascular diseases (CVD) account for increased morbidity and mortality in individuals with type 2 diabetes [98]. CVD is not only frequently observed in individuals with diabetes compared to those without diabetes, but it also occurs about 2 decades earlier among [99, 100]. Women with diabetes are equally, or possibly even at higher risk, of CVD than men in contrast to the general population where the reverse holds good. Women with type 2 diabetes, have a five to sevenfold higher rate of CVD death, compared with age-matched women without diabetes, with an event rate comparable to that seen in men with type 2 diabetes [101]. According to Laakso et al. [102], more than 70% of individuals with type 2 diabetes die of CVD causes.

Table 1.3 Prevalence rates of diabetic neuropathy Refs. [80–93]

Author, year	Place	Neuropathy diagnosis	Participants with diabetes (n)	Prevalence of neuropathy (%)
Walters et al. 1992 [80]	UK	Symptoms + signs	1,077	17.2
Dyck et al. 1993 [81]	Rochester, Minnesota	Symptoms, NCS and sensory examination	278	45
Harris et al. 1993 [82]	USA	Symptoms	2,405	38
Franklin et al. 1994 [83]	Colorado, Arizona	History and neurologic examination	277	27.8
Herman et al. 1998 [84]	Cairo, Egypt	VPT[a]	384	21.9
Shaw et al.1998 [85]	Mauritius	VPT[b]	433	12.7
Tapp et al. 2003 [86]	Australia	NNS,NDS, PPT and Postural BP drop	398	13.1
Gregg et al. 2004 [87]	USA	Symptoms and monofilament test	419	28.5
Pradeepa et al. 2008 [88]	Chennai, India	VPT	1629	26.1
Karvestedt et al. 2011 [89]	Sweden	Monofilament, tuning fork, and VPT	152	43
Katulanda et al. 2012 [90]	Srilanka	Monofilament, tuning fork	528	24
Kiani et al. 2013 [91]	Hamadan, Iran	NSS and NDS	521	49.3
Wang et al. 2014 [92]	Saudi Arabia	NSS, VPT	552	19.9
Jane et al. 2016 [93]	Taiwan	MNSI	628	30.6

NSS Neuropathy symptom score, *NDS* Neuropathy disability score, *NCS* Nerve conduction Studies, *VPT* Vibratory Perception Threshold, *PPT* Pressure perception test, *MNSI* Michigan neuropathy screening instrument

[a]Compared to locally derived age specific normal values

[b]Compared to locally derived values for healthy young adults

A population study of 3.3 million individuals in Denmark reported that there was a substantial increase in risk of myocardial infarction (MI) and coronary death in individuals with diabetes (without previous MI) and this was almost same as non-diabetic individuals with a previous MI [103]. After an acute MI, a considerable number of diabetic subjects die within the first year [104].

The longitudinal, multigenerational cohort of the Framingham Heart Study (FHS), which assessed CVD and its risk factors has shown that the absolute risk of CVD decreased by 35% between the 1950s and 1990s in individuals without diabetes and by 49% in those with diabetes. However, the study also reported that the

Table 1.4 Prevalence rates of peripheral vascular disease in diabetes Refs. [86, 87, 113–117]

Author, year	Place	Age (years)	Participants with diabetes (n)	Prevalence of PVD (%)
Beach et al. 1988 [114]	Washington, DC	50–70	252	22
Katsilambros et al. 1996 [115]	Greece	All age groups	193	42
Premalatha et al. 2000 [116]	Chennai, India	≥20	631	6.3
Tapp et al. 2003 [86]	Australia	≥25	2436	13.9
Gregg et al. 2004 [87]	USA	≥40	419	9.5
Tavintharan et al. 2009 [117]	Singapore	40–80	634	10.4
Pradeepa et al. 2014 [113]	Chennai, India	≥20	1755	8.3

relative risk among those with diabetes to develop CVD has persistently remained approximately two fold higher compared with those without diabetes [105]. The FHS has also reported that the rising prevalence of type 2 diabetes, combined with a increased risk for CVD, translated into a 60% increase in the attributable risk ratio for CVD associated with diabetes [106].

1.3.2.2 Peripheral Vascular Disease

Peripheral vascular disease (PVD) is caused by the narrowing of blood vessels that carry blood to the arms and legs. It is characterized by a gradual reduction in the blood flow to one or more limbs secondary to atherosclerosis [107]. Disability and mortality associated with PVD has increased over the last two decades in developing regions of the world and exceeds the increases in developed nations. In addition, the burden of PVD is no longer confined to the elderly population, but now occurs at younger age groups [108]. While PVD is a major risk factor for lower extremity amputation, it often coexists with cerebrovascular disease and/or CVD, and therefore, it is associated with poor prognosis and increased risk of morbidity and mortality [109]. PVD occurs almost three times more frequently in individuals with diabetes compared to age and gender matched individuals without diabetes [110]. Subjects with asymptomatic PVD not only have a higher risk for developing gangrene and amputations but also an increased risk for cardiovascular deaths [111]. Women with type 2 diabetes also have a higher prevalence/incidence of PVD and the risk of PVD is increased by age, duration of diabetes, and presence of peripheral neuropathy [112, 113].

Table 1.4 presents the prevalence of PVD in diabetic population [86, 87, 113–117]. Earlier prevalence estimates for PVD among diabetic individuals from the U.S. and Europe have reported to be 9.5% and 42% respectively [87, 115]. In contrast, the prevalence of PVD in Asian diabetic populations has been reported to be

lower than that in Western populations [112, 117]. A systematic review conducted in 34 population studies conducted between 1990 and 2010, including 112,027 participants reported that the prevalence of PVD in high-income nations was 5·3% in women and 5·4% in men at age 45–49 years, and while it was 18·4% in women and 18·8% in men at age 85–89 years. Prevalence of PVD in low-income nations among men was 2·9% at 45–49 years and 14·9% at 85–89 years). In LMIC, rates were higher in women than in men, especially at younger ages (6·3%). This review estimated that in 2010, there could be globally, 202 million people with PVD, with the majority (69.7%) in low-income nations which includes 54·8 million in south-east Asia and 45·9 million in the western Pacific Region [118].

1.3.2.3 Cerebrovascular Disease

Strokes are the commonest cause of mortality in diabetic and represent a major health burden. Individuals with diabetes, are at least twice as likely to have a stroke than those without diabetes [119]. They are more likely to suffer from increased morbidity and mortality after a stroke. Moreover, diabetes dramatically increases the risk of stroke at younger ages as well as women. The mechanism of development of stroke secondary to diabetes may be due to cerebrovascular atherosclerosis, cardiac embolism, or rheologic abnormalities. According to international Diabetes Federation (IDF), the prevalence estimates of stroke among individuals with type 2 diabetes ranges from 4 to 12% in clinic-based studies and between 4% and 5% in population-based studies [15]. The Framingham Study reported that the incidence of stroke was 2.5- to 3.5-times higher among subjects with diabetes compared to those without [120].

1.3.3 Overlap Between Micro and Macrovascular Complications

Numerous studies have shown an association between micro and macrovascular complications among individuals with diabetes as they share common pathophysiological mechanisms. For example, DR has been shown to be associated with cardiovascular disease and mortality [121]. An association between DR and the intimal medial thickness of the internal carotid artery has been demonstrated in an urban south Indian type 2 diabetic population [122]. Cross-sectional [123] and longitudinal studies [124, 125] report a relationship between microalbuminuria, proteinuria and retinopathy. Another study reported that both diabetes and ESRD synergistically increase the risks of CV events [126]. A population-based study from South India showed that the risk of nephropathy and neuropathy was five times, and three times higher, among the subjects with sight-threatening DR compared to those

without DR [127] This suggest that all three diabetic microvascular complications should be simultaneous screened for [128].

1.4 Mortality Due to Diabetes Associated Complications

Globally, the excess mortality attributable to diabetes in adults was estimated to be controllable to 3.8 million deaths [129]. Nearly 50% of type 2 diabetic patients die prematurely of a CVD cause and approximately 10% die of renal failure. The results of the Nurses' Health Study indicate that among women, diabetes is associated with dramatically increased risks of death from all causes and fatal CVD. The combination of prior CVD and a duration of diabetes >15 years was associated with a 30-fold increased risk of fatal CVD [130]. The National Health Interview Surveys (NHIS) reported that individuals with diabetes have significantly higher risk of death from all-cause and CVD than those without diabetes [131]. Eschol et al. [112], reported that among individuals with diabetes, prevalent PVD was associated with 3 fold increased mortality compared to those without PVD.

Risk of stroke-related dementia and recurrence, as well as stroke-related mortality, is elevated in patients with diabetes [132]. A meta-analysis which assessed the relationship between cardiovascular autonomic neuropathy and risk of mortality among individuals with diabetes reported that cardiovascular autonomic neuropathy was associated with significantly increased mortality [133]. A follow-up of 4,713 participants from ten centres in the WHO Multinational Study of Vascular Disease in Diabetes (WHO MSVDD) showed that CVD was the most common underlying cause of death, accounting for 44% of deaths in type 1 diabetes and 52% of deaths in type 2 diabetes while, renal disease accounted for 21% and 11% of deaths respectively [134].

1.5 Conclusions and the Way Forward

As the epidemic of diabetes continues to grow globally, the rate of its complications is also parallely increasing. These complications not only significantly contribute to the excess morbidity and mortality associated with diabetes, but also to the ever-increasing costs due to diabetes. As diabetes is largely asymptomatic, early screening for, and detection of, diabetes is crucial to reduce its vascular complications. Treatment of diabetes related complications, is far most expensive than treating diabetes itself. This underscores the need for a multi-pronged approach at all levels of care, namely Primary, Secondary and Tertiary prevention of diabetes. The time to act is NOW!

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Part II
Pathogenesis

Chapter 2
Genetic Basis for Increased Risk for Vascular Diseases in Diabetes

Dwaipayan Bharadwaj and Anjali Singh

Abstract Over the last several decades, the global incidence and prevalence of diabetes mellitus has increased significantly. The raised incidence rate is projected to continue as greater numbers of persons adopt a western lifestyle and diet. Patients with diabetes mellitus are at heightened risk of both adverse microvascular and macrovascular complications. Moreover, once cardiovascular disease develops, diabetes mellitus exacerbates progression and worsens outcomes. The risk of cardiovascular diseases associated with diabetes is probably due to genetic determinants influencing both glucose homeostasis and development of atherosclerosis. Although many genetic factors for both CAD and diabetes have been discovered, bringing important insights towards pathogenesis of these diseases. But there is comparatively less progress in our understanding of genetic basis of diabetic vascular complications. Genome wide association studies are beginning to expand our horizon of understanding of genetic architecture relating to diabetic complications that might offer an opportunity for improved risk prediction along with development of new therapies.

Keywords Genetics • Diabetes • Atherosclerosis • Cardiovascular disease in diabetes • Genome wide association studies

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2.1 Introduction

As the incidence of diabetes is on rise, there is a proportionate rise in the complications that are associated with diabetes as well. The devastating complications of diabetes mellitus are linked to several vascular diseases divided mainly into macrovascular and micro-vascular complications. Diabetic complications can be either due to damage to small blood vessels (micro vascular) or damage to larger blood vessels (macro vascular). Microvascular complications include damage to eyes (retinopathy), to kidneys (nephropathy) and to nerves (neuropathy) and diabetic foot disorders. Atherosclerosis, stroke, peripheral artery disease (PAD), myocardial infarction and congestive heart failure etc. are few cardiovascular diseases encompassed under macrovascular complications. These vascular complications of type 2 diabetes account for majority of social and economic burden among patients and society. Though the number of vascular diseases related to diabetic complications is huge this chapter aims to comprehensively discuss genetic basis for increased risk of diabetic cardiovascular complications only.

Diabetes is a group of metabolic diseases growing alarmingly as a potential epidemic with 415 million globally and 78 million in the South-East Asian Region; India being home to more than 69,1 million people (2015) currently, compared to 50.8 million in 2010 as per statistics of International Diabetes Federation (IDF) [1]. Worldwide, the number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014 [2].

Reports from different parts of India have suggested a rising trend in prevalence of Diabetes [3–12]. The ICMR-INDIAB that is first of its kind in India to provide National and Regional counts of diabetes, pre-diabetes and also of cardiovascular factors covering all Indian states, National Capital Territory of Delhi and two Union Territories projected 62.4 million people with diabetes and 77.2 million people with pre-diabetes for whole of India in 2011 [13].

Several factors contribute to accelerated diabetes epidemic as the "normal-weight metabolically obese" phenotype; high prevalence of smoking and heavy alcohol use; high intake of refined carbohydrates (e.g., white rice); and dramatically decreased physical activity levels. Poor nutrition in utero and in early life combined with over nutrition in later life may also play a role in diabetes epidemic [14].

Also an upsurge in number of early-onset diabetes cases is responsible for the development of various diabetic complications due to longer disease duration, however data on prevalence of diabetic complications across the whole of India is scarce [5, 6]. An international study in 2013 stated that diabetes control in individuals worsened with longer duration of the disease (9.9 ± 5.5 years) [5], with neuropathy the most common complication (24.6%) followed by cardiovascular complications (23.6%), renal issues (21.1%), retinopathy (16.6%) and foot ulcers (5.5%) [9]. These investigations are strongly similar with the prevalence of diabetic retinopathy (17.6%), diabetic neuropathy (26.1%), coronary artery diseases (21.4%) and peripheral vascular diseases (6.3%) to the results from South Indian population [8, 10–12]. But, in a latest study on central Indian population there was highest prevalence of

nephropathy (47%) followed by retinopathy (30%) followed by neuropathy (23%) [16]. Furthermore, this trend may translate to millions of people in India with each of the complications of diabetes and many with multiple complications.

Furthermore, Indians are more prone to progression of complications of diabetes at an early age (20–40 years) compared to Caucasians (>50 years) as they are genetically predisposed to the development of coronary artery diseases due to dyslipidemia and low levels of high density lipoproteins; emphasizing the need for careful screening and monitoring of patients regardless of their age within India [7]. As the age (40 years or more) increases the prevalence of diabetes also, gets higher compared to IGT (Impaired glucose Tolerance) that is significantly more prevalent in younger generation (under 40 years of age) [9]. In Indian diabetic population, poor glycemic control has also been observed as a factor that is responsible for micro- and macro-vascular changes related with diabetes [8].

This major lifestyle disease is undoubtedly the most challenging public health problem of twenty-first century mainly driven by sustenance, lifestyle and demographic evolution, increasingly changing diets and physical inactivity, in the background of genetic predisposition. India currently faces an uncertain future in relation to potential burden that diabetes may impose upon the country with meteoric increase in numbers of patients and related complications and create significant healthcare burden on family and society both. At this very crucial stage awareness and education on part of people and administration about diabetes is very essential. Expenditures apart, there is large requirement for government interventions such as funding community programs for public awareness about the diabetes risk reduction, availability of diagnostic services, medicines to all and one of community along with combined efforts of doctors, podiatrists and trained workforce paramedical workers [15].

The implementation of screening and early detection programs for pre-diabetes, diabetes prevention, self-management counseling, therapeutic management of diabetes, continuing education programs for general practitioners may yield positive health outcomes if rendered in society at ‘grass roots’ level to confront the new-age diabetes pandemic in our country. Interestingly, this has been proved by a study done a decade ago in a residential area in Chennai through mass awareness programs like public lectures, video clippings and distribution of educational pamphlets for 3 years continuously [17]. A follow up study was done 7 years after baseline study showing tremendous (277%) increase in proportion of walkers from baseline to follow up. The proportions of individuals who exercised increased from 14.2 to 58.7% [18].

2.2 Clinical Presentations of Type 2 Diabetes

Type 2 diabetes is most rampant form of diabetes in adults (>90%) and typically makes its appearance later in life. It is characterized by hyperglycemia resulting from insulin resistance or impairment in insulin-mediated glucose disposal and

malfunctioned secretion of insulin by pancreatic β-cells that are primary metabolic causes of type 2 Diabetes. The majority of patients are asymptomatic and hyperglycemia is noted on routine laboratory evaluation, prompting further testing. Classic symptoms of hyperglycemia include polyuria, polydipsia, nocturia, blurred vision and, infrequent weight loss that are usually noted in retrospect, after a blood glucose value has been shown to be elevated.

The Expert Committee on Diagnosis and Classification of Diabetes Mellitus in 2003, classified group of individuals whose glucose levels do not meet criteria for diabetes, and are higher than those considered normal as having impaired fasting glucose (IFG) [fasting plasma glucose (FPG) levels 100 mg/dl (5.6 mmol/l) to 125 mg/dl (6.9 mmol/l)], or impaired glucose tolerance (IGT) [2-h values in the oral glucose tolerance test (OGTT) of 140 mg/dl (7.8 mmol/l) to 199 mg/dl (11.0 mmol/l)] [19]. It should be noted that the World Health Organization (WHO) and numerous diabetes organizations define the IFG cutoff at 110 mg/dl (6.1 mmol/l) [20].

Individuals with IFG and/or IGT have been referred to as having pre-diabetes, indicating the relatively high risk for future development of diabetes. IFG and IGT should not be viewed as clinical entities in their own right but rather risk factors for diabetes as well as cardiovascular disease. IFG and IGT are associated with obesity (especially abdominal or visceral obesity), dyslipidemia with high triglycerides and/or low HDL cholesterol, and hypertension. Indians are generally having higher levels of LDL and triglycerides. Also, HDL levels are very low when compared to western population attributable to traditionally starch rich Indian diet that have been associated with higher levels of fats or triglycerides [21].

2.3 Vascular Diseases

2.3.1 Microvascular Diseases

Microvascular abnormalities and dysfunctions are systemic disease in diabetes. The microvascular diseases of diabetes beset long-term complications classically divided into nephropathy, neuropathy and retinopathy. In diabetic nephropathy, an increase in both intra-glomerular pressure and extracellular matrix proteins in glomerulus results in basement-membrane thickening, mesangial expansion, and glomerular hypertrophy. These changes reduce glomerular filtration area and function, and can progress to glomerulosclerosis. Diabetic retinal and glomerular vascular changes differ substantially from vasculopathies associated with ageing—which suggests their mechanisms are dissimilar. Roughly about 35% of patients of 18 years duration with type 1 diabetes will have signs of diabetic renal involvement [22]. Beginning of dialysis therapy in up to 35% of new patients was further diagnosed with type 2 diabetes [23]. Diabetic nephropathy can be divided into four phases: microalbuminuria, macroalbuminuria, nephrotic syndrome, and chronic renal failure. Microalbuminuria being a risk marker for atherothrombosis can be interestingly

explained by endothelial dysfunction in both Type I and II diabetes. Microalbuminuria (urine albumin 30–300 mg/day or <300 mg/g creatinine) is first clinical sign of diabetic damage to kidney [25]. Not only is microalbuminuria an indication of progressive kidney damage, but its existence also reflects an elevated risk for CVD [24, 25]. Macroalbuminuria (urine albumin >300 mg/day or >300 mg/g creatinine) typically denotes significant diabetic nephropathy and will be supervened by a decline in glomerular filtration rate (GFR). The majority of patients with diabetes who have macroalbuminuria also have hypertension [26]; in these patients, control of hypertension slows the decline in GFR [27]. Some diabetic patients develop nephrotic syndrome (urine protein >3 g/day); diabetic dyslipidemia in such patients often is compounded by nephrotic dyslipidemia, most notably by higher cholesterol levels. The nephrotic syndrome usually heralds progressive renal insufficiency; thereafter, ESRD (End-stage renal disease) ensues and dialysis and/or transplantation become necessary to sustain life. Mortality rates in patients with diabetes who are on renal dialysis probably exceed 20% per year [23]. CVD is the leading cause of death among patients with ESRD if diabetes is present [28, 29]. The prevalence of nephropathy in India was less (8.9% in Vellore [30], 5.5% in Chennai [31]) when compared with the prevalence of 22.3% in Indians living in UK though in European population it was 12.6% [32].

A strong familial clustering of diabetic nephropathy in Indian type 2 diabetic patients was noted [33]. The study showed that proteinuria was present in 50% and microalbuminuria in 26.7% of the siblings of probands with diabetic nephropathy. In contrast, the prevalence of proteinuria and microalbuminuria among the siblings of probands with normoalbuminuria was 0 % and 3.3 % respectively. Diabetic nephropathy is one of the leading causes of chronic renal failure in India. Among 4,837 patients with chronic renal failure seen over a period of 10 years, the prevalence of diabetic nephropathy was 30.3% followed by chronic interstitial nephritis (23%) and chronic glomerulonephritis (17.7%) [34].

Diabetic neuropathy is a group of conditions characterized by nerve dysfunction. The classification of neuropathy includes focal, diffuse, sensory, motor and autonomic neuropathy according to the nerves affected. Clinically, diabetic microangiopathy leads to retinopathy and glomerular dysfunction, and possibly contributes to neuropathy. Globally the estimates on prevalence of neuropathy vary widely from 13.1 to 45.0% [35, 36] that could be attributed to different types of diabetes, demography of the study population and different diagnostic criteria employed such as pin-prick perception, clinical signs and symptoms, quantitative sensory tests or electrodiagnostic tests [35]. A cross-sectional population-based study among urban south Indian Type 2 diabetic subjects showed 26.1% prevalence of Diabetic Neuropathy and is significantly associated with in addition to time-related variables as age, duration of diabetes [11].

Another microvascular complication, diabetic retinopathy occurs when diabetes damages the tiny blood vessels in the retina. Non-proliferative retinopathy and proliferative retinopathy are two main categories of Diabetic retinopathy. Proliferative retinopathy sometimes serves as a progression of non-proliferative retinopathy. Non-proliferative retinopathy is associated with pericyte loss trailed by formation of

micro aneurysms thus increased vascular permeability, development of retinal hemorrhages, capillary closure, venous loops, hard exudates, and soft exudates. These anomalies can lead to areas of non- perfusion and ischemia. Proliferative retinopathy on the other hand is defined as existence of new blood vessels with or without vitreous hemorrhage. Almost two-third of all Type 2 and almost all Type 1 diabetics are expected to develop diabetic retinopathy (DR) over a period of time [37, 38]. Prevalence of retinopathy among the South Indian type 2 diabetic subjects is 34.1% [39] that is higher than some studies done later on in South Indian population where the prevalence of diabetic retinopathy ranged from 10.6 to 26.2% [10, 40–43]. The global prevalence as per recent systematic analysis of 35 population-based studies showed the prevalence of DR, proliferative diabetic retinopathy (PDR), diabetic macular edema (DME), and VTDR among individuals with diabetes as 34.6 %, 7.0 %, 6.8 %, and 10.2 %, respectively [44].

2.3.2 Macrovascular Diseases

Macrovascular diseases include coronary artery disease, cerebrovascular disease, peripheral artery disease, non-significant carotid stenosis and polyvascular disease. Alarmingly, globally about 50–80% of all individuals with diabetes die of cardiovascular and cerebrovascular disease [45, 46]. In India also almost same trend prevails with more than 65% of patients with T2DM die of cardiovascular diseases; of these, nearly 80% are attributable to coronary artery diseases (CAD) [47]. The presence of T2DM seems to confer a three to four times higher risk of cardiovascular disease to Indian individuals [48] as compared to Europeans and mortality after an acute coronary event is also 40% higher in Indian patients [49].

This higher prevalence of cardiovascular disease in diabetic patients might be due to clustering of the patients together as a heterogeneous group with CVD, without separation into subgroups according to the macrovascular disease type. In fact, in a scientific statement released on ahajournals.org, the AHA (American Heart Association) asserts that from cardiovascular medicine point of view, it may be appropriate to say, “Diabetes is a cardiovascular disease.”

Peripheral vascular disease (PVD) is another macrovascular complication that is fortunately rare among patients with diabetes mellitus in India. Younger age of onset and relatively low prevalence of smoking are perhaps responsible for low prevalence of PVD. In the CURES cohort [50], the prevalence of PVD was 8.6%, compared with 23.5% among patients with T2DM in UK [51] and 20–30% in USA [52]. Increased age, female sex and duration of disease were all related to increased incidence of PVD [50].

Some previous studies in urban Indians exhibited central adiposity, obesity, hyperinsulinaemia, dyslipidaemia, hypertension and glucose intolerance along with diabetes as the factors that are involved in aetiology of macrovascular diseases [53, 54]. These are the central features accelerating athero-thrombotic cardiovascular disease (CVD). Atherosclerosis is the central pathological mechanism in

macrovascular disease. It leads to narrowing of arterial walls throughout the body. In response to endothelial injury and inflammation, oxidized lipids from LDL particles accumulate in the endothelial wall of arteries. Angiotensin II may promote the oxidation of such particles. Monocytes then infiltrate the arterial wall and differentiate into macrophages that accumulate oxidized lipids to form foam cells. Once formed, foam cells stimulate macrophage proliferation and attraction of T-lymphocytes. T-lymphocytes, in turn, induce smooth muscle proliferation in the arterial walls and collagen accumulation. The net result of this process is the formation of a lipid-rich atherosclerotic lesion with a fibrous cap. Rupture of the lesion leads to acute vascular infarction [55]. Besides, this general pathogenesis of atherosclerosis some factors specific to diabetes are worth mentioning here. Clinically, dyslipidemia is highly correlated with atherosclerosis, and up to 97% of patients with diabetes are dyslipidemic [56]. In addition to the characteristic pattern of increased triglycerides and decreased HDL cholesterol found in the plasma of patients with diabetes, abnormalities are also seen in the structure of lipoprotein particles. In diabetes, the predominant form of LDL cholesterol is the small, dense form. Small LDL particles are more atherogenic than large LDL particles because they can more easily penetrate and form stronger attachments to the arterial wall, and they are more susceptible to oxidation [57]. Because less cholesterol is carried in the core of small LDL particles than in the core of large particles, subjects with predominantly small LDL particles have higher numbers of particles at comparable LDL cholesterol levels [57].

2.4 Pathophysiology of Diabetic Complications

The pathophysiology of the link between diabetes and cardiovascular disease (CVD) is complex and multifactorial. Understanding these profound mechanisms of disease can help clinicians to identify and treat CVD in patients with diabetes, as well as help patients prevent these potentially devastating complications. Micro- and macrovascular disease pathways in type 2 diabetes are likely to share "common soil" hypothesis (Fig. 2.1). Both insulin resistance and hyperglycemia lead to oxidative stress and mitochondrial overproduction of superoxide and activate damaging pathways of protein kinase C, formation of glycation end-products and accumulation of sorbitol through aldose reductase pathway leading to diabetes complications [58, 59]. A unifying hypothesis has been proposed, with generation of reactive oxygen species (ROS) as a key central theme linking these different pathogenetic mechanisms [60]. Reactive oxygen species (ROS) are produced by glucose autooxidation, glucosamine formation, oxidative phosphorylation and can directly damage endothelial cells as well as by oxidizing LDL and AGEs. ROS formed by hyperglycaemia inhibits nitric oxide (NO) production, leading to elevated FFA (free fatty acids) levels and in course prevents the migration of vascular smooth muscle cells into plaques that is the necessary step for stabilization of the plaque. The unstabilized plaques are more vulnerable to rupture leading to thrombosis and hence

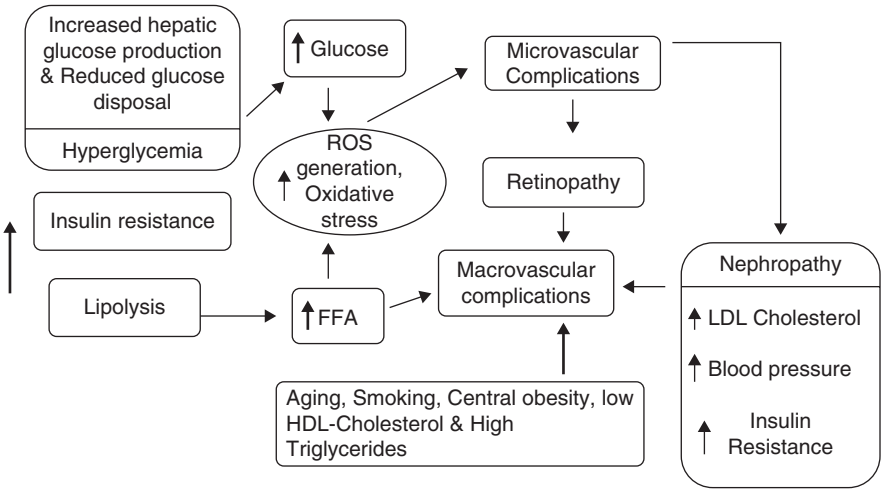

Fig. 2.1 'Common Soil' hypothesis of diabetic complications

atherosclerosis. Advanced glycation end products formed as a result of non-enzymatic reaction between glucose and arterial wall protein accelerate atherosclerosis by directly interfering in the arterial wall.

There are other important mechanisms implicated in the development of diabetic complications, including, for example, in relation to coexisting hypertension and hyperlipidemia, activation of renin–angiotensin system, adipokines production, protein folding and post-translational modifications, such as O-Glc-NAc modifications, inflammation and growth factors [59, 61].

In addition to pathways activated by hyperglycemia, recent studies have highlighted the importance of endogenous protective pathways such as insulin, platelet-derived growth factor, vascular endothelial growth factor and activated protein C that could provide new candidate genes for studying genetic factors protective against diabetic complications, as well as being targets for potential therapies to reduce diabetic vascular complications [62].

2.5 Risk Assessment in the Diabetic Patient

Risk estimation in diabetic patients must consider major risk factors as hyperglycemia, dyslipidemia, elevated blood pressure, cigarette smoking, and predisposing risk factors such as excess body weight, abdominal obesity, physical inactivity and family history of CVD along with ethnic origin. Identification of risk factors is a major first step for developing a plan for risk reduction in persons with diabetes. This should include a thorough medical history, careful physical examination, and appropriate laboratory measurements. Lipoprotein analysis should draw a clear

distinction between elevated LDL cholesterol concentrations and atherogenic or diabetic dyslipidemia as manifested by elevated triglycerides and small LDL and low HDL cholesterol levels. Even borderline-high-risk LDL cholesterol levels (130–159 mg/dL) are of concern in patients with diabetes and warrants aggressive intervention. A fasting triglyceride level of ≥150 mg/dl (≥1.70 mmol/l) is one of five accepted criteria for defining individuals at high risk for cardiovascular disease and type 2 diabetes, arguably termed the "metabolic syndrome" [63]. The quality of glycemic control can best be assessed by periodic measurement of hemoglobin A1c.

Though there is dearth of nationwide, comprehensive, prevalence data focusing on obesity as risk factor for diabetes from India [3] but interestingly, in spite of having lower overweight and obesity rates India has a higher prevalence of diabetes compared to western countries suggesting that diabetes may happen at a much lower body mass index (BMI) in Indians compared to Europeans [3, 4]. Hence, comparatively lean Indian adults with a lower BMI may be at equal risk as those who are obese in western countries. These types of findings have led to the concept of a specific 'Asian Indian Phenotype or Paradox', a collection of clinical and biochemical features that dispose Indian people to a higher risk of T2DM [64, 65]. Although genetic factors undoubtedly predispose Indian people to the development of T2DM but the environmental factors seem to have a far more important role in the development and propagation of the T2DM epidemic in India.

Several studies [66–69] indicated that the predisposing cardiovascular risk factors like cigarette smoking (Patients with diabetes who are smokers are at double risk), obesity and high serum cholesterol—continue to act as independent contributors to CVD in patients with diabetes thus acting as covariate risk factors. Thus, suggesting that the predisposing factors exacerbate the major risk factors: dyslipidemia, hypertension, and glucose tolerance; and they may cause CVD and diabetes mellitus through other pathways as well. To a large extent, both CVD and diabetes may be prevented through control of predisposing risk factors. Modification of life habits is at the heart of public health strategy for prevention of CVD and diabetes mellitus. High priorities are prevention (or treatment) of obesity and promotion of physical activities. Thus interventions to alter BMI, lipid levels, and blood pressure may decrease incident of diabetes and cardiovascular diseases [70].

2.6 Risk Factors for Development of Diabetic Vascular Complications

2.6.1 Insulin Resistance

Insulin resistance is defined as decrease in ability of insulin to promote glucose uptake in skeletal muscle and adipose tissue and to suppress hepatic glucose output that may be present for many years before development of any abnormality in plasma glucose levels [71]. It is associated with a number of classical risk factors for

cardiovascular diseases like central and general obesity, elevated blood pressure, elevated levels of triglycerides, activation of rennin-angiotensin system, increased reactive oxygen species (ROS) production, low levels of high density lipoprotein (HDL) cholesterol and glycotoxicity; each of them is an independent cardiovascular risk factor [72, 73]. In various multi-ethnic cross-sectional studies, significant relationships have been shown between insulin resistance and individual cardiovascular disease risk factors and markers of inflammation. Such as, insulin resistance is related to dyslipidemia in Indians [74] and Japanese adults [75], to blood pressure in Indians [74] and Chinese adults [76], to markers of inflammation in Caucasian [77], Hispanic, and African American adults [78], and to central obesity in Caucasian [79] and Japanese [75] adults. Insulin resistance advances from obesity and physical inactivity, acting on a substrate of genetic susceptibility [80]. It usually predates onset of type 2 diabetes and is accompanied by other cardiovascular risk aspects such as hypertension, dyslipidemia and prothrombotic factors [81]. Insulin secretion declines with advancing age [82] that may further be accelerated by genetic factors [83].

2.6.2 Atherogenic Dyslipidemia

Atherogenic dyslipidemia is characterized by three lipoprotein abnormalities: elevated very-low-density lipoproteins (VLDL), small LDL particles, and low high-density-lipoprotein (HDL) cholesterol (the lipid triad). The lipid triad occurs frequently in patients with premature coronary heart disease (CHD) and appears to be an atherogenic lipoprotein phenotype independent of elevated LDL cholesterol [84, 85]. Most patients with atherogenic dyslipidemia are insulin resistant [85, 86] thus is often called as diabetic dyslipidemia. Many patients with atherogenic dyslipidemia also have an elevated serum total apolipoprotein B [87]. Together they represent a set of lipoprotein abnormalities besides elevated LDL cholesterol that promote atherosclerosis. An elevated concentration of serum LDL cholesterol is a major risk factor for CHD. In fact, some elevation of LDL cholesterol appears to be necessary for initiation and progression of atherosclerosis. In population having very low LDL cholesterol levels, clinical CHD is relatively rare, even when other risk factors—hypertension, cigarette smoking, and diabetes—are common [88]. In contrast, severe elevations in LDL cholesterol can produce full-blown atherosclerosis and premature CHD in complete absence of other risk factors.

In Indians, triglyceride/HDL ratio of 3 has been proposed to be used as a surrogate marker for small LDL particles as these are associated with both CAD and diabetes [89]. Thrombus formation and dissolution of plaque are the most dangerous steps in CAD, resulting from the defect in various fibrinolytic and coagulation factors. The fibrinolytic and coagulation cascade consisting of activators and inhibitors play a major role in pathological mechanisms leading to CAD [90]. Finally, studies have also documented significantly higher prevalence of atherogenic small, dense LDL-C in Indians as compared to white Caucasians [91]. The higher

prevalence of atherogenic dyslipidemia in Indians can be attributed to environmental as well as genetic factors [92]. Not only the prevalence of dyslipidemia is high among Indians, it has been increasing steadily over past few decades. Degree of dyslipidemia increases with increase in age in both genders. Female are more prone to diabetic dyslipidemia and hence, have more risk of developing atherosclerosis with increasing age [93].

2.6.3 Hypertension

Hypertension is a well-established major risk factor for cardiovascular disease (CVD) and metabolic disorder [94]. Hypertension accounts for an estimated 54% of all strokes and 47% of all ischemic heart disease events globally [95]. Various investigators [96, 97] have reported a positive association between insulin resistance and hypertension suggesting elevated blood pressure to be listed among the components of metabolic syndrome. A reasonable justification could be the role of insulin resistance; its onset brings out clinical hypertension in a person who is genetically predisposed to elevated blood pressure [98]. When hypertension coexists with overt diabetes that it commonly does, the risk for CVD, including nephropathy, is doubly increased. A systematic review on prevalence of HTN (hypertensionin India, for studies published between 1969 and July 2011, reported a range between 13.9–46.3% and 4.5–58.8% in urban and rural areas of India, respectively [99]. A region-specific systematic review and meta-analysis in 2014 showed pooled prevalence of hypertension for rural and urban north Indian population 14.5% (13.3–15.7) and 28.8% (26.9–30.8), east Indian population 31.7% (30.2–33.3) and 34.5% (32.6–36.5), west Indian population 18.1% (16.9–19.2) and 35.8% (35.2–36.5) and south Indian population 21.1% (20.1–22.0) and 31.8% (30.4–33.1), respectively concluding that about 33% urban and 25% rural Indians are hypertensive [100].

2.6.4 Elevated Plasma Glucose

Typically, after onset of insulin resistance for many years, fasting and postprandial glucose levels are normal. As during this period, pancreatic β-cells are able to increase insulin secretion in response to insulin resistance and thereby maintain normal plasma glucose levels. But hyperglycemia develops when insulin secretory capacity declines and serum insulin falls to a level at which it cannot adequately overcome peripheral insulin resistance. Not only does increased peripheral insulin resistance promote hyperglycemia, but chronic overstimulation of pancreatic β cells that is typical of insulin resistance, may impair the capacity to secrete insulin. But in few individuals may be due to genetic factors insulin secretion reduces with aging, and elevated glucose concentrations appear. Hyperglycemia, which is characteristic of noninsulin-dependent diabetes, appears to be an independent risk factor

for coronary artery disease. Thus, hyperglycemia resulting from prolonged insulin resistance can be added to the list of mechanisms whereby insulin resistance increases the risk for coronary artery disease [98]. The first abnormality in plasma glucose in patients with insulin resistance is impaired fasting glucose (IFG) [fasting plasma glucose (FPG) levels 100 mg/dl (5.6 mmol/l) to 125 mg/dl (6.9 mmol/l)], or impaired glucose tolerance (IGT) [2-h values in oral glucose tolerance test (OGTT) of 140 mg/dl (7.8 mmol/l) to 199 mg/dl (11.0 mmol/l)] [101]. But further analyses that assessed diabetic complication retinopathy, helped to inform a new diagnostic cut point of ≥126 mg/dl (7.0 mmol/l) for FPG and confirmed the long-standing diagnostic 2-h PG value of ≥200 mg/dl (11.1 mmol/l) because certain cross-sectional epidemiologic studies demonstrated glycemic levels below that there was little prevalent retinopathy and above that the prevalence of retinopathy increased in an apparently linear fashion among the population [101].

Individuals with IFG and/or IGT have been referred to as having pre-diabetes, indicating the relatively higher risk for future development of diabetes and its presence usually accompanied with long-standing insulin resistance. Several studies [102, 103] display IFG as a risk factor for CVD; the degree of independence as a risk factor, however, is uncertain, because IGF usually coexists with other components of metabolic syndrome. Nonetheless, a patient with IFG must be considered at risk for both CVD and type 2 diabetes. As already indicated, once categorical hyperglycemia develops, it counts as an independent risk factor for CVD [94].

2.6.5 Inflammatory Mediators

There is increasing evidence that inflammatory processes and specific immune mechanisms are involved in atherogenesis, and inflammatory markers are reported to be higher among subjects with insulin resistance and diabetes [104]. Inflammation is considered to be a part of insulin resistance syndrome [105], and this, at least partly, explains the higher risk for CAD among diabetic subjects. Inflammatory changes could take place near the rupture of plaque, leading to instability in fibrous tissue in the plaque. One key inflammatory marker is C-reactive protein. C-reactive protein is main human acute phase protein produced in liver that is an extremely sensitive marker of systemic inflammation and tissue damage [107]. More recently, Serum concentration of hs-CRP is a good biomarker of chronic low-grade inflammation and is an established prognostic marker in acute coronary syndrome. Mild elevations of C-reactive protein within the normal range have been observed in subjects with confirmed atherosclerosis, impaired fasting glucose, impaired glucose tolerance, insulin resistance, diabetes, and metabolic syndrome [106, 107]. Low-grade systemic inflammation is associated with type 2 diabetes as indicated by elevated high-sensitivity C-reactive protein levels in Indian population [108]. Furthermore, hsCRP predicts the risk of MetS, independent of obesity and insulin resistance [109]. Elevated hsCRP level is again found to be associated with risk of pre-diabetes (IFG and IGT) in Indians and is independent of effect of traditional risk factors of hyperglycemia [110].

Some observational studies showed that elevations in plasma C-reactive protein are associated with increased incidence of diabetes and increased cardiovascular risk [106, 111]. C-reactive protein levels seem to be higher in migrant Indians compared to other ethnic groups [112]. CRP levels were 17% higher in Indians compared with white Europeans. C-reactive protein also had a strong association with cardiovascular risk factors like obesity, insulin resistance, and lipids [103]. Also, hs-CRP is found to be related with silent myocardial ischemia, and might help to detect silent myocardial ischemia in diabetic patients [113].

Besides CRP, studies on pro-inflammatory markers have revealed that cytokines like tumor necrosis factor α (TNF- α) and interlukin-6 are also strongly associated with CAD. In T2D, activation of inflammation results from obesity and insulin resistance. Due to this a large number of inflammatory and pro-inflammatory cytokines are released from adipose tissue [105, 114]. Adipocytes release cytokines (TNF-α, IL-6, PA-1), adipokines (adiponectin and leptin) that are associated with CVD. Among them adiponectin has anti- inflammatory role and hence protects against atherosclerosis [114].

2.6.6 Homocysteine

Hyperhomocysteinaemia is associated with both micro- and macro-vascular disease, and with death in people with the condition [115]. Homocysteine hits hard in people with diabetes and is known as an independent risk factor for atherosclerosis [116]. Like many other vascular risk factors, it seems to be a stronger risk factor in people with diabetes than in those without the condition. Several cross-sectional and case control studies have pointed towards a clear correlation between total serum homocysteine and the incidence of coronary, carotid, and peripheral vascular disease [117]. Homocysteine can mediate the formation of cardiovascular disease by several different mechanisms such as its adverse effects on vascular endothelium and smooth muscle cells with resultant alterations in subclinical arterial structure and function [118]. Given the high cardiovascular risk in diabetes, and the fact that hyperhomocysteinaemia can easily and safely be improved by folic acid, people with diabetes in particular should be considered for screening and treatment of hyperhomo-cysteinaemia.

2.7 Approaches to Identify Genetic Factors for Diabetes

Strategies to search for genetic susceptibility factors such as candidate gene approach and Genome wide association studies (GWAS) for type 2 diabetes and diabetic complications have evolved over years with improved molecular technology as well as better understanding of the genetic architecture. During earlier times the approach mainly utilized linkage analysis in families with clustering of cases,

and examined the co-segregation of parts of the genome (marked by microsatellite markers) with the disease of interest such as diabetic complications. Identification of linked loci found through linkage analysis is usually followed up by fine-mapping of the confirmed loci and examining candidate genes within that linked region in functional studies. Earlier candidate gene studies rely on prior knowledge and understanding of pathogenesis of diabetic complications to look for an association between genetic variants in genes implicated in these pathways and presence of diabetic complications. Although a large number of candidate-gene association studies have been published, many of these studies have been plagued by relative lack of replication for reported association. More recently, publication of the HapMap and advances in manufacturing of genotyping arrays have made possible a hypothesis-free approach utilizing genome–wide association studies (GWAS). GWAS have been highly successful globally in identification of common genetic variants for complex diseases, and in case of type 2 diabetes, have led to identification of over 100 genetic variants [119], including a study in Indian population suggesting that common susceptibility variants for T2D are largely the same across population and also revealed a newly identified population-specific locus (2q21 mapped toTMEM163) thus providing further insights into genetic architecture and etiology of T2D [120].

This approach is now also beginning to bear fruit on search for genetic factors for diabetic complications. There are also ongoing studies using other technologies, such as next-generation sequencing, and relative merits of different techniques in discovery process will partly depend on the frequency and effect size of the risk alleles being sought.

For candidate gene analysis, candidate genes of known sequence and locations are identified that may be involved in disease pathogenesis and these are often selected on basis of their physiological functions. In contrast, genome-wide screens are more powerful approach that can be used to screen whole human genome for gene linkage or association with a disease without making any assumptions regarding disease pathogenesis [121, 122]. These types of approaches have been used successfully to identify susceptibility genetic loci for diabetic cardiovascular complications (DCC). Genetic linkage analysis often consists of following steps: identifying linked loci, confirming linked loci in another set of independent population, fine mapping of confirmed loci and then testing genes in the linked region in functional studies [123].

2.8 Genetics of Type 2 Diabetes

There is strong evidence for a genetic component of T2D risk. First, the observation of a wide range of diabetes prevalence in different ethnic groups, from very low levels of around 1% in some population, such as tribes of Mapuche Indians or Chinese that live in rural areas, to extremely high levels, as found in Nauru and Pima Indians in Arizona [124]. A part of this ethnic variability can be attributed to

non-genetic environmental and cultural factors. However, the observation that disease prevalence varies substantially among ethnic groups who share same environment, supports the hypothesis that genetic factors contribute to disease predisposition [125]. Familial aggregation studies that compared the disease prevalence within family members of a proband according to that expected in general population showed the importance of the genetic factors. A greater prevalence in family members is thought to be due to an increased number of genes shared among them, including genes that play a role in disease predisposition [125]. According to Swedish registry that recorded 21,004 twins born between 1886 and 1925 and examined major independent variable, age at death from CHD there is a 13.4-fold increase in relative hazard of CHD death. Twin studies clearly show that genes play a major role. They do not isolate which genes are involved in CHD susceptibility, but this methodology is very powerful for assessing the presence of genetic factors [126].

T2D occurs more frequently among individuals who have first-degree relatives with diabetes: data from the Framingham Offspring Study reveal that children of one parent with T2D have a 3.5 times greater risk of developing the disease compared with an individual from the general population, and 6.1 times, when both parents have T2D [127]. The Isfahan Diabetes Prevention Study found a 10.3% higher diabetes prevalence among first-degree relatives of T2D patients compared with 6.0% for a control population of the same age [128]. Also, there is significant concordance in twin studies [129]. In addition, studies conducted in past few years have reported that the incidence of T2DM in Indian people is among the highest in the world, exceeded only by some isolated and homogenous population such as Pima Indian people and Pacific Islanders of Nauru [130].

Several lines of evidences suggest that genetic factors might be implicated in heritability of diabetic microvascular, as well as macrovascular complications. An estimate of heritability for ischemic heart disease in diabetes is approximately 50% [131], whereas a heritability estimate of carotid intima-medial thickness, a well-validated marker of subclinical atherosclerosis, was reported to be 0.41 in type 2 diabetes [132]. Collectively, these and earlier studies support a role for genetic factors in pathogenesis of both diabetic microvascular and macrovascular complications.

2.8.1 Current Status in Indian Population

There is significantly higher genetic diversity within India, compared with Europe and East Asia [133] due to diverse caste and tribal groups, with intergroup gene flow impeded by a hierarchical caste system, geographical dispersal, and subdivision of the country into different linguistic regions [134]. Well powered GWASs had provided novel insights into genetic effects underlying T2D susceptibility in highly differentiated Indian population. The first T2D-GWAS ever conducted exclusively in 12,535 Northern- Indians revealed a new type 2 diabetes associated locus at 2q21,

with the lead signal being rs6723108 (odds ratio 1.31; P = 3.3 × 10−9) [120]. Imputation analysis refined the signal to rs998451 (odds ratio 1.56; P = 6.3 × 10−12) within TMEM163 that encodes a probable vesicular transporter in nerve terminals. TMEM163 variants also showed association with decreased fasting plasma insulin and homeostatic model assessment of insulin resistance, indicating a plausible effect through impaired insulin secretion. Forty-nine of 56 previously reported signals showed consistency in direction with similar effect sizes in Indians and other previous studies, and 25 of them were also found to be associated (P < 0.05) to T2D. Known loci and the newly identified 2q21 locus altogether explained 7.65% variance in the risk of T2D in Indians [120]. The associated single-nucleotide polymorphisms (SNPs) also showed association with fasting plasma insulin levels in same population but were not associated with T2D in a large European sample, potentially due to differences in risk allele frequency or linkage disequilibrium. A novel locus at 13q12 in the SGCG gene (rs9552911, P = 1.82 × 10−8) was identified as associated with T2D susceptibility in the GWAS and multistage meta-analysis done in Punjabi Sikhs from Northern India. Thus, provided new information on previously unknown regions associated with T2D and also demonstrated a putative population-specific association that could lead to additional biological insights into T2D pathogenesis that to in spite of low obesity rates, ∼50% vegetarianism, and strict tobacco abstinence [135]. A current study provided a strong evidence for independent association between T2D and SNPs for in TCF7L2 (rs7903146) and SLC30A8 (rs13266634). MDR analysis showed statistically significant interactions among four SNPs of SLC30A8(rs13266634), IGF2BP2 (rs4402960), HHEX (rs1111875) and CDKN2A (rs10811661) genes demonstrating that independently non-significant variants may interact with one another resulting in increased disease susceptibility in Indian population [136].

Also, replication of Type 2 Diabetes candidate genes variations in three geographically unrelated Indian population groups has been conducted. The study suggests TCF7L2, HHEX, IDE, ENPP1 and FTO as commonly associated T2D susceptibility genes in the three Indian populations. Interaction analyses have shown an increased effect in associations suggesting the importance of gene and pathway based interaction between multiple functionally important genes [137].

2.9 Link Between CAD and Diabetes

T2D and CAD share remarkable relationship in their pathogenesis, due to coexistence of common effectors. Thus, hypothetically all candidate genes for diabetes are potential candidate genes for CAD. There are numerous studies on association of various genes with T2D but it is not clear that how many of them are responsible for increased CAD risk. Apart from genes related to insulin resistance and hyperglycemia, genes from other pathways are also important as various metabolic derangements are common between both conditions. Patients with diabetes have approximately two to fourfold increased risk of coronary heart diseases [72]. The

association between diabetes and incident of cardiovascular disease was most notable in a meta-analysis, for peripheral artery disease, ischemic stroke, stable angina, heart failure and coronary heart disease [138].

In United Kingdom Prospective Diabetes Study, elevated low-density lipoprotein cholesterol, reduced high-density lipoprotein (HDL) cholesterol, elevated triglyceride, glycated hemoglobin, systolic blood pressure, fasting blood glucose and smoking are main clinical risk factors found associated with the development of CHD in diabetes [139]. Though in studies from Asia, the major predictors of CHD in diabetes among Hong Kong Chinese were increasing age, male sex, smoking status, duration of diabetes, lowered estimated glomerular filtration rate (eGFR), increasing albuminuria and non-HDL cholesterol [140]. In Japanese Diabetes Complications Study, the main predictors for cardiovascular complications among patients with type 2 diabetes were identified as non-HDL cholesterol, total cholesterol/HDL-cholesterol ratio and low-density lipoprotein cholesterol [141], and elevated triglyceride was noted to be a particularly important risk factor for incident CHD [142].

Although hyperglycemia plays an important role in development of vascular complications in patients with both type 1 and type 2 diabetes but recent insights from clinical trials suggest a different risk–benefit ratio with regard to the role of intensive glucose lowering and the prevention of cardiovascular complications just proving the link. Meta-analysis of glucose-lowering trials in type 2 diabetes suggested a small reduction in CHD with intensive glucose lowering [143], and beneficial effects of glucose lowering on CHD might only emerge after a prolonged period of follow up [144]. Recent data from Look AHEAD Trial, whereby participants were randomized to intensive lifestyle intervention group had 31% lower incidence of chronic kidney disease (CKD), but no reduction in CHD, suggest that although strategies to reduce cardio-metabolic risk factors are important, the benefit in reducing cardiovascular complications could take a long time to occur [145, 146]. Many genes have been studied comprehensively both in diabetes and cardiovascular diseases across the globe to find out the genetic relationship between coronary artery diseases and Type 2 diabetes based on candidate gene analysis. They are as follows:

2.9.1 PPARγ

Peroxisome proliferator activated receptor gamma (PPARγ or PPARG) present on 3p25 is a transcription factor. It is highly expressed in adipose tissue and macrophages, where it is involved in adipocyte differentiation, triglyceride synthesis, glucose homeostasis and fatty acid trapping [147]. It also regulates the release of various adipokines including tumour necrosis factor α, angiotensinogen (AGT), interleukin- 6(IL6) and plasminogen activator inhibitor 1(PA-1) [148]. It also has an effect on vasculature by its expression in endothelial cells (decrease in endothelin 1, lox-1, NO release), vascular smooth muscle cells (decrease in MMP-9), Macrophages (increase in cholesterol efflux, decrease in cytokines and matrix metalloproteinases: MMP-9), and T cells (decrease of cytokines) [149]. Pro12Ala and C1431T are most

extensively studied polymorphisms in various population with conflicting reports for their association with T2D. Ala 12 allele for Pro12Ala polymorphism was found to confer protection in Japanese [150], Iranian [151], Danish [152] and American Caucasian [153] population. Risk reduction was highest in Asia with association of Pro12Ala being the strongest predictor of T2D as confirmed in Indian Sikhs [154]. C1431T has been studied less extensively in relation with T2D and metabolic syndrome. T allele has been reportedly associated with reduced risk of CAD [155].

2.9.2 TCF7L2

TCF7L2 (Transcription factor 7 like 2) is one of the most important candidate genes present on chromosome 10q25.3. It plays a major role in blood-glucose homeostasis and β cell function. It is expressed in various tissues including placenta, lung, brain, kidney, pancreas, heart and adipocytes and has a very important role in Wnt signalling pathway [156]. Strong association of TCF7L2 with T2DM was initially found in Icelandic population which has been subsequently replicated in Danish, U.S Indian population [157]. The three TCF7L2 SNPs (rs7390146, rs12255372 and rs11196205) that were strongly associated with T2DM in this study were consistently replicated in along with other SNPs of TCF7L2, in a huge meta-analysis [158] and various European and non-European population (including Indian and Japanese population) [159–161]. Association of *TCF7L2* with type 2 diabetes to the largest effect size was confirmed in a study on Indian population [162]. Consistent with these observations, a strong association of *TCF7L2* with HOMA-B and a nominal association with FPG and 2-h PPG confirmed the physiological role of *TCF7L2* in glucose homeostasis [162]. Further combined analysis of Indo-European samples revealed strongest signal at rs7903146 of *TCF7L2* [120]. Polymorphisms rs7903146 and rs680 were found independently to be significantly associated with T2DM risk in Indian adults [163].

In Indian population the replicative studies of this candidate gene were confined to Pune, Punjab, Haryana, Himachal Pradesh, Delhi, Jammu and Kashmir and Chennai and showed strong association of TCF7L2 with T2DM, but given vast ethnic, cultural, geographic, genetic heterogeneity, large population size and relatively high prevalence of diabetes, very small and insignificant number/proportion of Indian population were hitherto studied [160, 162, 164–166]. Polymorphisms in TCF7L2 and near CDKN2A/B genes seem to be of great importance since they appear to modulate both conditions T2D and CAD, and they are not necessarily related to insulinemia, or hyperglycemia, for CAD development [167].

2.9.3 ACE (Angiotensin 1 Converting Enzyme)

ACE gene is located on long arm of chromosome 17q23. It is a zinc metallopeptidase distributed widely on surface of endothelial and epithelial cells and has a very important role in rennin-angiotensin system, making it a strong candidate gene for

CAD and T2D both. Among all variants in this gene insertion (I)/deletion (D) [I/D] polymorphism has been extensively studied in context of T2D. In a meta-analysis, including 24 studies in 15,166 subjects, the D allele was associated with 14% increased risk of T2D relative to I variant [168].When subgroup analysis was done, a significant association in Caucasian and East Asians was observed, however lack of association was observed in Turkish Groups. In another meta- analysis, of 14 studies it was found that presence of D allele conferred a significant increased risk for T2D [169]. Indian studies, reported a strong association of ACE gene polymorphisms with T2DM in northern India [170] and southern India [171, 172].

2.9.4 TNF α

It is a pro-inflammatory cytokine, present on chromosomal location 6p21.3 within the MHC region [173]. It has a very important role in lipid metabolism and is widely implicated in insulin resistance as it is involved in down regulation of genes involved in insulin signalling, induction of elevation of free fatty acids and negative regulation of PPAR γ [174]. Among reported genetic variations in TNF α promoter region such as −238, −308, −857 and −1,031, the −308 and −238 polymorphisms have been extensively studied. Most of the meta- analysis failed to show any significant association with T2D [175, 176]. But a TNF-induced protein 3 (*TNFAIP3*, a negative regulator of NFκB) polymorphism was identified to modulate the risk for coronary artery disease in type 2 diabetics [177]. In Indian population also the variants of type 2 receptor for TNFα (TNFR2) are not associated with T2D. Hence concluding that *TNFRSF1B* (encoding TNFR2) gene though being an important biological candidate, the polymorphisms studied are not a major contributing factor to the genetic risk of type 2 diabetes, its associated peripheral neuropathy and hypertension and related metabolic traits in North Indians [178] and Indo-Europeans from North India [179].

2.9.5 Adiponectin

It is one of the most abundant proteins derived from adipose tissue and encoded by adiponectin gene (ADIPQQ) located on chromosome 3q27. It has protective role in T2D and CVD as it has anti-atherogenic, anti-inflammatory and insulin sensitizing properties. The serum concentrations of adiponectin are heritable [180, 181] thus, making it a strong candidate gene for T2D and CAD. The in vitro bioactivity of APN as an anti-inflammatory adipocytokine in atherosclerotic process is supported by increase of total APN or globular APN that attenuates progression of atherosclerosis in apoE knockout mice [182, 183]. Further, in a few reports APN knockout mice showed enhanced cardiac fibrosis following permanent ligation of left anterior descending artery or angiotensin II infusion [184, 185]. A study on role of 45 T/G

polymorphism [186] revealed that individuals with TG/GG genotype were at nearly fourfold increased risk of T2D in accordance with reported results in Iranian population [187]. Furthermore, the studies on association of Adiponectin gene in CAD indicate association of SNP 45 T>G with increased risk and +276 G>T with decreased risk.

2.9.6 IRS1

Insulin receptor substrate-1(IRS-1) is located on 2q36 and is found to have an important role in insulin action in skeletal muscle, adipose tissue and pancreatic β cells [188]. It has also been found to be associated with regulation of insulin secretion by pancreatic β cell [189]. Further investigations in 2013 explained whether genetic variation at 2q36.3 locus might also affect CHD risk via subclinical atherosclerosis in a sample of 2,740 participants in Framingham Heart Study [190]. Interestingly, compelling evidence exists for the association between cardiovascular events and the candidate functional variant *IRS1*G972R (rs1801278) [191].

2.9.7 Other Genes

Apart from the above mentioned major candidate genes of CAD and T2D, some other genes that are found to be widely associated with T2D have also been studied as candidate genes for CAD. These are Calpain10, FAB4, GST, IL-6, IL-10 and Paraoxonase. Paraoxonase is a glycoprotein; coded for by the PON set of genes—PON1, PON2 and PON3—located on the long arm of chromosome 7 in humans. It is bound to high-density lipoproteins (HDL) that prevents oxidative modification of low-density lipoproteins (LDL) in vitro was identified as a genetic risk factor for cardiovascular disease (CVD) [192]. A strong association was found between Met54Leu polymorphism of PON1 and diabetic retinopathy in adolescents with Type 1 diabetes [193]. Genetic variants in calpain-10 might affect insulin sensitivity [194], or insulin secretion [195], or relation between the two [196]. Also, single nucleotide polymorphism (SNP) in the gene for calpain-10 (*CAPN10*), a non-lysosomal cysteine protease of unknown function, has been demonstrated to be related to insulin resistance and subclinical atherosclerosis as defined by carotid intimal thickening [197]. Genetic variation near or in P2-promoter of MODY-1 gene or HNF4α gene (chromosome 20q) has been proposed to relate to common type 2 diabetes [198]. Also, for the first time HNF4α genetic variants were found to be associated with MetS and metabolic parameters in French Canadian children and adolescents suggesting HNF4α as an early marker for the risk of developing type 2 diabetes mellitus [199]. However, there is scarcity of reports for their association with CAD.

Furthermore, potential biomarkers such as CD36, PPAR-γ and YKL-40 may also play significant roles in insulin resistance and atherosclerosis in patients with T2DM. CD36 also known as Fatty Acid Translocase (FAT) is a multi-ligand scavenger receptor present on the surface of monocyte/macrophages. It binds and endocytoses oxidized LDL, and is implicated in formation of foam cells. Thus, CD36 plays a critical role in the development of atherosclerotic lesions [200]. Expression of scavenger receptor CD36 is increased in presence of peroxisome proliferator activated receptor γ (PPAR-γ) [201]. It is well documented that patients with coronary artery disease (CAD) express significantly higher levels of both PPAR-γ protein (approximately tenfold) and mRNA (approximately 60-fold) compared to healthy volunteers [202]. Several studies had showed that high levels of CD36 present in pre-diabetics, overt diabetics, polycystic ovary syndrome (PCOS), and impaired glucose tolerance strongly suggest that CD36 is involved in diabetes [203] and atherosclerosis pathogenesis and acts as inflammation biomarker [200, 204].

YKL-40 is a novel biomarker expressed and secreted by macrophages. YKL-40 mRNA expression is highly up-regulated on macrophages specifically those that infiltrate deeper in atherosclerotic lesion [205]. Recent studies have reported that elevated levels of plasma YKL-40 are proportional with HOMA-IR in T2DM subjects. This indicates that YKL-40 shows some correlation with insulin resistance and dyslipidemia.

Apart from these α-hydroxybutyrate (α-HB) [206], leptin [207], resistin [208], interleukin-18 [209], retinol binding protein-4 (RBP4) [210], Chemerin [211] are investigated potential biomarkers for Insulin Resistance in T2DM Patients with Coronary Artery Disease. Independently of genome wide association studies, the haptoglobin gene (HP) has been shown to play a role in diabetic atherosclerosis [212].

2.10 Genetic Factors for Coronary Heart Diseases in Type 1 Diabetes

There are relatively few studies that have investigated the role of genetic factors in development of CHD in type 1 diabetes. An early study that investigated the role of two functional polymorphisms in promoter of RAGE gene (−429T/C and −374T/A) and one in advanced glycation end-products binding domain (G82S) in 996 Finnish type 1 diabetic patients noted a reduced risk of coronary heart disease and myocardial infarction, as well as peripheral vascular disease in patients with AA genotype of −374 T/A polymorphism compared with those with TT+ TA genotype [213]. Another candidate gene study that examined the roles of genetic variants in renin–angiotensin system found that carriers of TT genotype in angiotensinogen (AGT) gene M235T polymorphism, the insertion/deletion (I/D) polymorphism at angiotensin converting enzyme (ACE) gene and AA/AC genotype in angiotensin type 1 receptor are at a significantly higher risk of progression of coronary artery calcification [214]. Many efforts are going on to utilize GWAS to advance the understanding of genetic factors underlying CHD in type 1 diabetes.

2.11 Genetic Factors for Coronary Heart Diseases in Type 2 Diabetes

2.11.1 Linkage Studies

Previous linkage studies have identified a few linked regions for cardiovascular disease-related traits in type 2 diabetes, including linkage signal in chromosome 19q region with elevated levels of triglyceride [215] and total cholesterol [216]. In Diabetes Heart Study, linkage of a locus on chromosome 3 with CVD in type 2 diabetes was noted [217].

2.11.2 Candidate Gene Studies

Several candidate-gene studies have been conducted exploring the link between the renin–angiotensin system and the adiponectin pathway. The D allele of ACE gene was first shown to be associated with increased risk of CHD in type 2 diabetes back in 1994 [218], with several studies also supporting this association, though a study in Chinese did not observe an association between the D allele and later risk of CHD in a prospective cohort [219]. Adiponectin secreted by adipocytes has anti-atherogenic effect, and is believed to be an important link between obesity and cardiovascular diseases [220]. In a meta-analysis of four studies, with 827 type 2 diabetes cases with CVD and 1,887 CVD-free control participants, +276T homozygote was significantly associated with a 45% reduction in the risk of CVD [221]. Several studies have examined the role of peroxisome proliferator-activator receptor γ (Pro12Ala) polymorphism in CHD risk, though results appear inconclusive [222, 223]. In a study on prospective cohort of Chinese patients with type 2 diabetes, variants in SCYA11 (eotaxin), PON2 (paroxonase 2) and ADRB3 (β3-adrenergic receptor) were independently associated with incident cardiac events including CHD and/or heart failure [224]. Variants in many candidate genes were extensively studied over past two decades, such as Gly972Arg polymorphism in IRS1, Gly1057Asp polymorphism in IRS2, Trp64Arg polymorphism in ß3 adrenergic receptor, −308 G/A promoter variant in TNF α, or variants in adiponectin gene.

Many candidate association studies have been completed to identify genes linked to cardiovascular disease (CVD) and/or diabetes mellitus and some gene associations have been consistently reported [225]. Such as, polymorphisms in genes related to lipid metabolism or fibrinolysis, including APOE, APOB, APOC, PON, CETP, and PAI1, have displayed increased risk of ischemic vascular disease in diabetic patients. Further, it is known that lipid factors and their oxidation effect the progression of diabetic metabolic syndrome and CVD. Also APOE, APOB, or APOC gene polymorphisms have been reported to associate with macrovascular complications of diabetes [131, 226], although these results have yet to be replicated.

Interestingly, an association between polymorphisms in Paraoxonase that is associated with high density lipoprotein (HDL) and risk of CVD has been consistently described in patients with T2DM from different ethnic backgrounds [227–230] and three polymorphisms (rs662, rs854560 and Q191R have been linked with the risk of CVD in patients with T2DM [231–233].

Patients with diabetes carrying the G allele of the rs662 polymorphism have been found to have more than doubled the risk of myocardial infarction (MI) than patients with other alleles [231]. Furthermore, the Q191R polymorphism was previously identified as an independent risk factor for CVD in patients with diabetes [233]. There is now a large body of evidences implicating this polymorphism as a genetic determinant for risk of ischemic vascular disease in T2DM [231–233]. An evidence of a weak association between rs662 and ischemic stroke risk, similar in magnitude to the corresponding association of the variant with coronary disease has been found [234].

CETP plays a key role in the metabolism of HDL that regulates uptake of cholesterol by hepatocytes, and *Taq*IB polymorphism of the CETP gene is a strong genetic predictor of macrovascular complications in type 2 diabetes [235]. Interestingly, the CETP rs1800774 polymorphism has been reported to associate with macrovascular disease in male T2DM patients independently of lipid levels [236–238]. It is well known that PAI-1 is main circulating inhibitor of fibrinolysis that causes thrombus dissolution. A single base insertion/ deletion polymorphism of rs1799889 in promoter of PAI-1 gene can partially determine the levels of PAI-1 [239] and a possible association between this polymorphism and risk of CVD in patients with T2DM has been reviewed in a meta-analysis [240]. In addition, two studies show apparently contradictory results regarding rs2227631 (−455G/A) polymorphism of fibrinogen gene. In one study, this allele was found to be associated with higher levels of fibrinogen and an increased risk of coronary disease in Chinese diabetic patients [241]. However, a second study conducted in an English T2DM population suggested that G allele was associated with an increased risk of coronary artery disease (CAD), without affecting circulating fibrinogen levels [242].

2.11.3 GWAS for Coronary Heart Diseases in Type 2 Diabetes

Results from several GWAS for CHD conducted in general population showed a potential relation to diabetic subjects [243, 244]. Twelve loci with genome-wide significance have been found to associate with either CAD or MI in general population. Two of 12 genes—LDLR and PCSK9 are mutated in Mendelian forms of hypercholesterolemia [245], as are genes in SLC22A3–LPAL2–LPA cluster, which includes the gene for atherogenic lipoprotein (Lp)(a). Moreover, variations at chromosome 9p21 have been found to significantly associate with CVD in general population [246, 247]. A previous, smaller study in patients with T2DM concluded that the 9p21 signal had a more marked influence on CAD risk among those with poor diabetic control [248]. For example, haplotype analyses have found an interesting

CVD association with a group of SNPs residing in a 60 kb region that includes ANRIL [249, 250]. A decreased risk associated with long to short variant ratio has been reported for this allele [251, 252]. ANRIL links to the CDKN2A and CDKN2B genes that are involved in controlled cell proliferation, cell aging and apoptosis [253, 254]. It is thought that several others of these 12 genes identified in general population can also influence the risk of CAD in diabetic population [255]. In addition, the chromosome 6p24 locus, which includes the PHACTR1 gene, has been found to promote CAD with a strong effect, second only to that of 9p21 locus [256]. Interestingly, receptor for advanced glycation end products (RAGE) gene has been found to associate with both diabetic nephropathy (DN) and diabetic retinopathy (DR); advanced glycation end products (AGE) gene associate with both diabetic nephropathy (DN) and diabetic cardiovascular complications (DCC), and vascular endothelial growth factor (VEGF) gene associate with diabetic retinopathy (DR) and diabetic cardiovascular complications (DCC). Unfortunately, no shared gene has been found to associate with the three complications.

First GWAS in patients with T2D of 1,517 CHD cases and 2,671 CHD controls were included in a three-stage genome-wide analysis, including subjects from the Nurses' Health Study and Health Professionals Follow-up Study, the Joslin Heart Study and the Gargano Heart Study. One novel variant in the vicinity of glutamateammonia ligase (GLUL) gene (rs10911021) associated with CHD at genome wide significance level was identified showing a significant association in all three stages and also genome-wide significance when all three stages were combined, with combined odds ratio (OR) 1.36 (95% confidence interval [CI] 1.22–1.51) [257]. Furthermore, carriers of the risk allele of GLUL showed decreased expression of the gene in endothelial cells and demonstrated lower plasma pyroglutamic-to glytamic acid ratio suggesting gamma-glytamic cycle as a mechanism by which variants the GLUL gene contribute to the risk for CHD [257]. Intergenic variants between GLUL and ZNF648 have been associated with CAD in patients with T2DM and an interaction with T2DM status has also been reported [257]. Preliminary findings obtained by combining data from SUMMIT and CARDIoGRAM*plus*C4D have provided early evidence that the effects of known variants for CAD at chromosome 9p21 near CDKN2A and at ADAMTS7 showed evidence for interaction with diabetes status [258]. One of the strongest signals that influences predisposition to PAD, first identified in samples not stratified for diabetes status, maps near CHRNA3 locus encoding one of several nicotinic receptors and is thought to act through its impact on smoking behavior [259]. The same variant is associated with smoking status and quantity [259]. Some evidence suggests that association between PAD and variants near CHRNA3 is less marked in subjects with diabetes [258]. One interpretation is that the potent direct impact of diabetes on the risk of PAD dilutes the indirect effect of CHRNA3 variants whose effects are mediated by differences in smoking behavior.

Interestingly, no association was found between rs10911021 and CHD for 737 non- diabetic CHD cases and 1,637 non-diabetic CHD-negative controls. This was consistent with the results of the interaction analysis, suggesting that the association between this variant and CHD appeared specific for type 2 diabetes patients.

Furthermore, among 22,233 CHD cases and 64,762 controls from general population included in the Coronary ARtery DIsease Genome-Wide Replication and Meta-Analysis (CARDIoGRAM) Study, this variant showed only a nominal significant association with CHD (OR 1.04, 95% CI 1.01–1.07, P = 0.01) and likely representing an association driven by small proportion of patients with type 2 diabetes included in CARDIoGRAM [257].

The variant was not associated with the risk of type 2 diabetes or insulin resistance, but instead was associated with plasma markers of glutamic acid metabolism and the γ-glutamyl cycle, thereby providing novel insights into the pathogenesis of CHD in patients with type 2 diabetes. Whether this variant is also associated with the risk of CHD in type 1 diabetes mellitus remains to be established.

A peculiar possibility is the relation of diabetes to imprinted genes—i.e., genes for which expression varies depending on the sex of the transmitting parent. The class III allele of the variable number tandem repeat near the insulin gene (Insulin gene variable number tandem repeat: chromosome 11p15) might relate to type 2 diabetes [260]. The class III allele is associated with decreased amounts of insulin mRNA. Only paternally transmitted class III alleles were found to be associated with diabetes in one study [261].

Interestingly, as per joint effort undertaken by CARDIoGRAM-CAD Consortium in 2013 the number of loci known to be associated with coronary heart disease at genome-wide significance level have reached 45 [262]. Also this study confirmed previous findings and discovered 15 new genome-wide significant loci and tested them by a thorough association analysis with traditional CHD risk factors [250, 263]. Out of that twelve loci *(*APOB, ABCG5-ABCG8, PCSK9, SORT1, ABO, LDLR, APOE and *LPA*) showed genome-wide significance for association with at least one lipid trait in the expected direction. Still, the overall spectrum of 65 T2D and 45 CHD genome-wide associated common variants explain only a small fraction (~10% each) of disease heritability, thus leaving a large unfilled space under the umbrella of the common variant/common disease hypothesis [264]. So the need of the hour is to fill in some unfilled spaces under the umbrella of the genetic basis of T2D and CHD by identifying less common or regulatory variants under these diseases. Figure 2.2 shows the metabolic pathways and genes involved in pathogenesis of diabetic complications.

In the Indian scenario as soon as National Human Genome Research Institute's GWAS database was made available, validation studies were initiated for most replicating and significant GWAS-identified SNPs specific to these complex diseases on Indians and Indian migrants living in the Western countries. The prominent findings of GWAS such as association of TCF7L2 gene with T2DM and CDKN2A/2B with both T2DM and CAD were replicated among them [265, 266]. However, some of the major T2DM genes of GWAS such as IGF2BP2 and SLC30A8, consistently replicated in other ethnic groups were not found to be associated with the disease in South Indians while they were in the case of North Indians [267]. These results suggest a lack of consistency in the pattern of association of disease-specific SNPs among the ethnic groups, both within India and elsewhere. The unique genetic predisposition toward complex diseases of Indians could be due to their unique genetic

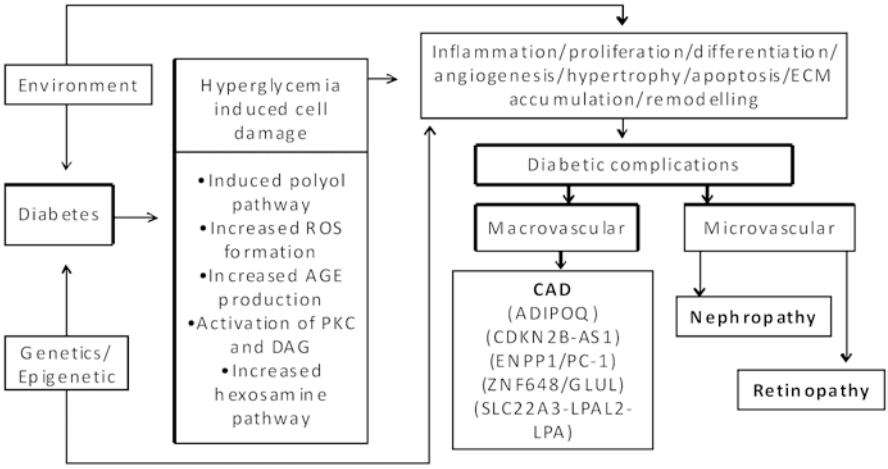

Fig. 2.2 Metabolic pathways and genes involved in the pathogenesis of coronary artery disease as a complication in diabetes

constitution as suggested by an earlier study [268], which observed a common MYBPC 25 bps deletion variant only in South Asians with chronic risk of heart failure [268].

2.11.3.1 Insights from GWAS for CHD

Recent GWAS have identified more than 40 variants associated with coronary artery disease [269, 270]. Among these, several regions appear to harbor variants that are also associated with type 2 diabetes [271]. For example, in chromosome 9p21 region identified to be associated with CHD [272], the cell cycle genes CDKN2A and CDKN2B have also been implicated in a GWAS for type 2 diabetes [273, 274]. In fact, in a genome search meta-analysis to look for shared genetic susceptibility between type 2 diabetes, CHD and obesity, two loci in 9p21.1-a21.32 region were identified to be shared by type 2 diabetes, CHD and obesity [273]. Other genetic factors associated with CHD in general population, such as variants in PCSK9 have also been found to be associated with CHD in type 2 diabetes [275].

Early replication studies of these GWAS-identified variants for CHD have suggested heterogeneity in genetic effects among individuals with or without diabetes. For example, it has been suggested that variants at the 9p21 locus have a larger effect on risk of CHD in patients with type 2 diabetes (compared to subjects without diabetes), particularly showing an interaction with poor glycemic control [248]. Furthermore, in a study in type 2 diabetes patients of variants associated with CHD (in non-diabetic individuals), just 5 out of 15 variants from 12 loci were found to show consistent association with CHD in type 2 diabetes. A genetic risk score (GRS) $\geq$8 composed of risk variants at rs4977574 (CDKN2A/2B), rs12526453

phosphatase and actin regulator 1 (PHACTR1), rs646776 cadherin EGF LAG seven-pass G-type receptor 2/praline/serine-rich coiled-coil 1/sortilin 1 (CELSR2-PSRC1-SORT1), rs2259816 hepatocyte nuclear factor 1 homeobox A (HNF1α) and rs11206510 proprotein convertase subtilisin/kexin type 9 (PCSK9) were associated with twofold increase in the risk of CHD in type 2 diabetes [275]. Another recent study that examined a GRS consisting of 13 or 30 SNPs identified from GWAS for CHD in general population noted an association between GRS score and prior cardiovascular disease, coronary artery calcification, and cardiovascular mortality in a cohort predominantly of African American descent [276].

Other than the SNPs mentioned above, SNPs from the following genes also reached the significance levels required in GWAS: WD repeat domain 12 (WDR12), LDL-receptor (LDLR), mitochondrial ribosomal protein S6/solute carrier family 5/potassium voltage-gated channel subfamily E member 2 (MRPS6-SLC5A3-KCNE2) [252], melanoma inhibitory activity family, member 3 (MIA3), chemokine (C-X-C motif) ligand 12 (CXCL12) [244, 250, 252], Ras-related protein M (MRAS) [243]. Also, two haplotypes from the SLC22A3-LPAL2-LPA gene cluster are associated to CAD [277]. Interestingly, most genes discovered in GWAS, and that appears to be involved with CAD, were not previously implicated in the etiology of atherosclerosis. Notable exceptions are: *LDLR*, which codes for the LDL receptor, PCSK9, which codes for a serine protease that is mutated in Mendelian forms of hypercholesterolemia [244], and the SLC22A3-LPAL2-LPA cluster that includes the gene for atherogenic lipoprotein Lp(a).

Several important insights have emerged from these studies. They highlighted some important differences in the genetic factors associated with risk of CHD in patients with diabetes compared to general population, although there is some important overlap. Therefore, there is the need to carry out studies to identify susceptibility genes for CHD specifically among patients with type 2 diabetes in order to identify susceptibility factors in diabetes, given the heterogeneity of effects when compared with studies carried out in non-diabetic individuals.

2.12 Epigenetics and Diabetic Complications

Epigenetics refers to the study of heritable patterns of gene expression and subsequent phenotypic changes that occur without alterations in DNA sequence [278]. Gene-environment interactions may play an important role in many common human diseases, such as diabetes and its complications, which might be due to epigenetic changes [279]. It would be worthwhile to assess whether lifestyle modifications such as exercise and healthy diets can reduce diabetic complications by altering epigenetic marks. A study showed the beneficial effects of exercise on epigenetic marks related to diabetes [280].

Epigenetic changes in chromatin that provides a crucial interface between genetics and environment, such as DNA methylation and histone modifications, have been linked to gene transcription [278]. Covalent marks on histones are preserved

even after cell division and changes in activating or inactivating histone marks represent a dynamic epigenetic mechanism by which glucose could influence expression of potentially damaging genes in target tissues [281, 282]. Exposure of cells to high levels of glucose can lead to epigenetic changes that affect the expression of genes and microRNAs leading to development of diabetic complications and the concept of metabolic memory that refers to persistence of diabetic vascular complications after glucose normalization [283]. Several studies have conferred that metabolic memory of vascular complications can be pre-dominantly due to harmful effects of hyperglycemia elucidated in cell culture and experimental animal models [284, 285].

Changes in DNA methylation and PTMs of histones have been related to coronary artery disease (CAD), heart failure, hypertrophic cardiomyopathy, dilated cardiomyopathy, arrhythmias, and other cardiovascular pathologies in general population [286]. Epigenetic modifications affect the expression of genes related to the extracellular matrix, TGF–β signalling pathway, and the renin–angiotensin system in glomeruli that have been implicated in pathogenesis of Diabetic Kidney Diseases (DKD) [287]. Also, there is evidence of hypermethylation of the UNC13B promoter region in epithelial cells isolated from patients with T1DM [288]. In large cohorts of patients that also include patients with diabetes miRNAs have been proposed as potential prognostic biomarkers for CAD [289–291], implying its role in diabetic CAD. For instance, miR-4513 rs2168518 polymorphism has been associated with higher prevalence of traditional CV risk factors such as fasting glucose, incidence of T2D, and poor survival of CAD patients [289]. Zampetaki et al. reported that miR-126 was a strong predictor of myocardial infarction in a 10-year follow-up of 832 patients [290]. Motawae et al. recently showed that miR-9 and miR-370 were significantly elevated in patients with CAD and T2D versus patients with diabetes or CAD separately [292]. Interestingly, miR-126 levels were low in circulating endothelial microparticles of diabetic patients with CAD [293].

Experimental studies have suggested that Histone methyl transferases including the activating Set7 and repressive Suv39h1 are responsible for metabolic memory as well has protective roles both for H3K9me3 and Suv39h1 against pre-activated state of diabetic vascular smooth muscle cells (VSMC) [294]. Dysregulation of epigenetic histone modifications may be a major underlying mechanism for metabolic memory and sustained pro-inflammatory phenotype of diabetic cells [294]. A Finnish study has also suggested an association of an exonic SNP in SUV39H1 histone methyltransferase gene with DR, and a trend toward an association with Diabetic DN and CVD [295]. Additionally, histone acetyltransferases (HATs) and histone deacetylases (HDACs) have been implicated in regulation of several key genes linked to diabetes and its complications [296].

Data on DNA methylation or histone acetylation in diabetic peripheral artery disease (PAD) is lacking [297]. Nevertheless, the potential of miRNAs for the diagnosis of PAD has recently been demonstrated. Initially, miR503 has been highlighted as a regulator of diabetic PAD in experimental studies as it is increased in ischemic limb of diabetic mice [298]. Of late, 12 different circulating miRNAs were identified and characterized in peripheral blood of patients with T2D and PAD

[299]. Although these findings were replicated in two separate samples of patients, the study was not designed to test the prognostic utility of the identified miRNAs in a longitudinal follow-up of diabetic patients with atherosclerotic PAD [299].

Because epigenetic changes are potentially reversible in nature, combination therapies with epigenetic drugs (epidrugs) [300] and antagomirs (miRNA inhibitors) [284] could be considered to supplement the current treatments for complications. Conversely, there are key challenges also, as epigenetic patterns are cell specific, data from heterogeneous tissue samples and biopsies could have problematic interpretation. Furthermore, apart from hyperglycaemia, other factors associated with diabetes, including insulin resistance, obesity, dyslipidaemia, environment, lifestyles and genetics, can work independently or co-operatively to also promote epigenetic changes in various affected target tissues. Thus evaluation of epigenotypes by epigenome-wide association studies (EWAS) can provide critical new information about the pathogenesis of diabetic complications and metabolic memory that in turn could identify futuristic newer therapeutic modalities and diagnostic biomarkers for early intervention.

2.13 Conclusions

Advancements in genotyping and DNA sequencing technologies have revolutionized the genetics of complex disorders by allowing identification of rare and common genetic variations that influence an individual's risk for these diseases. Worldwide, the high-throughput genomic approaches have resulted in the identification of over 2600 associated common risk alleles in more than 350 different complex traits [301]. Following the track investigations so far have identified a number of genetic variants related with cardiovascular complications, diabetic nephropathy, retinopathy and neuropathy, the number of variants associated with diabetic complications are rather limited compared with studies for genetic variants for type 2 diabetes or type 2 diabetes-related traits [119].This may be moderately related to scarcity of large well-characterized prospective studies to expedite identification of genetic variants for diabetic complications. The present GWAS approach to identify genetic factors, although successful in identifying genetic polymorphisms with association to disease of interest, are also limited by the incompetence to confer causality because of difficulty in finding the causal functional variants. Re-sequencing studies will help to identify functional variants within identified regions. Finally, the need for larger sample size of well-phenotyped subjects and the current costs associated with whole-genome genotyping or whole genome sequencing remain limitations for genetic studies, though these are likely to become less of a barrier in near future as sequencing costs are decreasing every day.

Additionally, a major limitation of genetic studies in diabetic complications relate to different classifications used and complications relating to case ascertainment. Diverse phenotypic criteria have been used for classification and diagnosis of

diabetic complications of coronary artery diseases, resulting in failure to differentiate between associated phenotypes and actual disease.

Though the potential source of variable findings is gene-gene or gene-environment interactions that differ between population but at the same time a better understanding of complex mechanisms like (Gene- gene, Gene- environment interaction) would give a clear picture of common variants in T2D and CAD [167]. Because if the effect of a variant were only manifest in population with a particular genetic or environmental background, then association would only be seen in population or subgroups with the appropriate genetic or environmental characteristics. This explanation is commonly invoked to explain differing results of association studies but is less frequently supported by direct evidences. Given the interrelationship between diabetes and cardiovascular complications, future genetic studies should also consider the potential overlap between these in terms of their underlying pathogenesis. Future studies utilizing trans-ethnic mapping might also help to narrow down functional variants within candidate gene regions identified through GWAS, as shown by the recent success utilizing this approach in studies of genetic variants for type 2 diabetes [302, 303].

Dissecting the precise functional role of these genetic factors in manifestation of complex diseases would help in developing better disease management strategies. Presently, much of the interest surrounding genetic association studies centers on the potential clinical application of polymorphisms that serve as markers for disease. This will further determine crucial genotypes accurately and predicting future health thus reducing the burden of diabetic complications.

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Chapter 3
Vascular Remodeling in Diabetes Mellitus

Srikanth Vallurupalli and Jawahar L. Mehta

Abstract Vascular complications of diabetes are a major source of morbidity and mortality. Diabetes induces vascular remodeling through various mediators and multiple pathways resulting in profound changes in the structure and function of all layers of the arterial wall. Vascular remodeling causes endothelial dysfunction, increased extracellular matrix formation, vascular smooth muscle cell proliferation and vascular calcification. This results in reduced vascular compliance and reduced perfusion while promoting atherosclerosis, a closely related complication. Despite the ubiquity of diabetes, precise knowledge of how diabetes causes these changes remains unknown. This remains a major obstacle to the development of targeted therapies to prevent vascular remodeling and its associated clinical complications.

Keywords Diabetes mellitus • Vascular remodeling • Oxidative stress • Nitric oxide synthase • Endothelial dysfunction • Vascular smooth muscle cell • Vascular calcification

3.1 Introduction

Cardiovascular disease is the leading cause of death in patients with diabetes mellitus (DM). The presence of DM is associated with a fourfold increase in the incidence of coronary artery disease, a tenfold increase in peripheral vascular disease, and a three to fourfold higher mortality rate [1]. Accordingly, diabetes mellitusboth type I and II are considered coronary artery disease equivalent. The vascular effects of DM are seen in many organs including the brain, mesentery and kidney. The effects of diabetes on the vasculature starts with endothelial dysfunction and lead to changes in the structure of large and small blood vessels, a change which is referred to as vascular remodeling(VRM). While VRM shares some common pathways with atherosclerosis, VRM and atherosclerosis are interlinked yet separate processes.

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Despite the high incidence of vascular disease in DM, not all patients with DM develop vascular disease despite exposure to the same metabolic milieu. Genetic and epigenetic factors play an important though as yet undefined role in DM induced VRM. For example, in the 50 year medalist study, many survivors of 50 years of DM type 1 did not show evidence of vascular disease suggesting that metabolic factors alone cannot explain the pathogenesis of vascular disease [2]. It also underscores the fact that our knowledge of diabetes and its impact on vascular disease remains incomplete.

Despite the ubiquity of DM and its vascular complications, the mechanisms of DM induced vascular changes remain poorly understood. This is reflected in the lack of targeted therapies to fight DM induced vascular disease. In this chapter we will review the possible mechanisms of vascular remodeling in DM.

3.2 Overview of Vascular Remodeling

The term "vascular remodeling" (VRM) was originally coined to describe a complex set of vascular (arterial) changes induced by chronic hypertension, including altered phenotype and function of endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), as well as the extracellular matrix (ECM) structure and composition, leading to altered vessel wall-to-lumen ratio [3]. However, VRM is not exclusive to HTN. It occurs due to both physiological (aging) and other pathological processes such as DM and chronic inflammatory disorders and can affect specific vascular beds from local diseases (such as pulmonary vasculature in COPD). The vessel wall changes due to remodeling can be classified based on two major aspects – change in vessel wall mass (hypotrophic, eutrophic and hypertrophic) and the change in lumen diameter (outward or inward). Diabetic vascular remodeling is typically hypertrophic and ofteninward. In inward hypertrophic remodeling, outer lumen diameter is increased, however since the media/lumen ratio is increased, the inner lumen diameter is decreased.

Under normal conditions, vascular structure is maintained through the closely regulated equilibrium of production and degradation of various mediatorsthat influence the vascular tone and matrix. The normal vascular endothelial liningand basement membrane function as a barrier against the passage of cells, lipids, AGEs and other molecules from the lumen into the vessel wall. Vascular smooth muscle cells regulate vascular tone and maintain perfusion in response to tissue oxygen demands. Various extracellular matrix components promote vessel integrity. Abnormalities in blood flow, transmural pressure and metabolic factors force the vessel to adapt and remodel. By altering this delicate balance among these regulatory forces, pathological conditions such as DM and HTN cause abnormal VRM.

There are several key differences between VRM caused by DM compared to that by hypertension [5]. First, while mechanical factors (flow and pressure) play a major role in HTN-related VRM, metabolic factors such as hyperglycemia and oxidative stress play a major role in diabetic VRM. The major driver of VRM in DM is

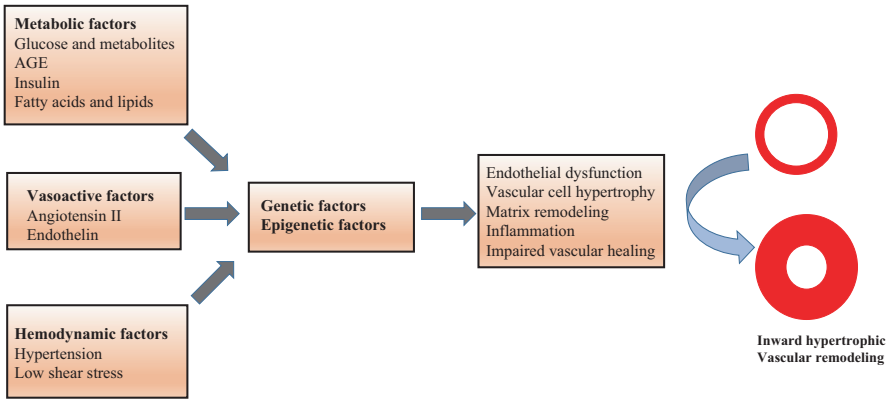

Fig. 3.1 Diabetes causes vascular remodeling through various biochemical and mechanical factors which are finally modulated by genetic factors. This explains why similar severity of diabetes causes different effects on vascular remodeling in human subjects. *AGE* advanced glycation end products

EC dysfunction and the complex signaling pathways that it activates, resulting in changes in all layers of the vessel wall. Second, concomitant remodeling risk factors such as HTN often co-exist in patients with DM type II. Thus DM represents a prototypical VRM state where flow, pressure and metabolism all play a role (Fig. 3.1). This results in a complex interplay of mechanisms resulting in VRM in DM which poses several challenges. Firstly, this impairs our ability to tease out the importance of each individual factor which then stymies effects to develop targeted therapies. Secondly, this complex interplay explains why VRM in DM cannot be completely attenuated by control of metabolic factors such as hyperglycemia alone. A final factor involves the importance of genetic and epigenetic influences on how these abnormalities produce VRM alone.

Despite multiple animal models of diabetes and increasing prevalence of the disease, there are several challenges in teasing out the exact mechanisms of diabetes induced remodeling. First, as mentioned above, various vascular remodeling factors such as hypertension and low shear stress co-exist. Secondly, diabetes produces a variety of metabolic abnormalities while many cell-based and animal studies focus on high glucose alone. This limits our understanding of vascular changes in this metabolic disorder in which hyperglycemia is just one marker. Thirdly, it is difficult to differentiate the effects of atherosclerosis and vascular remodeling since they share common pathways and occur simultaneously in DM. Finally, the widespread use of medications such as HMG-CoA reductase inhibitors, ACE inhibitors and anti-diabetic agents such as metformin, DPP four inhibitors interferes with our study of human diabetic vascular remodeling.

3.3 Mediators of DM Induce VRM

DM induces a number of metabolic and flow mediated abnormalities that activate biochemical pathways that result in vascular remodeling. (Fig. 3.1). Several interlinked pathways lead to the morphological and functional changes inherent to DM. High glucose levels itself is involved in the pathogenesis of DM induced VRM. Glucose metabolites, insulin resistance, increased free fatty acids and other DM associated abnormalities such as low shear stress, hyperlipidemia, hypertension and increased angiotensin II (Ang II) and endothelin 1 also play an important roles [5]. Here it is important to understand that though it is easy to study the effect of individual mediators in the in vitro setting, it is quite difficult to study their independent effect in animals or humans. It is likely that many of these mediators produce effects simultaneously and likely synergistically to mediate VRM.

3.3.1 Hyperglycemia and Related Metabolic Abnormalities

The most important metabolic abnormality is hyperglycemia which occurs in both DM type I and II. Excess glucose is toxic to a variety of cells in the body especially the vascular endothelium. Hyperglycemia-induced generation of superoxide anion ($O_2^{\bullet -}$) leads to DNA damage and activation of poly (ADP ribose) polymerase (PARP) as a reparative enzyme [5]. PARP-induced ADP ribosylation of glyceraldehyde phosphate dehydrogenase (GAPDH) then diverts glucose from its glycolytic path into alternative biochemical pathways leading to increase in advanced glycation end products (AGEs), hexosamine and polyol flux, and activation of classical isoforms of protein kinase C, that are considered major mediators of hyperglycemia-induced cellular injury.

3.3.1.1 Polyol and Hexosamine Pathway

Normally, most glucose is metabolized through the glycolytic and pentose shunt pathways [6]. Under conditions of hyperglycemia, the polyol and hexosamine pathways are activated. In the polyol pathway, sorbitol is produced through aldose reductase, the first and rate limiting step which leads to conversion of NADPH to NADP+. NADPH is an essential cofactor in glutathione production which is compromised by the polyol pathway. Sorbitol's conversion to fructose (through sorbitol dehydrogenase) results in increased NADH/NAD+ ratio which drives production of advanced glycation endproducts as well the de novo production of diacyglycerol (DAG). When excess glucose is shunted through the hexosamine pathway, glucosamine-6-phosphate and uridine diphosphate (UDP)-N acetyl glucosamine are produced (rate limiting enzyme – L-glutamine: D-fructose-6-phosphate amidotransferase (GFAT). Activation of the hexosamine pathway drives vascular

remodeling through its effects on inhibition of endothelial eNOS activity (through decrease in o-linked serine phosphorylation at residue 1177) as well as its effect on transcription factor Sp1which causes increased expression of transforming growth factor β and plasminogen activator inhibitor type I (PAI-I). TGFβ is a powerful mediator of fibrosis while PAI-I inhibitsfibrinolysis.

3.3.1.2 Diacyglycerol (DAG)

An increase in the glycolytic intermediate dihydroxyacetone phosphate occurs during hyperglycemia which is then reduced to glycerol-3-phosphate, which subsequently increases de novo synthesis of DAG [7, 8]. DAG activates protein kinase C which is considered a major unifying pathway in DM induced VRM [9] (see below).

PKC belongs to the serine-threonine kinase family and is an important player in cellular signal transduction for various cytokines [10]. There are three major classes of PKC isoforms- conventional (PKC-α, -β1, -β2, and -γ; activated by phosphatidylserine (PS), calcium, and DAG or phorbol 12-myristate 13-acetate (PMA), novel (PKC-δ, -ε, -θ and -η; activated by PS, DAG or PMA, but not by calcium) and atypical (aPKC; PKC-ζ and -ι/λ;; activated by neither PMA, DAG or calcium) [11].

PKC activation has been proposed as a final common pathway for diabetic VRM with DAG as a major (but not isolated) activator. PKC activationaffects multiple pathways (Fig. 3.2). By virtue of phosphorylation of threonine 497/495 at the calmodulin binding peptide of eNOS, PKC activation reduces NO production and causes endothelium dependent vascular dysfunction. In addition, it promotes vascular endothelial growth factor (VEGF) excess increasing the production of thromboxane, other cyclogenase-dependent vasoconstrictors and endothelin-1 (ET-1) while decreasing and decreases production of prostacyclin, a vasodilator. PKC activation directly increases the permeability of albumin and other macromolecules through barriers formed by EC via the expression of growth factors, such as VEGF/vascular permeability factor (VPF). Activation of PKC isoforms affects activity of various other intracellular signaling pathways such as MAPKs and early growth response 1 (Egr1) which is an important redox sensitive gene which mediates atherosclerosis. Severe PKC inhibitors have been tested in animals and humans to prevent various complications of diabetes. In one study, ruboxistaurin, a PKCβ inhibitor was found to improve brachial artery mediated flow dilation [11]. However, no single agent has been shown to improve clinical outcomes.

The effect of PKC in DM is modified by calpains which are nonlysosomal Ca 2+–dependent cysteine proteases expressed in a variety of cell types, including ECs. Calpains have been known to mediate endothelial dysfunction in DM [12]. One mechanism is oxidative stress which increases mu- Calpain; this acts as a cleavage activator of PKC especially in the presence of hyperhomocysteniemia (which commonly occurs in DM); this results in reduction of NO and endothelial dysfunction.

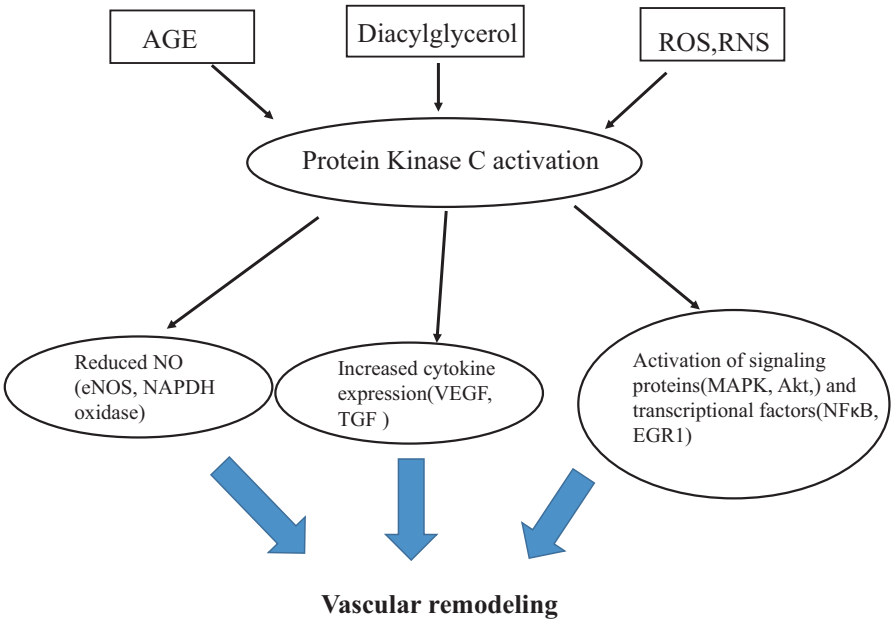

Fig. 3.2 A simplified diagram of the diacyl glycerol-protein kinase C pathway which has been proposed as a major mechanism of diabetes induced Vascular remodeling. *AGE* advanced glycation endproducts, *ROS* reactive oxygen species, *NO* nitric oxide, *NOS* nitric oxide synthase, *VEGF* vascular endothelial growth factor, *TGF* tissue growth factor

3.3.2 Advanced Glycation End Products (AGE)

AGEs are a large and heterogeneous group of compounds that in their classic form originate when the reactive carbonyl group of a sugar reacts with the nucleophilic amino group of an amino acid (the classic "Maillard reaction") [13]. We now recognizethat non-sugar entities such as the byproducts of lipid and protein degradation can provide the carbonyl group. The first, reversible step in their formation is the generation of an unstable "Schiff base" which then undergoes structural irreversible re-arrangements, to form stable keto-amines, called Amadori products. These Amadori products undergo further changes through oxidation and degradation to yield highly stable AGEs. Low molecular weight carbonyl compounds, such as the alpha-ketoaldehydes methylglyoxal (MGO) and glyoxal (GO), are formed under hyperglycemic conditions and behave as advanced glycation end product (AGE) precursors.

AGEs form under hyperglycemic conditions and chronically accumulate in the body. They likely mediate "glycemic memory" whereby glycemic control over many years rather than acute changes in blood glucose influences vascular complications. Though most research has concentrated on endogenously produced AGE's, the role of exogenous AGEs is now being recognized [13]. The two most common causes of

exogenous AGEs are the Western diet (processed foods and meats exposed to high heat) and tobacco smoke. Exogenous AGE intake correlates with endogenous AGE levels and is increasingly being recognized as a cause of vascular dysfunction. Here, it is interesting that the same diet that predisposes to the development of DM II can also propagate the adverse effects of diabetes even after it is established.

AGEs induce vascular remodeling by two different mechanisms.

3.3.2.1 Receptor Mediated Mechanisms

AGEs activate intracellular signals through several receptor- and nonreceptor-mediated mechanisms, leading to an increased production of reactive oxygen species and inflammatory cytokines [14]. One of the best-studied AGE receptors is RAGE. RAGE is a member of the immunoglobulin multi ligand receptor family involved in intracellular signal transduction. As a pattern recognition receptor, RAGE recognizes a diversity of ligands such as S100 calgranulins (expressed during tissue injury and inflammation) and modified LDL in addition to AGEs. RAGE activation promotes inflammatory responses, apoptosis, prothrombotic activity, expression of adhesion molecules, and oxidative stress. RAGE signaling activates several central transcription factors such as nuclear factor (NF)-ƙB, cAMP-response-element-binding protein (CREB)-1 (mediating vascular calcification), Egr1 and activator protein (AP)-1.

RAGE increases ROS and oxidative stress by multiple pathways. It suppresses eNOS phosphorylation and expression via the Pi3K/AKt/eNOS pathway as well as via NAD(P)H oxidase. Activation of the RhoA-ROCK pathway by AGEs regulates various pathways that eventually lead to vascular remodeling. Rho-associated protein kinase (ROCK) also belongs to the serine threonine kinase family and is the main downstream effector of RhoA, a small GTPase molecule. It plays a major role in the organization of the actin cytoskeleton and regulating cell movement [15, 16]. Adverse effects include oxidative stress leading to reduced NO production (via eNOS) as well as its effects on VSMC via actin polymerization and NF-κB activation [17]. Fasudil, a ROCK inhibitor is approved in some countries for treatment of angina but has not been clinically tested in diabetes.

RAGE also signals via MAPK pathwayswhich activate the redox-sensitive transcription factor NF-κB. Since RAGEs are present on the surface of different cell types: macrophages, adipocytes, ECsand VSMC, their activation produces profound remodeling effects throughout the vascular wall. In ECs, RAGE activation also triggers expression of adhesion molecules suchas VCAM-1 which promotes attachment and transendothelial migration of monocytes across the EC layer. RAGEs increase proliferation and migration of VSMCs via activation of ERK1/2 and suppression of adenosine monophosphate kinase (AMPK) activation.

3.3.2.2 Non-receptor Mediated Mechanisms

AGEs crosslink proteins directly thereby altering their structure and function. Glycation of extracellular matrix proteins such as collagen VI, laminin and vitronectin leads to increased thickening and fibrosis. Modification of the cell-binding domains of type VI collagen decreases endothelial cell adhesion to the basement membrane thus altering stability of the vessel.

3.3.3 Insulin Abnormalities

Insulin has many biochemical and physiological effects on the vascular wall. These effects are mostly mediated by its effect on the insulin receptor which results in activation of two different pathways: (a) PI3K/Akt – which results in increased eNOS activation (acutely) and production (chronically) and resultant increased NO, and (b) MAPK pathway – which mediates VSMC proliferation and synthesis of ECM proteins. Mitogen associated protein kinases (MAPK) mediate cellular response to extracellular stimuli, and play an important role in inflammation, cell development, cell differentiation and senescence. There are four distinct subgroups of MAPK: (1) extracellular signal-regulated kinases (ERKs), (2) c-jun N-terminal or stress-activated protein kinases (JNK/SAPK), (3) ERK/big MAP kinase 1 (BMK1), and (4) the p38 group of protein kinases. Of these, p38 and ERK mediated vascular effects of DM (mediated by upregulation of transcription factors such as NF-κB, AP-1 and others) are well recognized. High levels of plasma free fatty acids, pro-inflammatory cytokines, and/or glucose among other mediators activate p38 and ERK1/2 MAPKs which activate and injure vascular components – such as ECs, VSMCs and fibroblasts.

Under normal conditions, the effects of PI3K/Akt may be considered beneficial while that of the MAPK pathway detrimental to vascular health. PI3K/Akt pathway is active only at normal insulin concentrations. With excess insulin levels, activation of PI3K/Akt is reduced while MAPK is unaffected [18]. Similarly, with lack of insulin, lack of the beneficial anti-remodeling effects of PI3K/Akt are lost. This may explain the presence of VRM in both DM type 1 (insulin deficiency) and DM type 2 (insulin excess).

Recently, the role of Tribble 3 (Trb3), a cytosolic pseudokinase, in mediating hyperinsulinemia induced vascular remodeling has been studied. Trb3 is upregulated by insulin excess which causes decreased phosphorylation of Akt while activating the JNK/MAPK pathway [19]. Trb3 silencing restored Akt/MAPK balance and reduced aortic vascular remodeling in diabetic rats.

3.3.4 Excess Free Fatty Acid Production

DM type II is associated with excess production of free fatty acids (FFAs). FFAs cause mitochondrial dysfunction through oxidation of fatty acyl-COA and result in increased ROS and oxidative stress [20]. It also activates various cell signaling pathways (such as p38 MAPK) which influence VRM. In addition, it appears to cause increased formation of ox-LDL and activates LOX-1 and other scavenger receptors.

3.3.5 Increased Angiotensin II and Endothelin-1

Diabetes is associated with upregulation of renin angiotensin system (RAS) and elevated tissue Ang II. Ang II is a potent vasoconstrictor and inducesoxidative stress through generation of superoxide radicals. In addition, through upregulation of MAPKs, it activatesNF-κB. Plasma ET-1 is increased in patients with diabetes mellitus and ET-1 exaggerates diabetes-induced endothelial dysfunction. This is mediated by decrease in eNOS expression, increase in vascular oxidative stress, and decrease in antioxidant capacity. When diabetes is induced in mice overexpressing human ET-1in the endothelium, endothelial nitric oxide synthase (eNOS) and superoxide dismutase expression is reduced compared to wild type mice [21].

3.3.6 DM Associated Hyperlipidemia and Hypertension

Hypertension and dyslipidemia commonly co-exist with DM. Hypertension causes VRM due to increased transmural pressure and flow related abnormalities while dyslipidemia mediates VRM through lipids and their modified lipid fractions. Modified LDL especially ox-LDL plays an important role in mediating oxidative stress mediated endothelial dysfunction as well as abnormalities in VSMCs including vascular calcification [22].

Ox-LDL and the activation of lectin like oxidized LDL receptor-1 (LOX 1) appears to play a major role in DM induced VRM especially vascular calcification. LOX-1 as a scavenger receptor is activated by multiple ligands including AGE. AGE upregulates LOX-1 likely through the PI3k/Akt pathway.

3.3.7 Abnormal Shear Stress

Though most research efforts have concentrated on its metabolic effects, diabetes mellitus has long been recognized as a low shear stress state [23]. The low shear stress is caused by a combination of reduced erythrocyte deformability and increased

erythrocyte aggregation as well as changes in plasma proteins due to abnormal glucose metabolism. Endothelial cells (ECs) are the lining on the inside surface of vasculature and are capable of perceiving shear stress as a mechanical signal which is then transduced into various biomolecular responses. Mechanosensors in the endothelium include various ion channels and receptors and the glycocalyx. The endothelium interacts with circulating blood through the surface glycocalyx layer, which serves as a mechanosensor/transducer of fluid shear forces leading to biomolecular responses. Hyperglycemia impairs mechanotransduction in bovine aortic endothelial cells (BAEC). Heparan sulfate content is reduced and this coincides with a significantly lower activation of eNOS after exposure to shear, and reduced cell alignment with shear stress [24].

Low shear stress is a potent mediator of endothelial dysfunction. It reduces NO availability while upregulating prostacyclin and endothelin which are potent vasoconstrictors. Low shear stress induces MMP-9 expression through integrins-p38 MAPK and ERK1/2-NF-κB signaling pathways which in turn alter the extracellular matrix (Fig. 3.3). In addition, low shear stress promotes VSMC apoptosis and proliferation. Atherosclerosis localizes typically in regions of low or disturbed shear-stress, but in diabetics, the distribution is more diffuse, suggesting that low shear stress is a generalized phenomenon in DM and that there could be a fundamental difference in the way diabetic cells sense shear forces.

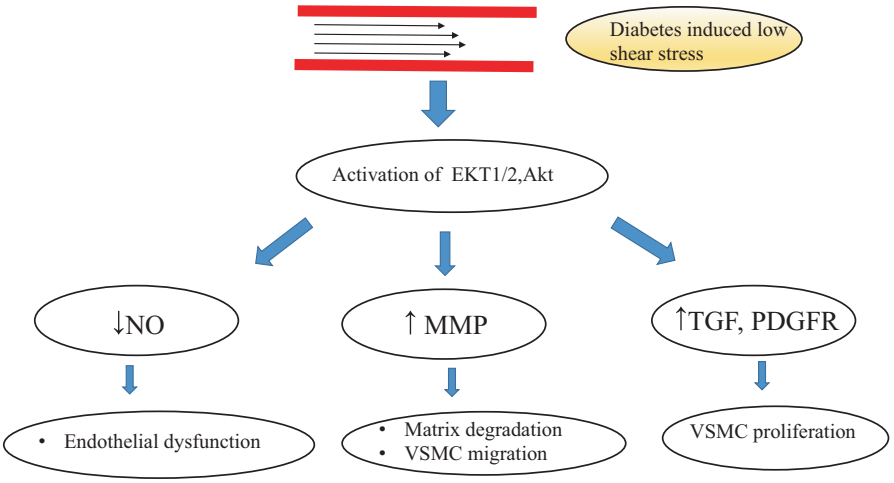

Fig. 3.3 Effects of low shear stress, an important mechanical trigger of vascular remodeling in diabetes. *NO* nitric oxide, *MMP* matrix metalloproteinases, *VEGF* vascular endothelial growth factor, *TGF* tissue growth factor, *PDGFR* platelet derived growth factor receptor

3.4 Pathophysiological Mechanisms of Diabetes Induced Vascular Remodeling

The mechanisms by which DM induces VRM remain incompletely understood. Of all the described mechanisms, oxidative stress and production of ROS is probably the most important pathway.

3.4.1 Oxidative Stress and Endothelial Dysfunction

In normal physiology, the key mediator of endothelial cells (EC) regulated vascular homeostasis is nitric oxide (NO) [25]. NO is an important mediator of cell signaling and cell-cell communication. Due to its low molecular weight and its lipophilic properties it diffuses easily across cell membranes. NO is produced by the action of nitric oxide synthases (NOS) on L-arginine in the presence of tetrahydrobiopterin (BH4) as a cofactor converting it to L-citrulline. Of the three known NOS isoenzymes, constitutive endothelial NOS(eNOS) and inducible NOS (iNOS, expressed in macrophage and endothelial cells due to the effect of pro-inflammatory cytokines) play an important role in vascular endothelial function. eNOS, the predominant NOS isoform in the vasculature, is responsible for most NO production under normal conditions. NO crosses the endothelial intima and reaches VSMC where it causes cGMP mediated smooth muscle cell vasodilation. NO also plays a key role to maintain the vascular wall integrity by inhibition of inflammation, cellular proliferation, and thrombosis. This is mediated in part by the s-nitrosylation of cysteine in various proteins including the transcription factor NF-κB. iNOS is activated under situations of stress and inflammation and produces NO in concentrations that are thousands of times higher than eNOS; this excess NO by itself is toxic.

NO is inactivated by reactive oxygen species (ROS) and reactive nitrogen species (RNS). The most common ROS are superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$) while the most common RNS is peroxynitrite (ONOO-). Under normal conditions, ROS such as superoxide ion (O_2^-) are formed in minute quantities in the mitochondria during electron transport which is crucial in the process of ATP generation to sustain the ongoing needs of living cells in minute amounts and are buffered by native anti-oxidant defense mechanisms such as superoxide dismutase, catalase and glutathione. In normal conditions, electron transfer through complexes I, III, and IV extrudes protons outward into the inter membrane space, which generates a proton gradient that drives ATP synthase (complex V). Excess electrons are donated to molecular oxygen resulting in the formation of superoxide ion. Superoxide inactivates NO to form $ONOO^-$ a reactive nitrogen species (RNS). $ONOO^-$ oxidizes BH4 and also activates Rho-ROCK pathway, thus mediating endothelial dysfunction.

$O_2^-$is a relatively short-lived species; it dismutates to hydrogen peroxide, a step mediated by superoxide dismutase (SOD) enzyme). H2O2 is a more stable ROS and

high levels promote vasoconstriction and cause oxidative damage to vasculature. Intracellular H_2O_2 levels are tightly regulated through (i) a direct involvement of catalase, peroxiredoxin and thioredoxin enzyme networks, and (ii) an indirect involvement from uncoupling proteins and Nrf-2 expression. Therefore, endothelial function is regulated though a complex network of regulation in NO production, O_2^- production and dismutation, and peroxide clearance.

Three major mechanisms play a role in DM induced ROS damage: (a) derangement of electronic transport (b) induction of various enzymes such as NAD(P)H oxidase family enzymes (NOX), xanthine oxidase, uncoupledeNOS (due to reduced GTP cyclohydrolase I causing reduced BH4), induction of iNOSand (c) depleted antioxidant mechanisms such as reduced glutathione (Fig. 3.4).

Under conditions of hyperglycemia, electron donors (NADH and FADH2) are available in excess, this results in a block in the electron transport chain at complex III and donation of excess electrons to O2 through coenzyme Q. Another major source of superoxide ion and hydrogen peroxide (H2O2) occurs through membrane-bound, nicotinamide-adenine-dinucleotide- (NADH-) dependent oxidase (NOX). NOX generates superoxide by electron transfer from NADPH to molecular oxygen to yield $O2^-$. In addition, it leads to uncoupling of eNOS(due to dissociation of the ferrous-dioxygen complex) which then produces O2- instead of

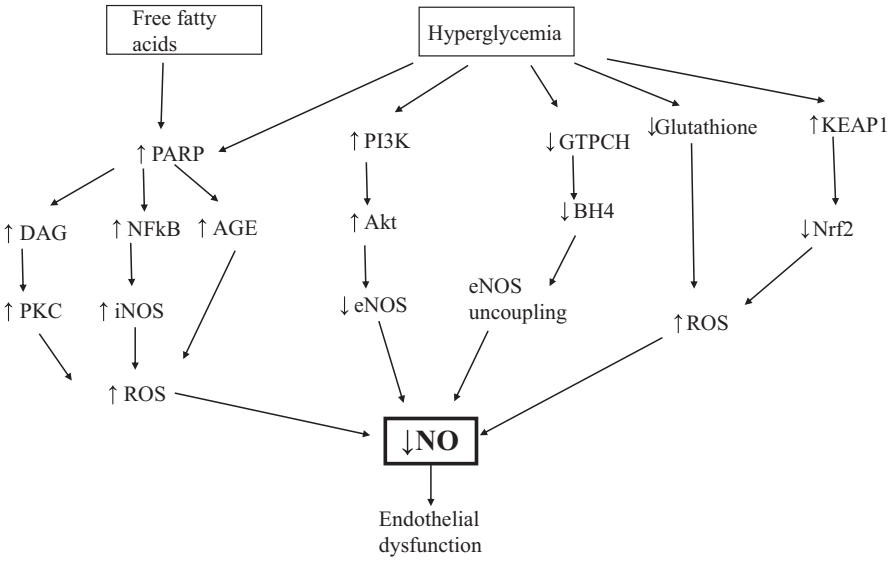

Fig. 3.4 Different NOS mediated oxidative stress mechanisms in diabetes. In addition to reducing NO bioavailability and production by reducing eNOS, induction of iNOS and eNOS uncoupling (by reducing tetrahydrobiopterin4) occur. *PARP* poly ADP ribose polymerase, *DAG* diacylglycerol, *AGE* advanced glycation endproducts, *PKC* protein kinase C, *ROS* Reactive oxygen species, *eNOS* endothelial nitric oxide synthase, *iNOS* inducible nitric oxide synthase, *GTPCH* GTP cyclohydrolase I, *BH4* tetrahydrobiopterin, *KEAP 1* Kelch-like-ECH-associated protein 1, *Nrf2* Nuclear factor-like 2, *NO* nitric oxide

NO. Caveolin-1 (Cav-1), an anchoring protein in the plasma membrane caveolae in ECs and vascular smooth muscle cells (VSMCs), attenuates endothelial NO production by occupying the calcium/calmodulin (Ca2+/CaM) binding site of eNOS. Diabetes leads to increased Cav-1 expression; Cav-1 then binds and inactivates eNOS resulting in reduced NO production [26].

An example of DM related depletion of antioxidant mechanisms is its effect on the KEAP1-Nrf2 pathway [27]. The redox sensitive transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) protects against oxidative stress via the induction of phase II and antioxidant enzymes. Under low oxidative stress conditions, Nrf2 is sequestered by its cytosolic binding protein Kelch-like ECH Associated Protein 1 (Keap1) and targeted for proteasomal degradation. Keap 1 levels are increased in DM resulting in reduced Nrf2 and unchecked ROS production. Activators of Nrf2(such as sulforaphane) are being tested in diabetes and its related complications though their effect on VRM are unknown.

The effects of oxidative stress are felt far beyond the endothelium. Oxidative stress causes increased matrix production through its effect on matrix metalloproteinases which reduce elastin and increase collagen production. ROS also increase proliferation and migration of vascular smooth muscle cells likely through induction of cyclophilin A [28]. Finally ROS causes activation of pro-inflammatory cytokines which potentiate several remodeling pathways and accelerate atherosclerosis.

3.4.2 Endoplasmic Reticulum Stress and Autophagy

The endoplasmic reticulum (ER) plays essential roles in physiologic regulation of many cellular processes such as protein folding, lipid synthesis, and regulation of the intracellular calcium balance. Perturbations of the normal function of the ER trigger a signaling network called the Unfolded Protein Response (UPR) pathways. Three UPR sensors namely, activating transcription factor-6 (ATF6), inositol requiring protein-1 (IRE1), and protein kinase RNA-like ER kinase (PERK) sense misfolded proteins and initiate UPR. UPR can be adaptive in acute ER stress and apoptotic in chronic ER stress.

Secretory and membrane proteins, which are synthesized in ER, undergo proper folding in the ER lumen. A key ER chaperone that is essential for proper protein folding is BiP or glucose-regulated protein 78 kDa (GRP78). In normal conditions, BiP is bound to the molecules of the three UPR sensors. In ER stress, BiP dissociates from and activates the UPR sensors and results in induction of UPR. Excessive and prolonged ER stress mediated by hyperglycemia and ox-LDL is associated with increased inflammation and apoptosis [29].

Autophagy is a crucial cell maintenance mechanism which involves degradation of abnormal proteins and cell organelles. It appears to play a key role in mediating normal endothelial function. In DM, preliminary studies underscore the importance of normal autophagy in protection of endothelial cells against high glucose induced injury [30, 31].

3.4.3 Inflammation

Diabetes is a pro-inflammatory state. Systemic inflammation plays an important role in activation of various adverse remodeling pathways both by itself and by potentiating the effects of other mediators.

3.4.4 Vascular Calcification

Vascular calcification of the medial (VCm) is a characteristic feature of diabetes mellitus and is caused the deposition of highly crystallized calcium hydroxyapatite in the tunica media. These crystals bind to the extracellular matrix (specifically to elastin). The prevalence of VCm in patients with newly diagnosed T2D was 17% and among patients with established T2D receiving oral anti-diabetics, the prevalence of VCm was as high as 41.5% [32]. Vascular calcification is a well-recognized marker for cardiovascular complications and has several functional implications. Arterial wall stiffening causes an increase in pulse wave velocity and increase in pulse pressure. This can affect organ perfusion and mediate end organ damage. Augmented afterload due to vascular stiffening results in left ventricular hypertrophy and could result in development of heart failure with preserved ejection fraction.

3.4.4.1 Mechanisms of Vascular Calcification

Long considered a passive degenerative process, we now recognize that vascular calcification is an active process which involves the interplay between multiple molecular cascades which are regulated by genetic and metabolic factors. Hyperglycemia, AGEs and ox-LDL appear to be major mediators of DM induced VCm. High glucose levels enhance BMP activity (BMP-2 and 4) in endothelial cells. Bone morphogenetic protein-2 (BMP-2) appear to be the key initiator of vascular calcification; it increases expression of core binding factor alpha-1 (CBFA-1, or RunX2) which upregulates the production of osteoblast proteins within vascular smooth muscle cells (VSMCs) promoting a phenotypic switch of contractile VSMCs to an osteoblast-like phenotype. Alkaline phosphatase (ALP) and bone sialoprotein (BSP) have been demonstrated to be early markers of osteoblast activity, while markers, such as osteopontin (OPN) and osteocalcin, are upregulated later in VCm. Their primary function is to enhance the formation and deposition of hydroxyapatite, which is composed of type I collagen and other noncollagenous proteins. ALP cleaves pyrophosphate to phosphate to promote hydroxyapatite deposition and mineralization within the bone. BSP is responsible for the nucleation of hydroxyapatite mineral. OPN is also linked to hydroxyapatite deposition and can serve as a mediator of cell attachment and signaling. OPN up regulated in medial VSMCs also enhances adventitial myofibroblast (osteoprogenitor) migration, proliferation and

matrix metalloproteinase (MMP)-dependent matrix turnover. Hydroxyapatite size and shape are mediated by osteocalcin through a vitamin K dependent mechanism.

Oxidative stress in diabetes increases VCm via upregulation of NF-κB which in turn activates CBFA1An emerging regulatory pathway for vascular calcification in diabetes involves the receptor activator for nuclear factor κB (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG). RANKL increases vascular smooth muscle cell calcification by binding to RANK and increasing BMP4 production through activation of the alternative NF-κB pathway. OPG (member of the TNF-related family) binds to RANKL as a soluble decoy receptor and prevents calcification. Inactivation of OPG in miceleads to profound vascular calcification [33]. In diabetes, increased RANKL and decreased OPG levels lead to increased vascular calcification.

Another intriguing mechanism by which diabetes effects VCm is through matrix Gla protein (MGP). MGP, a vitamin K dependent protein, is a calcification inhibitor expressed by many cell types including VSMC. Activation of MGP requires post-translational carboxylation and serine phosphorylation. Though the mechanism is not completely understood, diabetes effects MGP phosphorylation which in turn could potentiate VCm [34].

Substantial evidence also supports the role of AGEs as the major mediator of DM induced vascular calcification [35]. Mechanisms include upregulation of various pathways including BMP2 and through increased oxidative stress.

The role of ox-LDL in promoting VCm is increasingly being recognized. Diabetes promotes the increased formation of oxidized LDL whose role in mediating VC in DM is not completely understood but is being recognized. The major mechanism appears to be through (a) promotion of oxidative stress and upregulation of NF-κB which inturn activates CBFA1 and (b) upregulation of BMP2 which activates CBFA1 via the Smads pathway. Other mechanisms include the upregulation of vascular peroxidase 1 (via the production of hypochlorous acid) [36] and activation of PI3K/AKT, ERK1/2, and P38 MAPK/Runx2 pathways) and calpain 1 (through disordered pyrophosphate metabolism).

3.4.5 Genetic and Epigenetic Effects on Diabetic VRM

Despite widespread advances in genomics, genetic markers of accelerated diabetic VRM remain poorly understood. Among the epigenetic modifications, the role of microRNA (miRNA) is increasingly being recognized in DM. miRNAs are a family of small (18–25 nucleotide), noncoding single-strand RNA molecules that modulate various physiological and pathological pathways via post-transcriptional inhibition of target gene expression. They bind to messenger RNA (mRNA) leading to mRNA degradation or suppression of translation. There is an explosion of knowledge of miRNAs but their role in DM induced VRM is unclear. They appear to modulate endothelial a functional and angiogenesis which is important in wound healing. In vascular smooth muscle cells (VSMCs) cultured from T2DM *db/db* mice, miR-125b

appears to be upregulated by hyperglycemia, this downregulates of its predicted target Suv39h1, a histone-lysine N-methyltransferase and causes enhanced inflammatory gene expression [37]. In human subjects, microarray profiling has shown an altered profile of miRs expression in subjects with T2DM [38]. For example, the pro-angiogenicmiR 126 is significantly downregulated in DM. Hyperglycemia and AGEs downregulate miR221 and mIR222 with impaired endothelial and endothelial progenitor cell (EPC) proliferation and angiogenesis, which are important in response to vascular injury [39]. As knowledge accumulates, one hopes that targeting specific miRNAs would help alleviate vascular remodeling.

3.5 Overview of Morphological Changes in DM Induced VRM

The normal artery consists of the following layers- endothelium, intima, the internal elastic lamina, the media, the external elastic lamina and the adventitia. The endothelial cell layer is probably the most important and dynamic structure in the vessel wall that serves as the interface between circulating blood and the vessel itself. It senses different physical or chemical stimulus that occur inside the vessel, and endothelium is capable of producing a large variety of molecules that maintain vascular function.

As a result of the various mechanisms illustrated above, DM results in ultrastructural changes in all components of the arterial wall which include (a) macroscopic changes such as increase in intimal thickness, vascular smooth muscle cell hypertrophy and calcification and adventitial inflammation and thickening and (b) functional changes such as reduced vasomotor contractility and reduced arterial compliance (Fig. 3.5).

3.5.1 Endothelial Cell Alterations

The initial target of DM induced VRM appears to be the endothelium whose morphology and function are effected. There are fourmajor of DM on endothelial cells (a) Increased endothelial cell layer permeability due to reduced density of tight and adherence junctions which can occur as soon as 4–6 weeks after initiation of diabetes (b) Cell death via senescence and apoptosis and (c) Abnormal signaling resulting in increased inflammation and resultant effects on the rest of the vascular wall (d) Impaired vascular relaxation through reduction in NO production. In addition, impaired endothelial cell migration prevents neovascularization in response to injury and leads to impaired wound healing.

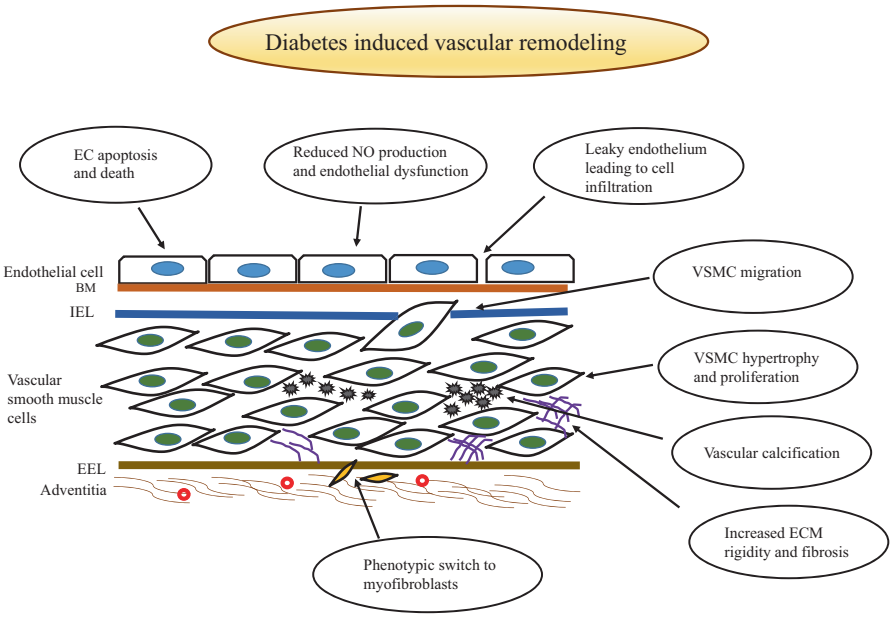

Fig. 3.5 This figure illustrates the major morphological changes in arterial wall due to diabetes. All layers of the wall are effected and cell-cell interactions play a major role in initiation and propagation of remodeling. *EC* endothelial cell, *NO* nitric oxide, *VSMC* vascular smooth muscle cell, *ECM* extracellular matrix, *IEL* internal elastic lamina, *EEL* external elastic lamina

3.5.2 Extracellular Matrix (ECM) Alterations

ECM displays a very dynamic equilibrium where there is constant synthesis, degradation and reorganization of various components. Turnover of vascular ECM proteins such as collagen type 1 and 3, fibronectin and thrombospondins is regulated by matrix metalloproteinases (MMPs) and its inhibitors (TIMPs) [40]. Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes that degrade various components of ECM and mediate ECM remodeling in both physiological and pathological processes. Though different pathways (oxidative stress, activation of ERK and p28 MAPK by shear stress and Ang II), diabetes causes various changes in the ECM through MMPs and TGF β. Increased levels of MMP-2 and MMP-9 are present in DM along with reduced TIMP 2. Thebasement membrane and ECM thicken and this leads to vessel wall rigidity.

3.5.3 Vascular Smooth Muscle Cell Alterations

Vascular smooth muscle cells form the bulk of the vascular wall and are important in maintaining vascular tone. The four major alterations of VSMC in DM include cell proliferation, cell migration into the tunica intima, conversion to osteoblasts leading to vascular calcification and abnormal vascular tone (inability to relax).

In DM, vascular smooth muscle cells proliferate and contribute to vascular hypertrophy. VSMC migrate to the tunica intima where they contribute to atherogenesis. Diabetic vascular smooth muscle cells display abnormal contractile responses which is mediated by the activation of the Rho-ROCK pathway and subsequent PKC/CPI-17 (C-kinase potentiated Protein phosphatase-1 Inhibitor Mr = 17 kDa) phosphorylation [41]. Phosphorylated CPI-17 inhibits myosin phosphatase which in turn, increases myosin phosphorylation and smooth muscle contraction in the absence of increased intracellular Ca^{2+} concentration.

3.5.4 Adventitial Alterations

Long considered mere scaffolding for the artery, the adventitial layer is now known to be effected by DM [42]. Adventitial fibroblasts express adventitial NADPH oxidase and contribute to ROS formation and cytokines. Under their influence, adventitial fibroblasts to undergo a phenotypic switch into myofibroblasts, which migrate to the medial and intimal layer where they mediates monocyte migration and atherogenesis. In addition, BMP2 expression in adventitial myofibroblasts also mediates VCm along with increased cell proliferation and thickening.

Thus even though diabetic VRM is long considered an "inside out" phenomenon (endothelium initiated), increasing evidence of the role played by the adventitia has led to an "outside in" hypothesis where adventitial ROS generation and its effects can be considered the initiating factor.

3.6 Impaired Vascular Healing

Diabetes is associated with impaired and abnormal vascular healing [4]. This leads to a variety of clinically important complications such as a higher rate of restenosis after coronary artery stenting to a higher rate of lower extremity amputations. VEGF plays an important role in neovascularization. DM impairs healing through various effects on VEGF. For example, AGEs appear to reduce endothelial angiogenesis RAGE-mediated, peroxynitrite-dependent and autophagy-induced vascular endothelial growth factor receptor 2 (VEGFR2) degradation.

Diabetes also effects the function of endothelial progenitor cells (EPC) which are produced by the bone marrow and recruited to areas of vascular injury where they

mediate endothelial proliferation and repair. Multiple studies have shown reduced number and impaired number of circulating EPCs in patients with DM. High glucose impairs cell cycling and migration while increasing apoptosis in EPCs. [43].

3.7 Future Directions

While tremendous progress has been made in delineating the molecular mechanisms of DM, a lot remains unlearned in the area of VRM. Our knowledge of the mechanisms of VRM is limited by evidence that is mostly from cell and animal studies which may or may not be applicable in human subjects. The complex interplay of metabolic, flow and vessel pressure abnormalities inherent to DM make it tough to assess the importance of each individual factor in causing VRM and thus develop targeted therapies. In addition, commonly used drugs (antidiabetic agents, HMG-CoA reductase inhibitors) affect pathways that modulate vascular remodeling. These competing effects make it challenging to understand the mechanisms involved in VRM. In spite of these difficulties, studies in humans should be pursued. Noninvasive imaging (especially molecular imaging such as positron emission tomography) is crucial in understanding long term effects of VRM as well as the modifying effect of various therapies such as control of glucose and lipid lowering. Continued investigation into the pathophysiology of VRM is important to improve our ability to develop such therapies.

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Chapter 4
Pathogenesis of the Plaque Vulnerability in Diabetes Mellitus

Vikrant Rai and Devendra K. Agrawal

Abstract Atherosclerosis leads to narrowing of vessels and acute coronary syndrome resulting in ischemic events due to either vasospasm or decreased blood flow. Atherosclerosis and acute coronary syndrome are more common in diabetes mellitus. Hyperglycemia and hypercholesterolemia in diabetes predispose the arteries to plaque development. Smoking, hypertension, male sex, and family history or genetic susceptibility are other predisposing factors for plaque development. Depending on the size, morphology, and symptoms of the patients, plaques can be classified as stable and unstable plaques. Unstable plaques are characterized by the presence of thin fibrous cap, necrotic core, and proliferation of vascular smooth muscle cells, angiogenesis and calcification. Plaque formation initiates with fatty streak and progresses through atheroma, atheromatous plaque to fibroatheromatous plaque. Fibroatheromatous plaques with thick fibrous cap are stable plaques. Thinning of the fibrous cap makes a plaque unstable, prone to rupture and thrombus formation. Mechanisms such as increased inflammation, foam cell deposition, impaired repair mechanism, endothelial cell dysfunction, vascular smooth muscle cell proliferation, angiogenesis, intra-plaque hemorrhage, and calcification which facilitate the plaque rupture are increased in diabetes mellitus. Thus, diabetes mellitus increases the prevalence of plaque formation and rupture. Diabetes mellitus affects various cellular and molecular effectors involved in plaque development and rupture. Understanding these cellular and molecular effectors and involved mechanisms in association with diabetes mellitus is essential for the development of potential therapeutic strategies. This review is a critical overview on the effect of hyperglycemia in diabetes mellitus on the pathogenesis of plaque formation and rupture.

Keywords Atherosclerosis • Stable and unstable plaque • Diabetes mellitus • Hyperlipidemia • Hyperglycemia • Fibrous cap • Plaque rupture

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4.1 Introduction

The luminal narrowing due to plaque formation or precipitating thrombus in atherosclerosis results in adverse cardiac events (myocardial infarction, angina), brain injury (ischemic stroke) and peripheral vascular disease. Coronary artery disease (CAD) is the most common of all these, resulting in myocardial infarction and angina pectoris. An increased serum level of low-density lipoprotein (LDL) is sufficient to induce the atherosclerotic changes. The facilitating factors such as smoking, hypertension, diabetes mellitus, male sex, and family history or genetic susceptibility further add nuances to the disease presentation [1]. Coronary arteries, carotid bifurcations, abdominal aorta, iliofemoral arteries, the branch points of arteries, and the artery near the curvature are the common sites for atherosclerotic lesion due to the presence of low or oscillatory endothelial shear stress [2]. Depending on the clinical symptoms, the atherosclerotic plaques may be asymptomatic (subclinical disease), obstructive (stable angina, transient ischemic attack, amaurosis fugax), and symptomatic (acute thrombosis leading to acute coronary syndrome, stroke) [1, 3].

CAD is more common in diabetes mellitus type II (T2DM), or the individuals with persistent hyperglycaemia are more prone to CAD due to increased blood glucose and atheroma formation [4]. Further, it has also been documented that elevated glycosylated haemoglobin [5] and genetically driven hyperglycemia distinctly from T2DM also increases the risk of CAD [6], suggesting hyperglycemia as a major risk factor for CAD and can affect the pathogenesis as well as the stability of the plaque. Obesity is a chronic inflammatory disease and results in obesity-induced insulin resistance or impaired insulin secretion resulting in hyperglycemia, which further leads to functional and structural alterations of the vessel wall and culminates with diabetic vasculopathies [7]. Hyperlipidemia is another major risk factor for atherosclerosis. Deposition of LDL in the intima initiates the process of atherosclerosis. T2DM is associated with elevated triglycerides, decreased high density lipoprotein (HDL) and increased low density lipoprotein levels collectively characterizing the hyperlipidemia [8].

Hyperlipidemia and hyperglycemia are the risk factors for atherosclerosis, and increased deposition of lipids and inflammatory cells resulting in necrotic core within the atherosclerotic plaque renders the plaque vulnerable. Nearly 75 % of all acute coronary events and 90 % of all carotid plaques causing ischemic stroke results due to atherosclerotic plaque rupture [9]. Increased infiltration of inflammatory cells, thin fibrous cap, large necrotic core, and increased angiogenesis are the mechanisms involved in plaque rupture [10]. Since hyperglycemia and hyperlipidemia, both associated with T2DM causes the functional and structural alterations of the vessel wall, the core morphological alteration behind atherosclerosis, diabetes mellitus can affect the pathogenesis and process of atherosclerotic plaque formation and rupture. This chapter is focused on the effect of hyperglycemia on the various aspects of plaque pathogenesis and vulnerability.

4.2 Pathogenesis of Plaque Formation and Plaque Rupture

The process of plaque formation consists of adaptive intimal thickening with smooth muscle cell (SMC) proliferation, lipoprotein retention, intimal inflammation with inflammatory cell recruitment, foam cell formation, apoptosis and necrosis,matrix synthesis, calcification, angiogenesis, and arterial remodelling. Fibrous cap rupture results in thrombus formation and ischemic events [1]. LDL binding with the proteoglycans in the intima is important for initiation of the plaque formation [11]. The oxidation and aggregation of LDL lead to chronic innate and adaptive immune response resulting in induction of endothelial and smooth muscle cells. This results in expression of various adhesion molecules, chemo-attractants, and growth factors leading to enhanced homing, migration, and differentiation of the monocytes into macrophages and dendritic cells [12, 13]. Oxidized LDL also aids in pro-inflammatory macrophage (M1) predominance [14]. Further, macrophage and dendritic cells act as the deposits of the LDL. Deposition of LDL and foam cell formation leads to xanthoma formation, which further progresses to atherosclerotic lesion with the pathological intimal thickening involving deposition of acellular lipid-rich material in intima [15]. Deposition of collagen and extracellular lipid pools results in formation of fibroatheroma, characterized by the presence of a necrotic core, angiogenesis and fibrous cap. Calcification occurs in progressive atherosclerotic lesions which increase with age, and apoptotic cells, extracellular matrix, and necrotic core material in fibroatheroma act as nidus for calcification increasing the calcium deposits [16, 17] (Fig. 4.1).

The precipitating factor for acute coronary syndrome (ACS) is luminal thrombus or a sudden plaque hemorrhage within the atherosclerotic plaque. The ACS is not necessarily accompanied with concomitant vasospasm. Plaque rupture is the most frequent cause of thrombosis. Plaque rupture results in the exposure of highly thrombogenic, red cell–rich necrotic core material to the blood [18]. Plaque rupture mainly occurs in thin-cap fibroatheromas having an extremely thin fibrous cap. Infiltration of the foam cells or macrophages in the intima results in thinning of the fibrous cap, mainly in the cap margin or shoulder region. Thinning of the fibrous cap is mediated by gradual loss of SMCs from the fibrous cap and degradation of the collagen in fibrous cap via infiltrating macrophages/foam cells secreting proteolytic enzymes such as plasminogen activators, cathepsins, and matrix metalloproteinases (MMPs). Thrombus formation can also occur in the areas of plaque erosion, most often in the areas of pathological intimal thickening. Coronary vasospasm is the frequent event responsible for plaque erosion and rupture [19]. Emotional stress or increased physical activity may be the precipitating event in plaque rupture [1, 18, 19]. Thin fibrous cap, thrombus formation, large necrotic core, neovascularisation, hemorrhage within the plaque, adventitial or perivascular inflammation, and spotty calcification characterize the vulnerable plaque (Fig. 4.1).

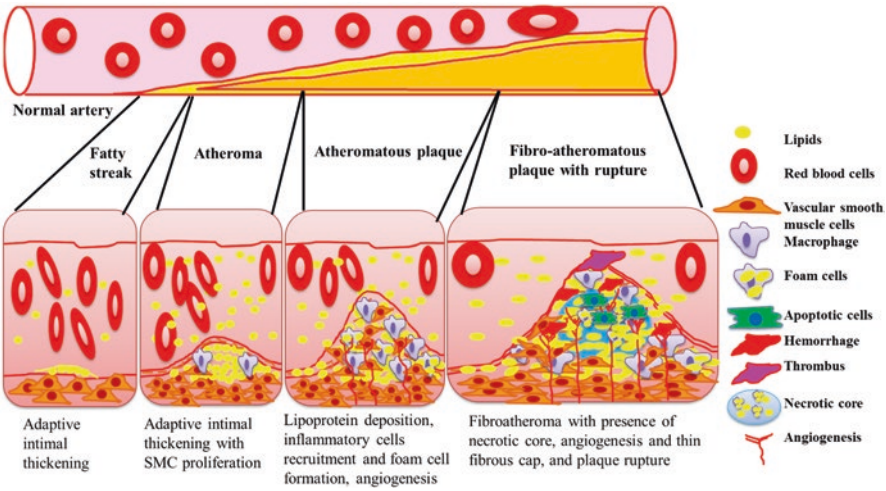

Fig. 4.1 Pathogenesis of plaque formation and plaque rupture. Deposition of the lipids in the intima leads to fatty streak formation and adaptive intimal thickening. Infiltration of inflammatory cells, lipid deposition, and vascular smooth muscle cells (VSMCs) proliferation results in progression of the fatty streak to atheroma and atheromatous plaque. Formation of the necrotic core due to increased apoptosis and necrosis in plaque; increased lipid deposition and angiogenesis and thinning of the fibrous cap results in the development of vulnerable fibroatheromatous plaque

4.3 Diabetes Mellitus and Atherosclerosis

Diabetes mellitus is one of the major risk factors for atherosclerosis and cardiovascular disease in the United States. Hyperglycemia increases the risk for atherosclerosis by a cumulative effect of various mechanisms (Fig. 4.2) discussed elaborately in the literature [7, 8]. Briefly, oxidized LDL enhances the oxidative stress in the intima which leads to activation of inflammatory cascade involving inflammatory receptors [receptor for advanced glycosylation end products (RAGEs), toll-like receptors (TRLs), and triggering receptor expressed on myeloid cells (TREMs)], downstream signaling kinases [protein kinase C (PKCs), c-Jun NH_2-terminal kinase (JNK), ERK, mitogen-activated protein kinase (MAPK) etc.] and pro-inflammatory cytokines [interlekin (IL)-6, tumor necrosis factor (TNF)-α]. This leads to increased monocytic infiltration, M1 macrophage predominance and foam cell formation, which further enhances the inflammation and vascular smooth muscle cells (VSMCs) proliferation resulting in atherosclerosis (Figs. 4.2 and 4.3) [3]. Further, research studies have also demonstrated the involvement of inflammatory surface markers (TREMs and TLRs) [20–22] and pro-inflammatory cytokines (IL-6 and TNF-α) [22] in plaque vulnerability.The increased secretion of these pro-inflammatory cytokines [23], and increased expression of the inflammatory surface marker [24, 25] involved in the pathogenesis of atherosclerotic plaque formation and rupture suggest that persistent hyperglycemia in diabetes have a potential role in plaque formation and rupture.

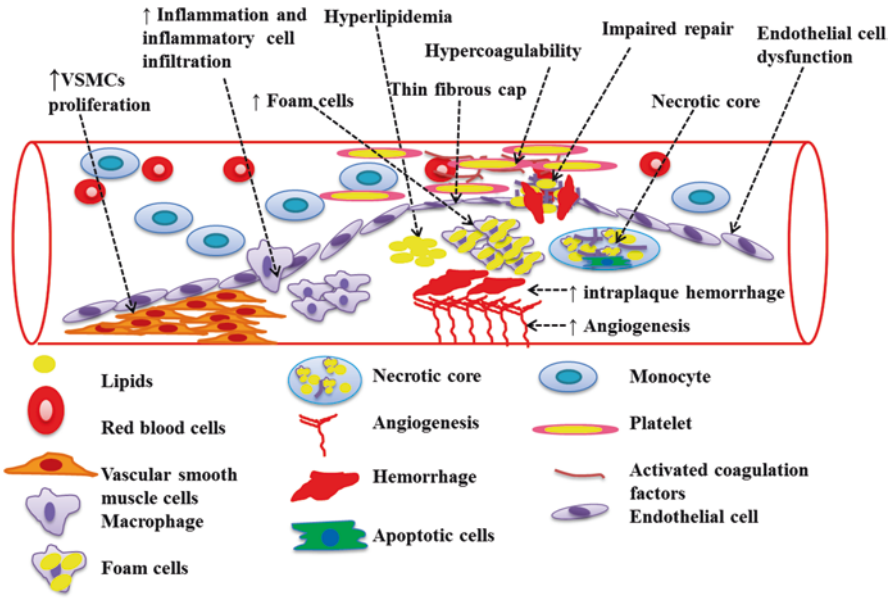

Fig. 4.2 Pathogenesis of atherosclerosis and plaque vulnerability in diabetes mellitus. Persistent hyperglycemia in diabetes mellitus potentiate several mechanisms such as hyperlipidemia, increased angiogenesis, intra-plaque hemorrhage, proliferation of vascular smooth muscle cells (VSMCs), infiltration of inflammatory cells, foam cell formation, necrotic core formation due to oxidative stress, and hypercoagulability enhancing the thrombus formation along with thinning of the fibrous cap by altering the collagen content. These mechanisms make the plaque prone to rupture and thereby prevalence of acute coronary syndrome

4.4 Diabetes and Plaque Vulnerability

4.4.1 Diabetes and Fibrous Cap

Fibrous cap of the plaque faces the lumen of the vessel and is responsible for the integrity of the plaque. Normally, fibrous cap is composed of VSMCs embedded in a collagen type I and III rich matrix. Thinning of the fibrous cap is associated with the plaque rupture and ACS. Studies have suggested the role of MMPs and macrophages in autolysis of the matrix content resulting in thinning of the fibrous cap, however, the exact mechanism underlying the MMP activity in under research. It has been found that symptomatic plaques have increased macrophage density, higher expression of MMPs, decreased VSMCs density, and decreased expression of collagens compared to asymptomatic plaques, and increased expression of MMPs with a decreased collagen expression is associated with plaque vulnerability [3, 20–22, 26]. Further, the association of diabetes with a higher prevalence of macrophage infiltration and thin-cap fibroatheroma suggest the proneness of plaque

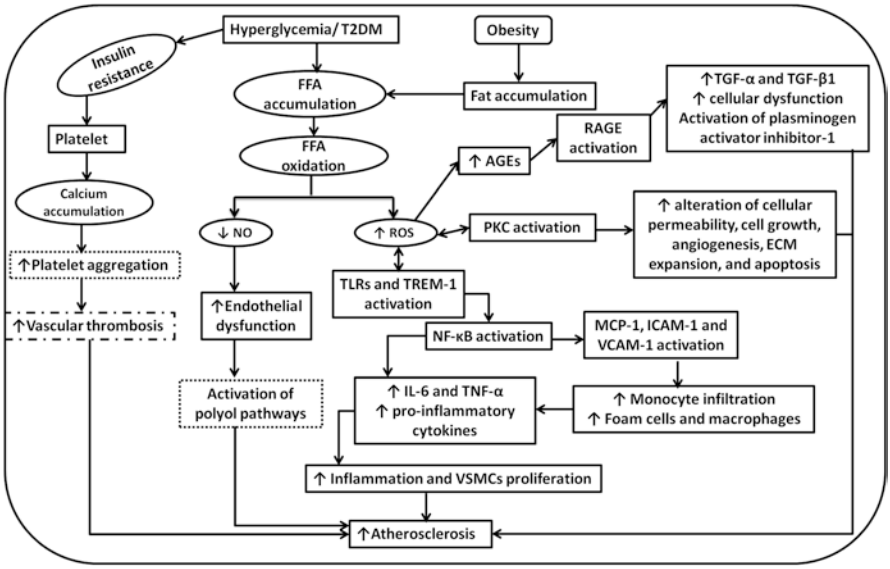

Fig. 4.3 Schematic representation of the factors involved in the development of atherosclerosis in diabetes mellitus. Hyperglycemia in diabetes mellitus leads to increased free fatty acid (FFA) accumulation and oxidation. This results in increased oxidative stress due to the generation of reactive oxygen species (ROS) and the decreased availability of nitric oxide (NO). This leads to activation of various inflammatory pathways resulting in increased prevalence of atherosclerosis. Advanced glycosylation end products (AGEs); diabetes mellitus type 2 (T2DM); extracellular matrix (ECM); interleukin (IL)-6; Intercellular Adhesion Molecule (ICAM) 1;monocytes chemoattractant protein (MCP)-1; nuclear factor-kappa beta (NF-kB); protein kinase C (PKC); receptor for advanced glycosylation end products (RAGE);toll-like receptors (TLRs); triggering receptor expressed on myeloid cells (TREM); transforming growth factor (TGF)-alpha (α)-beta (β); tumor necrosis factor (TNF)-α; vascular cell adhesion protein (VCAM)-1;vascular smooth muscle cells (VSMCs)

rupture in T2DM, as well as the role of persistent hyperglycemia in thinning of fibrous cap [27] (Fig. 4.2).

The mechanisms involving and promoting the thinning of the fibrous cap remains incompletely understood. The alteration of ECM matrix, impaired collagen content, and accumulation of lipids remains the cornerstone of the plaque rupture and thinning fibrous cap forming the rupture-prone plaque. A study on the diabetic and hypercholesterolemic swine reported that coronary regions exposed to low endothelial shear stress favour the collagen-poor, thin-capped fibrous plaque formation compared to high endothelial shear stress. This thinning of the fibrous cap was accompanied by reduced intimal SMC content; decreased procollagen-I gene expression; increased (MMP)-1, -8, -13, and -14 expression; and reduced collagen content [28]. Increased collagen loss in the fibrous cap suggests increased activity of MMPs in the symptomatic plaque whose prevalence is high in diabetics. Upregulated expression of IL-6, IL-8, and monocytes chemoattractant protein (MCP)-1 and the activities of MMP-2 and MMP-9 and downregulated expression of

tissue inhibitor of metalloproteinase (TIMP)-2 with hyperglycemia via hyperglycemia-induced glycosaminoglycans alterations in the cell surface perlecan as well as in the extracellular matrix (ECM) have been reported [29], however, lower concentration of MMPs (MMP-2 and MMP-9) in T2DM has also been documented suggesting the complexity of the role MMPs [30]. Similarly, Fiaschi et al. [31] reported that hyperglycemia in association with angiotensin II enhances the collagen I production and deposition in ECM involving signal transducer and activator of transcription (STAT)3 in cardiac fibroblasts.

4.4.2 Diabetes and Lipid Deposition

T2DM and insulin resistance has been associated with reduced HDL cholesterol, a predominance of small dense LDL particles, and elevated triglyceride levels [8]. Deposition of the lipids in the intima initiates the process of atherosclerosis, and a large deposition of lipids (lipid core) within the fibroatheroma characterizes the vulnerable atherosclerotic plaque [26] (Fig. 4.2). Increased lipid deposition and larger lipid index have been reported in coronary plaques of diabetics compared to non-diabetics [27]. These results suggest that increased LDL levels are associated with increased atherosclerosis and plaque rupture. However, elevated plasma triglyceride levels but not the elevated plasma cholesterol levels have been reported with diabetes in hyperlipidemic pigs in association with increased atherosclerosis [32]. This suggests that diabetes is associated with hyperlipidemia (hypertriglyceridemia but not hypercholesterolemia) and increased prevalence of atherosclerosis, however, it has also been reported that isolated hypertriglyceridemia alone in not associated with increased CAD, but hypertriglyceridemia in association with hypercholesterolemia have a synergistic effect on CAD development [33]. Thus, increased LDL in diabetes is associated with increased prevalence of atherosclerosis, and increased lipid deposition in plaque is associated with plaque rupture correlates increased prevalence of plaque rupture with T2DM.

4.4.3 Diabetes and Inflammation

Infiltration of the inflammatory cells (monocytes, macrophages, dendritic cells etc.) is a key mechanism involved in fibroatheromatous plaque development (Figs. 4.2 and 4.3). Studies have suggested the association of increased infiltration and density of these cells in symptomatic plaque compared to asymptomatic plaque [21, 22, 26]. Further, it has also been suggested that inflammation not only enhances atherosclerosis and plaque formation but also the thrombus formation by affecting platelet function, coagulating factors and clotting mechanism, potentiated by diabetes being a chronic inflammatory disease [34]. Increased inflammation in the atheroma is also associated with hyperlipidemia in diabetic patients suggesting the synergism

between hyperlipidemia and inflammation in diabetes resulting in enhanced vulnerability of plaque [1].

4.4.4 Diabetes and Calcification

Calcification is a histological feature of fibrous atherosclerotic plaque. Minimally oxidized LDL and T2DM are the risk factor for increased calcification in the pathogenesis of plaque development. Hyperglycemia influences the calcification in the vessel wall through ROS production. Increased expression of Cbfal (transcription factor for bone formation) and bone morphogenetic protein (BMP)-2 and enhanced calcification of VSMCs is associated with high serum glucose. Increased levels of BMP-2 exert pro-inflammatory and proatherogenic effects of BMP-2 induce the oxidative stress and endothelial dysfunction, leading to enhanced plaque calcification by inducing osteogenic phenotype in VSMCs [35, 36]. Avogaro et al. [37] have discussed various mechanism in association with diabetes such as upregulation of runt-related transcription factor 2 (Runx2), osterix, osteopontin (OPN), osteocalcin, and downregulation of smooth muscle-specific genes in VSMCs trans-differentiating it to more bone-forming cells. Increased calcification and higher prevalence of calcification have been reported in coronary plaques of diabetics compared to non-diabetics [27]. These studies suggest that diabetes is associated with increased calcification in the intima, which in turn is associated with increased prevalence of plaque rupture.

4.4.5 Diabetes and Thrombus Formation

Diabetes is a hypercoagulable disease due to an imbalance of pro- versus anticoagulation, and is associated with increased numbers of endogenous pro-coagulant triggers bearing circulating microparticles. Hypercoagulability in diabetes increases the risk of atherosclerosis and peripheral vascular disease [38]. Increased association of inflammation and oxidative stress with hypercoagulability state in diabetes has been established which leads to endothelial dysfunction, plaque formation, progression and rupture [39]. Further, increased thrombus formation in diabetes due to inflammation by affecting the platelet function, coagulating factors and clotting mechanism has been reported [34]. Higher prevalence of thrombus formation has been reported in coronary plaques of diabetics compared to non-diabetics [27]. These studies suggest that hypercoagulable state of diabetes promotes inflammation and thrombus formation (Fig. 4.2).

4.4.6 Diabetes and Intra-plaque Hemorrhage

The structural type I collagen, the predominant structural collagen, in the vessel wall is produced by smooth muscle cells and fibroblasts in the vascular media and intima. Type III collagen is present as a minor component in vessels. However, Purushothaman et al. [40] while comparing the diabetic and non-diabetic subjects reported that diabetes is associated with increased type III collagen instead of type I collagen, a feature of progressive atherosclerotic plaque, accompanied with inflammation, neovascularization, and intraplaque hemorrhage (IPH). Similar trends in collagen reversal in association with diabetes have been documented by various other studies [41–43]. Further mature plaques have a rich network of small vessels called as '*vasa vasorum*' within the matrix of plaque, and rupture of these vessels within the plaque leads to IPH. Higher prevalence of IPH has been associated with symptomatic plaques [44], and plaque rupture [45] (Figs. 4.2 and 4.3).

4.4.7 Diabetes and Angiogenesis

Angiogenesis is a morphological feature of fibroatheroma. Lipid deposition and inflammation causes oxidative stress and increased ROS. Increased oxidative stress is a precursor for angiogenesis and arteriogenesis. Similarly, the toxic metabolites in metabolic syndrome and diabetes induce angiogenesis via oxidative stress, which further accelerates the progression of atherosclerosis [46]. Increased infiltration of inflammatory cells and angiogenesis increases the size of the necrotic core and IPH rendering the plaque prone to rupture [47]. A greater degree of plaque intimal neovascularization and inflammatory infiltrate leading to plaque vulnerability has been reported in diabetic subjects compared to non-diabetic subjects [48]. Although, the distribution, density and the role of *vasa vasorum* have been discussed in the context of plaque progression, atherosclerosis, and IPH, the causative or the only reactive role of *vasa vasorum* in atherogenesis needs to be elucidated (Figs. 4.2 and 4.3).

4.4.8 Diabetes and Impaired Endothelial Repair

Inflammation of the atheroma and plaque rupture is the cornerstone of the ACS. Plaque rupture occurs at the thinnest part of the fibrous cap, which is due to ECM and collagen loss. ECM and collagen loss leading to thin fibrous cap occurs due to impaired repair mechanism. Edsfeldt et al. [10] studied the carotid endarterectomy specimens in T2DM and non-diabetic patients analyzing the plaque structure, connective tissue proteins, inflammatory cells, and inflammatory markers, and reported the increased proneness of the atherosclerotic plaques to rupture in subjects with T2DM because of impaired repair responses rather than to increased vascular

inflammation. The plaques in T2DM patients had lower collagen and elastin content, decreased levels of the VSMC growth factor, platelet-derived growth factor (PDGF), decreased levels of inflammatory cells and decreased levels of MMP2. However, Ruiz et al. [49] reported the increased proliferation of VSMCs and decreased apoptosis leading to enhanced arterial remodeling in diabetic patients with upregulated expression of Bcl-2 gene with glucose (Figs. 4.2 and 4.3).

4.5 Conclusion

Unstable plaques and plaque rupture precede the acute coronary syndrome. Unstable plaque is characterized by the presence of necrotic core, lipid deposition, angiogenesis and thin fibrous cap. Inflammation in the atheroma, infiltration of inflammatory cells, angiogenesis and collagen loss renders a stable plaque to unstable plaque. Diabetes mellitus is a chronic inflammatory disease, associated with increased inflammation, oxidative stress, hyperlipidemia and increased angiogenesis, thereby increasing the prevalence of atherosclerosis as well as plaque vulnerability. Higher prevalence of atherosclerosis and plaque vulnerability has been reported in various studies along with the improved modalities of imaging to assess the plaque volume and its proneness to rupture. Although, there are reports about the most common arteries involved in atherosclerosis, diabetes induced structural changes in intima and intimal thickening, predominance of collagen III instead of collagen I, and presence of *vasa vasorum* and IPH, still further studies are needed to elucidate the molecular mechanism more clearly underlying these changes in diabetes to develop a potential therapy for plaque stability in diabetes mellitus.

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Chapter 5
Endothelial Dysfunction in Diabetes

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Abstract Cardiovascular diseases (CVD) are a leading cause of mortality and morbidity world-wide. The instances of CVD are multi-fold higher in type -2 diabetic patients. The initiating cause of these diseases is endothelial dysfunction, due to presence of multiple risk factors such as, insulin resistance, dyslipidemia, inflammation and hyperglycemia. This chapter summarizes our current understanding of molecular mechanisms that contribute to endothelial dysfunction in diabetes.

Keywords Diabetes • Endothelial dysfunction • Hyperglycemia • Inflammation • Dyslipidemia • Insulin resistance

5.1 Introduction

Endothelium, the innermost lining of a blood vessel which is in direct contact with the flowing blood, by virtue of its position, plays an indispensable role in vascular homeostasis. It strikes a balance between fibrinolysis and coagulation, leukocyte diapedesis and inflammation; in addition to regulating the vessel tone, nutrient permeability and tissue perfusion [1–4]. Endothelial cells covering the entire span of vasculature, starting from endocardial lining of the heart, through aorta, all the way to tissue capillaries, veins and the valvular linings, account for a $7m^2$ worth of surface area for nutrient exchange in adults. Hence, it is not surprising to note that they exhibit versatility in terms of their phenotype as well as function [5].

Endothelial cells work through a complex, but, a robust and, an interconnected system of molecular cross-talk with other cell types, to bring about vascular homeostasis. For instance, by secreting gaso-transmitters such as nitric oxide (NO), they bring about relaxation of underlying smooth muscle cells and thus, increase the local diameter of the blood vessel independent of the neuronal regulation [6–8]. Additionally, NO acts on circulating immune cells to prevent their adherence to

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the vessel wall [4, 9]. Endothelium derived NO is also anti-atherogenic as it prevents migration and proliferation of smooth muscles. In contrast, endothelium derived endothelin-1 (ET-1) brings about vaso-constriction [3, 10, 11]. It is worth noting that endothelial cells also respond to other circulating, paracrine, autocrine and endocrine mediators, such as thrombin, bradykinin, angiopoietins and vascular endothelial growth factor (VEGF). Among these, angiopoietins and VEGF members play a crucial role in vessel remodelling and angiogenesis [12–14]. Hence, under normal physiological conditions, a tight balance between regulatory and counter-regulatory endothelium-derived mediators is of paramount importance for vascular homeostasis. Any deviation in their levels or modes of action, and/or sensing of these agonists by the endothelium, leads to endothelial dysfunction. The best characterized among these, is the decreased secretion or availability of endothelium derived NO in the vasculature. In this chapter we summarize our current understanding of endothelial function and how it is compromised in type 2 diabetes, in order to get an insight on diabetic vasculopathy.

5.2 Normal Endothelial Function

Endothelium derived chemical mediators include prostanoids, endothelin-1, NO, endothelium derived hyperpolarizing factor (EDHF), von Willebrand factor (vWF), Angiotensin-II (Ang-II), tissue plasminogen activator (tPA), plasminogen activator inhibitor (PAI), cell adhesion molecules (ICAM-1, VCAM-1, E-selectin), cytokines and, growth factors such as TNF-α and VEGF [2, 7, 11, 15]. Majority of these proteins are not only synthesized in the endothelium, they are also stored in specialized storage vesicles of the endothelium, referred to as Weibel-Palade bodies [16]. Upon suitable trigger, these stored auto-coids are released in blood, such as angiopoietins or histamine, to mediate required vascular effects. Similarly, stored cell adhesion molecules in WBPs are exposed on to the endothelial cell surface, allowing anchoring points to circulating immune cells to carry out routine regulatory functions. Such a tethering of immune cells onto the endothelial surface, provides them with an opportunity to invade the inflamed tissue for repair. Usually this is a transient process which resolves upon repair of the damaged tissue.

Controlled and balanced production of tPA and its inhibitor PAI, or that of coagulation cascade members thrombomodulin, protein C and tissue factor, ensures maintenance of blood fluidity as well as vascular repair [2, 4]. Through the release of other mediators such as AngII, platelet-derived growth factor (PDGF) or NO, endothelium also modulates differentiation and proliferation of smooth muscle cells present underneath, and thus, regulate vascular remodelling [7]. Endothelium is also a good sensor of fluid shear stress exerted by the circulating blood onto the lateral walls of the vessels [17]. So much so, that they can distinguish between magnitude, as well as the profile of blood flow. In the linear arms of bigger blood vessels, these cells largely experience laminar shear stress, and in response to it, produce NO, a potent vasodilator. In contrast, at the points of vessel bifurcations or curvature, the

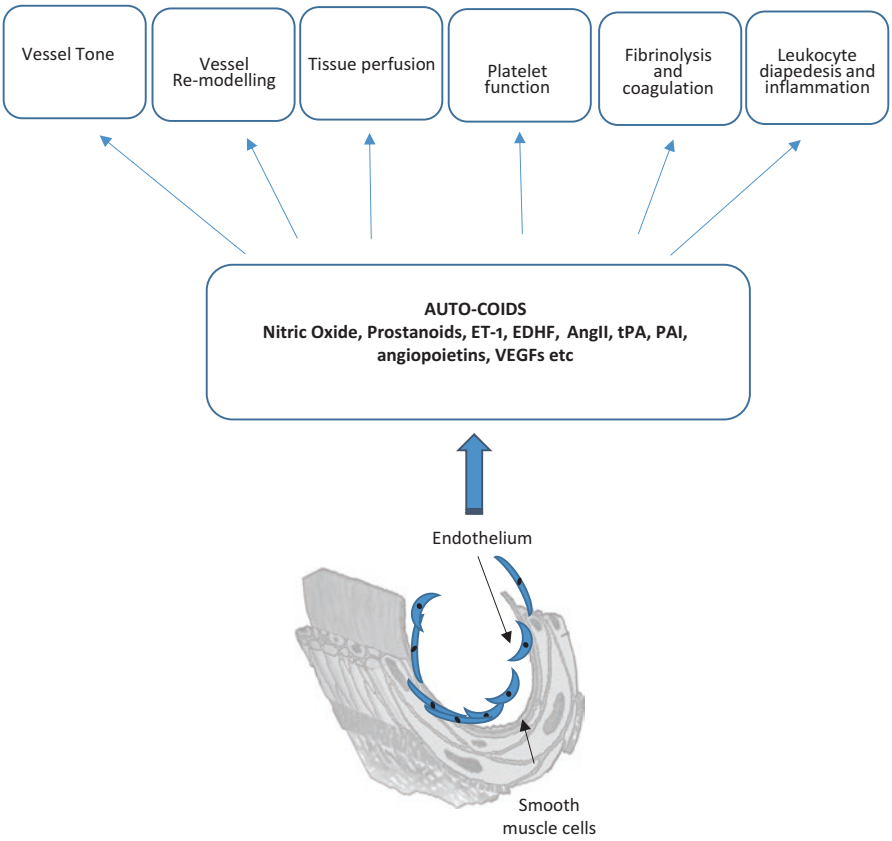

Fig. 5.1 Functions of vascular endothelium

resident endothelial cells experience disturb flow [17–19]. In the microvasculature or the tissue micro-capillaries, the endothelium provides adequate surface for nutrient exchange between the incoming blood and the tissue fluid. Hence, endothelium being the first line of cells in contact with the circulating blood, not only senses biochemical or rheological changes in it, but, also responds appropriately to maintain vascular homeostasis and organ perfusion. These functions of the endothelium are summarized in Fig. 5.1.

5.2.1 NO Generation by the Endothelium

Without a doubt, the best characterised endothelium derived vaso-mediator is nitric oxide. In the endothelium it is predominantly produced through enzymatic conversion of L-arginine to citrulline and NO, by constitutively expressed endothelial nitric oxide synthase (eNOS) [20]. Functional eNOS is a dimer and its activity is

regulated through multiple mechanisms such as, serine/threonine/tyrosine phosphorylation, sub-cellular trafficking, and through its interaction with other co-factors and proteins (Fig. 5.2). For instance, a functional dimer exists in association with calcium binding protein calmodulin (CaM), heat shock protein 90 (hsp90) and tetrahydrobiopterin (BH4) [20–23]. Among the serine threonine kinases which positively enhance the eNOS activity, the most potent one is PI-3 Kinase dependent Akt. This enzyme activates eNOS by phsophyrlating its Ser^{1177} residue [24]. Other kinases which phosphorylate this residue are protein kinase A (PKA), adenosine monophosphate-activated kinase (AMPK), protein kinase G (PKG) and CaM protein kinase II (CaMKII) [20]. Phosphorylation of eNOS at this residue increases its calcium-CaM sensitivity, allowing NO production even in presence of low intracellular calcium. In contrast phosphorylation of Thr^{497}, inhibits eNOS activity by interfering with CaM interaction. The phosphorylation of this negative regulatory site is largely mediated by protein kinase C (PKC). Among the tyrosine residues that regulate eNOS activity upon phosphorylation, are Tyr 83 and Tyr 657, which positively and negatively regulate the enzyme respectively [25–28].

Displacement of CaM by caveolin-1 however decreases NO synthase activity, and dissociates the dimer into individual monomers. These monomers in absence of CaM and other co-factors such as BH4, instead, start producing reactive oxygen species [20, 29]. Hence, coupling of two monomers of eNOS through appropriate protein-protein interactions is a must for sufficient NO production. The other negative regulators of eNOS derived NO production are Arginases, which competes with eNOS for their common substrate L-arginine due to greater V_{max} [30, 31].

Stimulation of endothelial cells by receptor dependent agonists such as, acetylcholine, serotonin or insulin increases eNOS activity. Another activator of eNOS is fluid shear stress exerted by the circulating blood on to the apical surface of the endothelium [20, 24]. The NO thus generated being lipophilic in nature, readily diffuses into the underneath layer of smooth muscle cells (SMC), where it activates NO dependent guanylated cyclase/PKG axis, to cause SMC relaxation and thus vasodilation. In this way at the local tissue level, endothelial derived NO regulates blood flow and vessel tone. NO also exhibits anti-inflammatory, anti-proliferative, anti-migratory, anti-platelet, anti-oxidant and anti-permeability properties [32]. By decreasing the cell surface expression of cell adhesion molecules onto the endothelial surface, NO prevents tethering of circulating immune cells to block vascular inflammation. Additionally, by inhibiting cytoskeletal rearrangements through Rho inhibition, it prevents migration of smooth muscle cells from media into lumen of a vessel during atherosclerotic plaque formation. Similalry, inhibition of platelet activation allows for attenuation of thrombosis [2, 4, 17, 18, 32]. Thus, all in all, endothelium derived NO exerts potent anti-atherosclerotic effects (Fig. 5.2) and any dysregulation in its expression or biological activity, will accelerate occlusive vascular diseases through promotion of endothelial inflammation, thrombosis, plaque formation, increased arterial stiffness and impaired arterial tone.

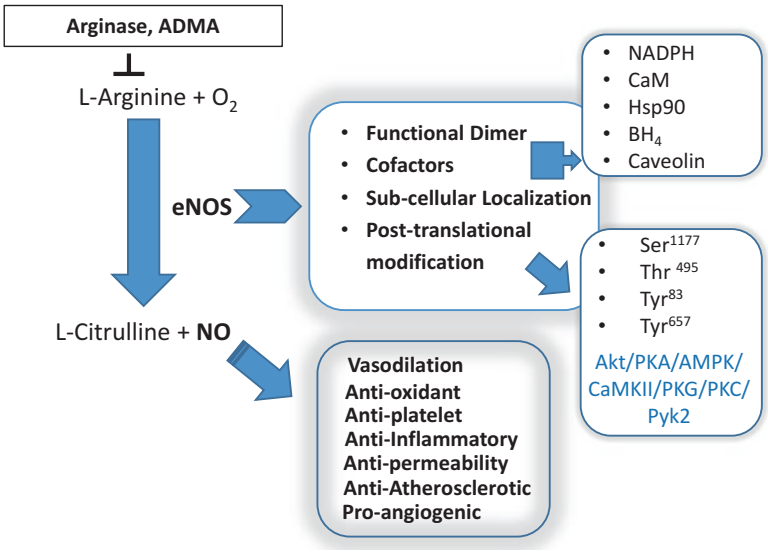

Fig. 5.2 Regulation of NO production and its vascular function

5.3 Endothelial Dysfunction, Its Assessment and Endothelial Repair

Endothelial dysfunction is defined as impairments in functional ability of the endothelium to maintain vascular homeostasis, be it for maintaining vessel tone or prevention of pro-atherosclerotic events such as, inflammation and thrombosis. It is largely attributed to either decreased production of endothelium derived NO or due to decreased bioavailability of NO. The latter effect, refers to inactivation of free NO, due to simultaneous presence of oxidative stress [33–35]. Superoxide anions seen during oxidative stress for instance, react with NO to form detrimental peroxynitrite [36]. Peroxynitrite is a potent oxidant which effectively inactivates numerous cellular enzymes. Alternatively, presence of other substrate analogues of eNOS, such as, asymmetric dimethyl arginine (ADMA) or enhanced expression of arginases, also reduces NO levels in circulation [20, 30, 37]. It should however be noted that endothelial dysfunction also reflects aberrant production and secretion of other vaso-active molecules by the endothelium such as increased levels of ET-1 [10].

In vivo estimation of circulating NO in blood is a challenging task due to its short half-life of few seconds and, due to lack of sensitive NO detecting reagents which can detect it in physiological range (pM). Hence, downstream vascular effects of NO are measured, to assess endothelial function. Clinically relevant assays measure endothelium-dependent vasodilatory effects, either through plethysmography, high resolution ultrasonography or through arterial tonometry to measure blood flow changes in peripheral circulation [3, 38]. In non-invasive flow mediated dilatation

(FMD), local ischemia, followed by hyperemia in the brachial artery is induced by inflating and deflating blood pressure cuff, to generate shear stress. The consequent endothelium dependent increase in vessel diameter is measured through high resolution Doppler ultrasonography [38]. Another emerging non-invasive tool to assess vasodilatory endothelial function, is to measure digital peripheral artery tonometry (Endo-PAT) [39, 40]. In contrast to these simpler assessments, non-invasive methods to assess coronary microvascular dilatations, are cost intensive such as Doppler echocardiography, positron emission tomography and MRI. These laborious and sometimes operator dependent assays have forced majority of the researchers to resort to economical, but surrogate circulating markers of endothelial activation, such as, sICAM-1, sVCAM-1, sE-selectin, ADMA, vWF, sTie2 and angiopoietins. Unfortunately majority of them also reflect ongoing systemic inflammation and thus, are poor in sensitivity and are rather non-specific. It is in this regard that researchers have turned their attention to measurement of circulating endothelial progenitor cells (EPCs) and endothelium derived micro-particles (EMPs) as likely markers of endothelial dysfunction.

5.3.1 Endothelial Progenitor Cells

Mature endothelial cells are quiescent in nature with a very low proliferative potential. Hence, for a long time it baffled scientists and clinicians alike, as to what repairs a damaged endothelium. The landmark discoveries by Ashara and others of bone marrow derived $CD34^+$ cells capable of recovering blood flow in rodent model of hind-limb ischemia, upon transplantation, through endothelial differentiation, challenged the long held dogma, that *de novo* formation of blood vessels is an embryonic event [41, 42]. Subsequently it was reported that numerous population of circulating mono-nuclear cells exhibit angiogenic potential. Although the exact marker definition of these progenitor cells is still unresolved, endothelial progenitor cells (EPCs) are broadly classified as bone marrow derived rare population of haematopoietic marker (CD34, CD133 or both) and VEGFR2 expressing circulating mononuclear cells, which can mediate endothelial repair. These cells under physiological conditions periodically mobilize from bone marrow into various tissues and organs, in response to stimuli such as VEGF, chemokine SDF-1α, estrogen or erythropoietin [43–45]. Some of these cytokines for instance, SDF-1α, are secreted in ischemic tissues by the hypoxic endothelium. Upon reaching their destination, EPCs repair the endothelium (Fig. 5.3), either by differentiating into endothelial cells, or by modulating the angiogenic ability of the resident endothelial cells through secretion of paracrine factors [46–48]. Intriguingly, mobilization of these progenitors in response to cytokines, requires involvement of PI-3/Akt pathway and eNOS derived nitric oxide [49]. Numerous studies have shown that in humans, circulating counts of EPCs significantly correlate with endothelial health, with reduced numbers of EPCs in blood predicting future cardiovascular events [50]. The

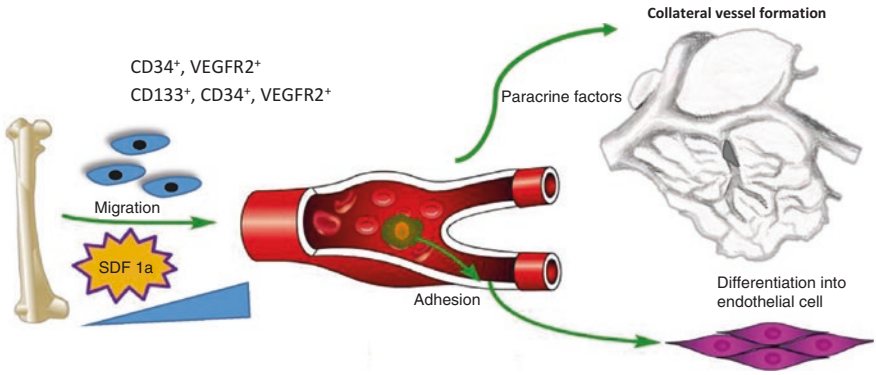

Fig. 5.3 Repair by endothelial progenitor cells

molecular reasons for similar aberrations seen in diabetic patients will be discussed in subsequent section of this chapter.

5.4 Diabetes and Mechanisms of Underlying Endothelial Dysfunction

Components of metabolic syndrome such as abdominal obesity, hypertension, dyslipidemia, diabetes and insulin resistance; are classical risk factors of cardiovascular diseases, with all of them being associated with endothelial dysfunction. Among these, the worst clinical out-comes are seen in diabetic patients, as one or most of the above listed risk factors are present in these patients. These risk factors not only contribute to CVD individually, but when present along with others, tend to potentiate each other's influence on the endothelium.

Hyperglycemia mediated endothelial dysfunction is well-understood both in humans and diabetic rodent models, however, recent clinical trials such as the ACCORD, ADVANCE, UKPDS and the Framingham Heart study, have reported disappointing results with regard to efficacy of combined therapy of insulin and hypoglycemic agents on cardiovascular outcomes. It was observed that despite tight glucose control, respite from macro-vascular complications was marginal, suggesting that hyperglycemia is just one of the many other factors contributing to vascular diseases [51–54]. This notion in part was also supported by the observations made in Helsinki policemen study and the NHANES trial, both of which, observed positive correlations between circulating levels of insulin and incidences of heart attack [55–58]. Similarly, Framingham and numerous others studies observed an inverse correlation between insulin resistance and brachial artery FMD. In fact, use of insulin sensitizers such as thiazolidinediones (TZDs) and metformin are consistently shown to improve endothelial dysfunction, both in diabetic and non-diabetic subjects having other risk factors such as dyslipidemia and hypertension [59–61]. These

observations indicate that the onset of endothelial dysfunction or rather vascular diseases is a pre-diabetic event, with the disease getting worse with subsequent appearance of hyperglycemia. In the following section we will summarise molecular mechanisms which link the above mentioned individual risk factors with endothelial dysfunction.

5.4.1 Insulin Resistance and Hyperinsulinemia

Prior to blood glucose levels spiking up in type 2 diabetic patients, the sensitivity of the metabolic tissues (liver/skeletal muscle/adipose) towards insulin is blunted, leading to insulin resistance. Insulin resistance is also characterized by increased circulating levels of free fatty acids (FFAs), pro-inflammatory cytokines (TNF-α and IL-6) and, compensatory hyperinsulinemia. On one hand the pro-inflammatory mediators attenuate insulin signalling by impairing tyrosine kinase activity of the insulin receptor, the 'Compensatory hypothesis', vouches for increased release of insulin by the β-cells to over-come insulin resistance. Thus, both insulin resistance and compensatory hyperinsulinemia co-exist during early stages of type 2 diabetes and metabolic syndrome. It is worth noting that insulin is a vasoactive hormone, as long as it is released periodically at physiological levels following meal intake, however, it's sustained presence and increased circulating levels have detrimental effects on the vasculature as reported in some of the recent findings.

Similar to its role in metabolic tissues, insulin activates the PI-3 kinase/Akt axis in endothelial cells to activate eNOS through serine phosphorylations [24, 62, 63]. The consequent NO release, particularly in skeletal muscles, improves blood flow for better glucose uptake. However, cell culture based studies performed on human umbilical vein derived endothelial cells (HUVECs), have shown that chronic hyperinsulinemia, promotes NFκB mediated surface expression of cell adhesion molecules such as, VCAM-1, to promote adherence of monocytes [64, 65]. Our own study has shown that sustained exposure of HUVECs to even low levels of insulin, promotes leukocyte adhesion, with the effect being enhanced multi-fold with increasing concentration of insulin [66]. This increase in endothelial inflammation was largely due to increased uncoupling of eNOS from a dimeric to a monomeric form and, due to increased activity of arginase II. Although we presently do not have a clear answer to what causes eNOS uncoupling in response to hyperinsulinemia in cell culture, the increase in arginase expression was due to insulin mediated p38MAPK activation [66]. Even in heterozygous knock-out mice for insulin receptor, which exhibit endothelial insulin resistance, despite normal glucose tolerance, eNOS uncoupling, decreased NO and increased ROS [67, 68].

As seen for mature endothelial cells, knock-out studies in mice have shown that both PI-3 kinase/Akt and eNOS are necessary for mobilization, differentiation and angiogenic ability of EPCs [47, 49, 69, 70]. Additionally cell culture studies demonstrate that insulin enhances clonogenic ability of EPCs through IGF-1 receptor dependent p38MPK and ERK1/2 pathways [71, 72]. Seminal observations were

made by Fadini et al. with regard to progressive decrease in circulating counts of fasting $CD34^{+}VEGFR^{+}$ EPCs with increasing severity of glucose intolerance [73, 74]. They also observed inverse correlation between EPC counts and glucose levels, following glucose challenge. Inverse association between EPCs and insulin resistance is also seen in South Asian Men, who are far more insulin resistant compared to western population [75]. In a pilot study performed by our group, although we did not see a decrease in fasting counts of $CD34^{+}$or $CD133^{+}CD34^{+}$ progenitor cells in pre-diabetic Asian Indian men, we did see abrogation of oral glucose nduced increase in their circulating counts [76]. Additionally, we observed that the cells obtained from the pre-diabetic subjects did show poor migratory potential towards SDF-1α [77]. Although a detailed understanding on dysfunction of EPCs during insulin resistance in humans is currently lacking, the existing clues point towards defective mobilization and differentiation of these progenitors.

5.4.2 Obesity and Dyslipidemia

Imbalance in HDL- to LDL-cholesterol and presence of abdominal adiposity are strongly associated with type 2 diabetes and consequent endothelial dysfunction. Adipose tissue being an endocrine organ itself, secretes numerous vasoactive cytokines and hormones, for example interleukin-6 (IL-6), TNFα, adiponectin, resistin, angiotensin II and leptin. Among these, adiponectin exerts anti-inflammatory effects on to the endothelium, by increasing NO production through AMPK and PKA mediated eNOS activation [78]. However, its circulating levels are decreased in T2DM. In contrast, angiotensin II causes endothelial dysfunction by decreasing PI-3 kinase activation and increasing JNK and MAPK activation. Ang-II is also reported to increase ROS production, endothelin-1 release, and surface expression of ICAM-1 [79]. Thus it is not surprising to observe that ACE inhibitors and Ang-II receptor type I blockers (ARB blockers) exhibit beneficial cardiovascular effects [80–82].

Imbalance in lipid profile and increased postprandial triglycerides also induce endothelial dysfunction by contributing to oxidative stress and by promoting endothelial apoptosis. Numerous randomized placebo-controlled trials with statins, as well as fenofibrates, have shown their beneficial effects on endothelial function as assessed through brachial artery FMDs [38, 83]. These treatments are also reported to mitigate endothelial inflammation by decreasing the expression of VCAM-1 and E-selectin on endothelial cells. Additionally, statins are reported to improve the circulating counts of endothelial progenitors [84]. However, other studies have not observed any improvements in microvascular functions as recorded with skin blood flow measurements. Readers are referred to an excellent review by Hamilton and Watts on effects of various lipid lowering therapies on endothelial dysfunction [38].

5.4.3 Inflammation

Occlusion of bigger vessels such as coronary artery or the carotids also referred to as atherosclerosis, is an inflammatory disease. Numerous epidemiological studies have shown that inflammation independently associates with endothelial dysfunction. Even acute inflammatory insults during vaccination or infusion of LDL particles, blunts endothelium function [85, 86]. These attenuations in endothelial function are largely due to decreased production or bioavailability of endothelium derived NO. Chronic inflammation in type 2 diabetic patients is characterized by increased circulating levels of pro-inflammatory cytokines, such as, TNF-α, IL-6, MCP-1, CRP and fibrinogen. Majority of these cytokines increase the adherence of leukocytes to the endothelial surface due to NF-κB mediated increased surface expression of cell adhesion molecules [E-selectin, ICAM-1 and VCAM-1] [2, 87, 88]. All these cytokines also decrease the expression of eNOS, thereby additionally enhancing vascular permeability and thrombus formation [89]. Among these cytokines, CRP decreases the expression of prostacyclin, while increasing the expression of vaso-constritor endothelin-1 [90]. Similarly, TNFα apart from promoting endothelial inflammation, induces microvascular endothelial apoptosis in diabetic patients. The latter effect of TNF-α, is associated with nephropathy and retinopathy [91]. Intriguingly, TNF-α blocking therapy reverts microvascular endothelial dysfunction and capillary recruitment in spondylitis patients exhibiting vascular inflammation [92]. Primed polymorphonuclear leukocytes (PMNs) also contribute to vascular inflammation during diabetes. These cells release superoxide due to decreased plasma glutathione levels [93]. These evidences suggest that anti-inflammatory treatments may improve diabetic-cardiovascular complications and currently two clinical trials (TINSAL-CVD and TINSAL-FMD) are underway to check the same [94–96].

5.4.4 Hyperglycemia

Multiple clinical and epidemiological studies on diabetic patients have observed association of hyperglycemia with increased oxidative stress and decreased bioavailability of nitric oxide [33, 34]. The latter is in part due to increased expression of caveolin-1, ADMA and enhanced arginase activity [20, 30, 37, 97]. All these three, negatively affect eNOS activity. In contrast, the positive modulators of eNOS such as Akt phosphorylation and Hsp90 mediated stabilization of eNOS dimer, are blunted in diabetic arterial tissue [98]. Some of the responsible players in hyperglycemia mediated endothelial dysfunction are listed below.

5.4.4.1 Oxidative Stress

The most prominent player is increased oxidative stress characterized by enhanced production of superoxide radicals. These radicals nullify the vasodilatory effect of NO either by forming peroxynitrite or by oxidizing its cofactor tetrahydrobiopterin [20]. In fact, supplementation of BH_4 improves endothelial dysfunction in type 2 diabetic patients [99]. Increased superoxide production also mediates lipid peroxidation, which in turn, inhibits receptor dependent eNOS activation. The major sources of reactive oxygen species in diabetes are: NADPH oxidase, the polyol/sorbitol/aldose reductase pathway or the uncoupled eNOS itself [20, 33, 34, 100]. Apart from decreasing the bioavailability of nitric oxide, increased oxidative stress also leads to increased production of methylglyoxal and advanced glycation end products [101]. Unfortunately, despite sufficient evidence on the role of oxidative stress and endothelial dysfunction, clinical trials with anti-oxidant therapies have met with limited success [102, 103]. While ascorbic acid treatment seems to improve blood flow, clinical trials with α-tocopherol were disappointing [104].

5.4.4.2 Diacylglycerol and PKC Pathway

Hyperglycemia induces activation of PKC-β isoform through generation of DAG from glycolytic intermediates such as dihydroxyacetone phosphate and glyceraldehyde-3-phosphate [105]. Enhanced PKC-β activity, inhibits PI-3kinase/Akt pathway, thereby preventing Ser^{1177} mediated activation of eNOS [20]. In humans, treatment with PKC-β inhibitor prevents glucose infusion mediated blunting of brachial artery flow mediated dilatation. Additionally, PKC-β activates the NFκB arm to promote endothelial inflammation. It also leads to increased gene expression of ET-1 and PAI-1. By increasing the thickness of basement membrane in response to hyperglycemia, the PKC-β signalling axis decreases vessel compliance [105].

5.4.4.3 Advanced Glycation Endproducts (AGEs)

These are formed through non-enzymatic glycation of proteins, through condensation of carbonyl group of free glucose with the amino groups of lysine or arginine in proteins. The resulting Schiffs base intermediate, undergoes Amadori rearrangement to form stable glycated proteins such as HbA_{1c} or fructosamine. In the endothelium, the major AGE is methylglyoxal, which leads to increased ROS generation [101, 106]. AGEs also promote NFκB signalling through AGE receptors (RAGEs) to promote endothelial inflammation. Modification of collagen in basement membranes through AGE affects the elasticity of the blood vessels, and, by modifying heparin sulphate, AGEs impair endothelial interaction with extracellular matrix and thus induce endothelial leakage. Similar to proteins, glycation of lipids such as LDL (gLDL) decreases their recognition by their cognate receptors, for uptake, and

consequently expose them to further oxidation. Interestingly, gLDL prevents NO production and L-arginine uptake in endothelial cells [107].

5.4.4.4 Hyperglycemia and Endothelial Repair

Similar to effects exerted on endothelial cells, hyperglycemia and observed oxidative stress attenuates the reparative ability of EPCs [69, 108, 109]. EPCs obtained from diabetic patients and rodents have decreased proliferative and chemotactic response towards mobilizing agents such as SDF1α [45, 47, 108]. Even the circulating levels of SDF1α are reduced in the injured tissues in hyperglycemia. Furthermore, the expression of CXCR4, the receptor responsible for sensing SDF1α, is also decreased in diabetes [110, 111]. It is worth noting that every aspect of EPC mediated endothelial repair, be it their mobilization, homing or differentiation into mature endothelial cells; are all dependent upon PI3-kinase/Akt/eNOS pathway [47, 49]. Unfortunately in diabetes this PI-3 Kinase/Akt mediated NO generation from eNOS is blunted due to concomitant presence of oxidative stress [69]. Although insulin and adipokines such as leptin are known to enhance tubule formation and colonogenic ability of EPCs, presence of chronic inflammation during diabetes abrogates their ability to suitably respond to these hormones, making them either insulin or leptin resistant [67, 112–114]. However, the molecular mechanisms altering the responses of EPCs towards these agonists are currently ill-characterized.

5.5 Conclusion

Endothelium, being in direct contact with circulating blood, is the ideal sensor of changes in blood chemistry, in addition to being a key regulator of vascular homeostasis. Any aberration in its physiological function is the triggering point for initiation and progression of vascular diseases. Unfortunately, multiple risk factors observed in diabetes, not only corrode the endothelium but, also attenuate the reparative mechanisms of the endothelial progenitor cells (Fig. 5.4). Understanding of molecular mechanisms contributing to endothelial dysfunction and blunted repair will thus identify suitable drug targets for mitigation of vascular diseases in diabetes and metabolic syndrome.

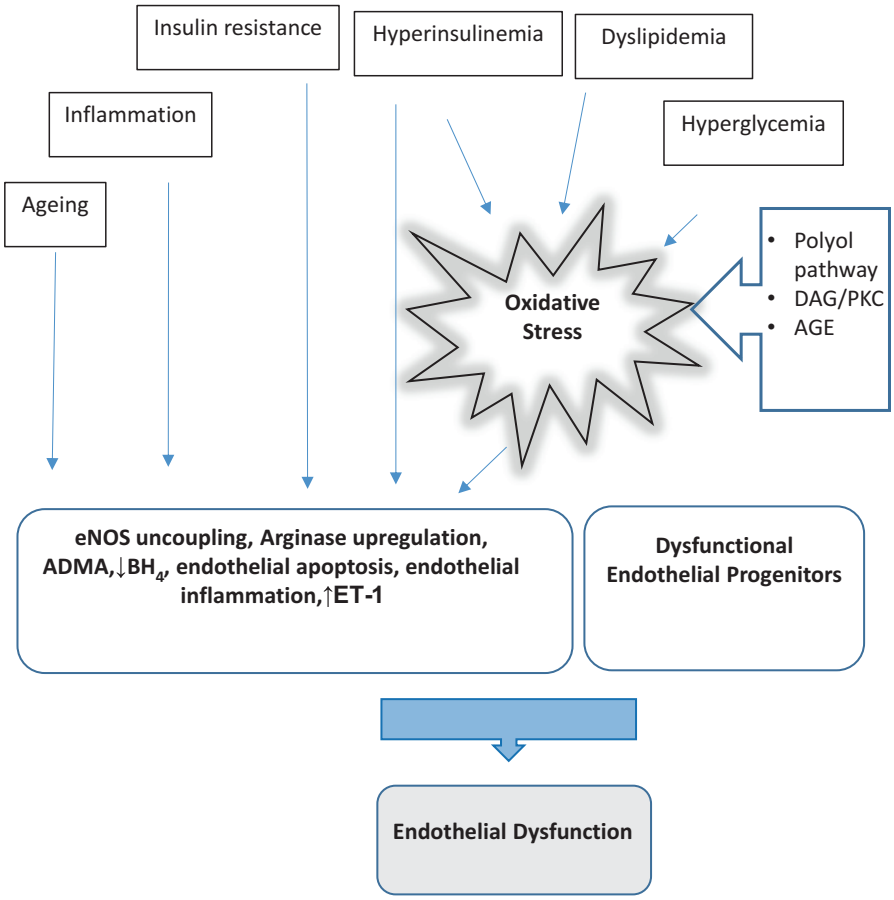

Fig. 5.4 Mechanisms causing endothelial dysfunction

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Chapter 6
Smooth Muscle Cells in Diabetes Mellitus

Uma Nahar Saikia and Suvradeep Mitra

Abstract Diabetes mellitus is a multisystem systemic disease with significant morbidity and mortality. The morbidity is often contributed by the microvascular disease whereas the macrovascular disease is significantly associated with the mortality in the diabetic patients. The macrovascular disease, also known as diabetes-accelerated atherosclerosis is promoted by the interplay of multiple factors. These biochemical and molecular parameters predominantly affect the endothelial cells and the smooth muscle cells. Both these cells actively take part in the diabetes-accelerated atherosclerosis. The smooth muscle cells evidently proliferate, accumulate and show phenotype shift in diabetes-accelerated atherosclerotic lesions. These properties are studied mainly in the animal models and therapeutic drugs can be targeted to reduce these complications.

Keywords Diabetes • Atherosclerosis • Smooth muscle cells • Vascular disease • Hyperglycemia • Dyslipidemia

6.1 Introduction

Diabetes mellitus is a disease of quantitative or qualitative deficit of insulin resulting in a state of chronic hyperglycemia. Type 1 diabetes mellitus is characterized by the progressive destruction of the β cell population of the islets of Langerhans from an immunological process leading to a quantitative deficit of insulin whereas type 2 diabetes mellitus results from insulin resistance and subsequent loss of the islets. Different body systems are profoundly affected by diabetes, especially the vascular bed. Both the types of diabetes affect both the microvascular and the macrovascular compartments leading to significant morbidity and mortality. The microvascular compartment includes renal glomerulus, vasa nervorum of the peripheral nerves and retina leading to diabetic nephropathy, neuropathy and retinopathy or diabetic

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triopathy if all the three compartments are involved. Macrovascular disease necessarily means diabetes-accelerated atherosclerosis involving the major vessels like aorta and its major branches. Diabetes leads to accelerated formation/progression of lesions of atherosclerosis affecting the vasculature. Myocardial ischemia and infarction, cardiovascular accident (stroke in common parlance) and limb ischemia or dry gangrene leading to limb amputation is the life-threatening or limb-threatening complications of the diabetic macrovascular disease. Needless to say that there has been a flurry of research around diabetes-accelerated atherosclerosis in recent times. The morphologic changes in the blood vessels followed by the pathobiology of the disease is discussed to understand the role of diabetes on the smooth muscle cells (SMCs).

6.2 Factors Affecting Smooth Muscle Cell Proliferation in Diabetes

Many biochemical factors are implicated in the SMC pathobiology. The main biochemical pathways involved are (1) polyol pathway, (2) hexosamine pathway, (3) advanced glycation/lipoxidation end-product (AGE/ALE) pathway and (4) protein kinase C (PKC) pathway [1]. These biochemical pathways portend in the generation of the reactive oxygen species (ROS) which forms the final common pathway [1]. Different studies have also shown that multiple parameters including chronic hyperglycemia, insulin resistance, hyperlipidemia etc. can affect the SMCs individually as well as concurrently. The basic pathogenetic scheme of the diabetes-accelerated atherosclerosis is depicted in Fig. 6.1.

6.3 Morphological Changes of Smooth Muscle Cells Secondary to Hyperglycemia and Dyslipidemia

The diabetic changes occur at a global scale. Variable research attempts have been made to pinpoint the site of the major brunt of the injury. Endothelial cell is undoubtedly one of the major sites to be inflicted with ROS. In addition to the endothelial cells, the micro and macrovasculature in the diabetic patients show smooth muscle cell (SMC) proliferation and morphological changes which help in the progression of atherosclerosis. The molecular biology of the SMCs in diabetes-accelerated atherosclerosis has been studied in the experimental models. In small animal models, infiltration of the monocytes followed by the activation and differentiation of these cells into lipid-loaded macrophages is seen in areas without pre-existing intimal thickening [2]. In culture, the most potent growth factors for SMCs are platelet derived growth factor B chain homodimer (PDGF-BB) and fibroblast growth factor-2 (FGF-2). SMC proliferation is also regulated by other factors i.e. components of

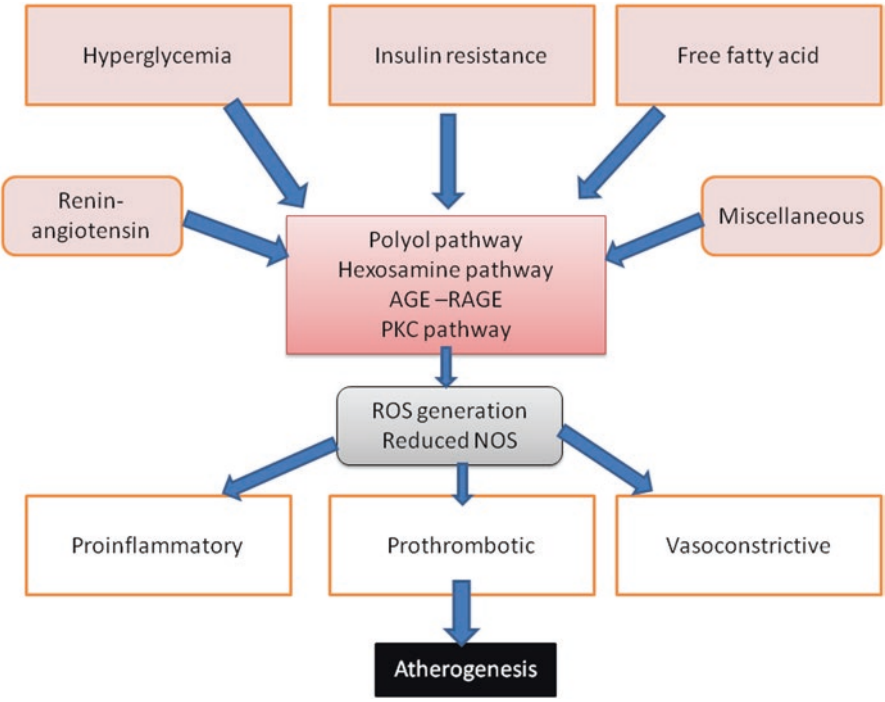

Fig. 6.1 Basic biochemical pathway of atherogenesis in diabetes

the extracellular matrix, and O2 tension [3]. Although these factors stimulate proliferation of cultured SMCs, one should bear in mind that in vivo and ex vivo effects may differ.

In humans, lipid-loaded macrophages are seen in areas with intimal thickening which is smooth muscle cell mass followed by an increased accumulation of lipid-loaded macrophages and extracellular lipid called as atheroma [4]. Formation of "atheroma" (type IV lesion) means the accumulation of lipid laden macrophages in the intima at a subendothelial location [4, 5]. The next step for progression of the lesion is increased accumulation of smooth muscle cells (SMCs) in the intima and formation of a fibroatheroma. "Fibroatheroma" (type V lesion) is the formation of a fibrous cap over the atheroma with a central core containing the lipid laden macrophages [4, 5]. Many of these lipid laden macrophages die releasing their content extracellularly forming the lipid rich core. The dead cells and their debris accumulate in the core due to ineffective clearance (efferocytosis) [6] making the core material more thrombogenic. The fibrous cap may rupture exposing the highly thrombogenic core and causing "plaque rupture" or may remain stable with accumulation of more material in the core thereby causing "progressive occlusion" of the vessel [7]. These lesions can become destabilized, possibly by thinning of the SMC-rich fibrous cap and/or increased macrophage death, leading to plaque rupture, thrombosis (Fig. 6.2a, b), and the acute clinical manifestations of atherosclerosis [2, 8].

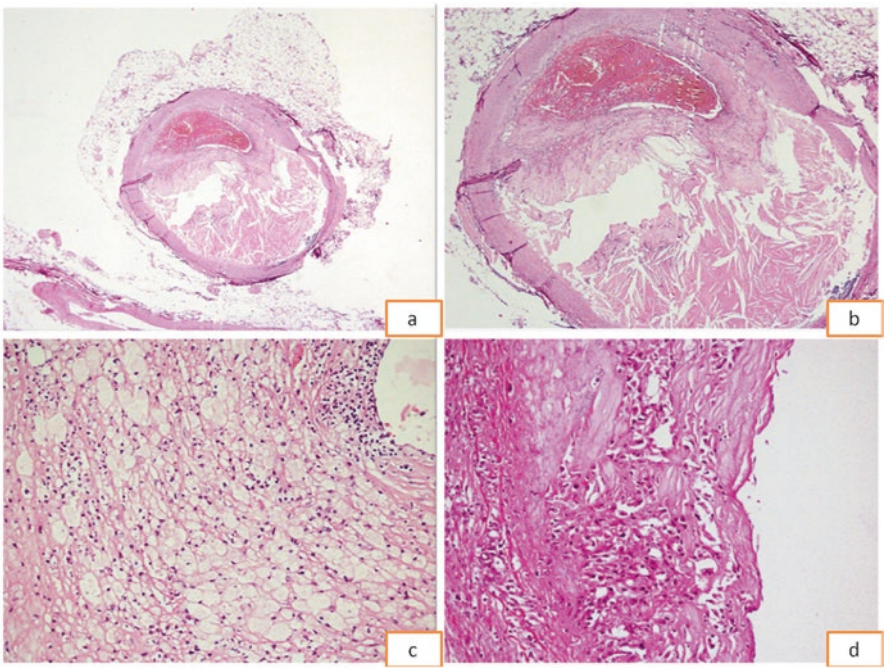

Fig. 6.2 (**a**) Scanner view of a medium sized artery (*Left* Anterior Descending Artery) in a patient with diabetes-accelerated atherosclerosis (Hematoxylin and Eosin, 20×); The artery is near-totally occluded. (**b**) The occlusion is because of the plaque rupture and thrombosis (Hematoxylin and Eosin, 40×); Note the fibrous cap is thinned out at one edge and numerous needle shaped cholesterol clefts in the atheroma core. (**c**) Numerous subintimal foam cell accumulation associated with sprinkling of the lymphocytes (Hematoxylin and Eosin, 200×). (**d**) Some of the foam cells show myoid type morphology with abundant deep eosinophilic cytoplasm (Hematoxylin and Eosin, 200×)

The atheroma formation necessarily begins with endothelial injury, a pro-inflammatory milieu and subsequent accumulation of subendothelial macrophages at the site of turbulence. The associated dyslipidemia promotes the accumulation of the lipid material within these macrophages. The circulating low density lipoprotein (LDL) particles in diabetes are small and dense. Diabetes associated hyper triglyceridemia often contributes to the generation of these small, dense LDLs. They offer higher penetration, increased susceptibility to oxidation and stronger avidity to the endothelium rendering them more atherogenic than the larger LDL particles [9]. The oxidation of the LDL particles is a crucial step as the oxidized LDL is antigenic and incites a chronic low grade inflammation at the site of atheroma formation with recruitment of the inflammatory cells which perpetuates the inflammation and subsequent endothelial injury by degranulation. Moreover, glycation of LDL increases the half life of LDL while the half life of the glycated high density lipoprotein (HDL) becomes shorter. In short, it means overproduction and perpetuation of LDL cholesterol and excess clearing of the protective HDL cholesterol. The atheroma formation occurs with the accumulation of lipid laden macrophages with subsequent intimal thickening. These macrophages recruit bone-marrow derived SMCs [10] as well as

promote the migration and proliferation of the intra-intimal and medial SMCs [11, 12]. Moreover, there is evidence that the macrophages themselves can differentiate and adopt myofibroblastic phenotype depending on the inflammatory milieu and the autocrine and paracrine factors released by the macrophages themselves and the endothelial cells. The increased accumulation of the SMCs in an atheroma portends the beginning of a fibroatheroma. The general belief of the migration of the medial SMCs, their proliferation and lack of apoptosis in the formation of fibroatheroma has been challenged by some authors in animal studies. Imperative to say, that different growth factors play roles in the formation of the fibrous cap.

The SMCs secrete collagen required for the formation of the fibrous cap strengthening the cap architecture. The increased apoptosis of the SMCs promote plaque rupture in two important ways. The first one is due to the relative lack of the collagen which is produced and secreted by the SMCs. The other cause is due to the release of pro-inflammatory cytokines related to the myocyte death which potentiates the instability of the plaque. The monocyte-macrophage system plays a crucial role in the plaque stabilization by influencing cell death of the SMCs. The advanced atherosclerotic lesions can undergo remodeling by an increase in the SMC content and reduction of the macrophage content as shown in the animal models. Also HDL can bring about similar changes and stabilize an atherosclerotic plaque [13]. The SMCs are recruited from the media and they migrate and accumulate in the vicinity of the plaque. The SMCs, native to the intima also undergo proliferation and accumulate in the atheromatous plaque. Some of these SMCs take up and accumulate the lipid within them similar to the foamy macrophages [14]. In fact, Katsuda et al. showed that the majority of the cellularity in the early atherosclerotic plaque is contributed by the SMCs. The foam cells also are mostly derived from the SMCs rather than from the macrophages (Fig. 6.2c, d), as was demonstrated by the immunohistochemistry [14]. These SMCs behave like the foamy macrophages and also undergo apoptotic death releasing the lipid material extracellularly. The extracellular lipid alongside the cellular debris forms the necrotic core. In addition, the SMCs also tend to contain this necrotic core within the subintima by forming a fibrous cap over it. This fibrous core is formed by the direct presence of the SMCs as well as by the secretion of collagen and elastin by the SMCs [7, 15].

Functionally, the SMCs can be divided into resident SMCs and migrating SMCs. The resident SMCs are native to the intima and is found normally within the intima, whereas the migrating SMCs are derived from the media and can only be seen in progressive atherosclerosis. The migration of the SMCs is stimulated by different mitogenic factors. The migrating and resident SMCs also receive proliferating signal to cause progression of the atherosclerotic process. Morphologically or ultrastructurally, these SMCs can be myofilament-rich or rough endoplasmic reticulum (RER)-rich. The myofilament-rich SMC is RER-poor and vice versa. The former serves a more contractile function and the later has synthetic functions. The contractile phenotype is also known as differentiated phenotype and is commonly found in the healthy blood vessels whereas the non-contractile/synthetic phenotype predominates in the diseased blood vessels [16]. It has been shown in animal models that the SMCs in the diabetes-accelerated atherosclerosis show a synthetic phenotype rather than a contractile phenotype along with a switch in the expression of the actin

isoform [16, 17]. The contractile alpha smooth muscle isoform converts to a non-muscle beta isoform in diabetes-accelerated atherosclerosis [17]. The advanced lesions of atherosclerosis (type III onwards) show visible ultrastructural difference [4, 5]. The basement-membrane around these SMCs is very thick giving it the name of basement-membrane-rich or pancake-like cell [18]. The diabetic SMCs show an abundance of cytoplasm and RERs on ultrastructure alongside an increased amount of extracellular material [17]. These phenotypic switch in diabetes-accelerated atherosclerosis is also termed as "phenotypic modulation" [17]. Moreover, studies have also pointed out a vascular bed specific remodeling in diabetes and differential phenotype of SMCs in different vascular beds in animal studies. As for example, the coronary SMCs are found to down regulate the expression of the contractile proteins with altered interaction with the extracellular matrix (ECM) as compared to the aortic SMCs. The phenotypic variation of the diabetic SMCs is also described in other studies. One such study concluded that the human SMCs isolated from the diabetic patients show a significantly higher rate of proliferation, adhesion and migration in addition to abnormal morphology in the culture medium [19]. This property of the SMCs is termed as "vascular hyperreactivity" by some authors and a change in the subcellular calcium ion distribution in activated SMCs has been postulated as one of the causes to bring about these changes [20]. The enzyme content (endothelial Nitric oxide synthetase), intracellular guanosine monophosphate (cGMP) levels also change in the diabetic SMCs leading to the hyperreactivity of the SMCs in the diabetic patients [16]. Indeed, calmodulin-stimulated cyclic nucleotide phosphodiesterase gets accumulated in the SMCs of both the phenotypes and takes part in the SMC proliferation and recruitment [21, 22].

It is also important to note that the SMCs in diabetic vessels do not show uniform phenotypic and/or functional change. In a seminal study by Boor et al., the enzymatic activity of the SMCs in the plaque region or underlying the plaque had been found to be different than the enzymatic activity in the vicinity. An isoenzyme of glutathione-S-transferase (GST), known as hGSTA4-4 is known to be associated with detoxification of the generated intracellular ROS [23]. Preferential expression of this enzyme in the SMCs underneath the plaque substantiates the involvement of SMCs in the pathogenesis of the diabetes-accelerated atherosclerosis. In addition, the role of ROS in the pathogenesis is also highlighted.

6.4 Biochemical and Pathobiologic Basis of Smooth Muscle Cell Proliferation in Diabetes

Despite the general and uniform agreement that SMCs proliferate and accumulate in diabetes-accelerated atherosclerosis, the true pathobiology of SMC induction is yet not elucidated. Many factors namely hyperglycemia, insulin, AGE, triglycerides and non-esterified fatty acids, hypertension and renin-angiotensin system and different paracrine molecules are all implicated with contradictory results in different studies. Similarly, the studies highlighting the factors affecting the stability of the

plaque are also not met with consensus agreement. Probably the SMC induction and plaque stability are the functions of multiple inter-related factors.

Different studies have concluded the effects of hyperglycemia on SMC proliferation differently. This ranges from stimulatory effect to no effect to inhibitory effect. However, few studies have shown that the glucose consumption by the SMCs in diabetes remains very high, so much so that they can be almost compared to the tumour cells in terms of glucose hunger. However, unlike the endothelial cells, the SMCs use glycolytic pathway for ATP generation even under aerobic condition, a paradoxical condition known as "aerobic glycolysis". Energetically infidel, aerobic glycolysis protects the SMCs from the oxidative stress [24]. There are some studies proposing the theory of induction of proliferation and accumulation of the SMCs by chronic hyperglycemia [16, 25–27]. Application of different inhibitor drugs on animal subjects have shown consequent blockade of the SMC proliferation and accumulation. For example, epalrestat, the inhibitor of aldose reductase enzyme (key enzyme of polyol pathway) has abolished the proliferative and migratory phenotypic and functional switch in diabetic animals proving the role of polyol pathway in the pathogenesis [27]. Similarly, the anti-insulin drugs are also found to nullify the SMC chemokinesis substantiating the role of free radical pathway in SMC pathobiology [26]. Advanced glycation end products (AGE)s are found to cause oxidative stress in the SMCs by the AGE-RAGE (receptor of AGE) interaction and the growth stimulatory effect of this ligand-receptor interaction. Also, the altered cell-cell and cell-matrix interaction promoted by the AGEs or ALEs (advanced lipoxidation end products) cause an aberrancy of cellular function. On the contrary, the stimulatory effect of chronic hyperglycemia on SMC has been challenged by a few authors [12, 28]. In this context, the study by Peiro et al. is noteworthy. These authors had shown a death promoting effect of chronic hyperglycemia on the SMCs by the activation of necrotic pathway. Moreover, hydrogen peroxide has been found to play a pivotal role in this necrotic cell death, as catalase enzyme had been found to abolish these effects. This necrotic cell death promotes the changes of diabetic vasculopathy [28]. Morphologically, the changes of both diabetic vasculopathy and accelerated atherosclerosis are well documented in the same organ. Hence, probably, chronic hyperglycemia has a complex interaction with the SMCs and the smooth muscle changes are function of multiparametric interaction.

There is evidence of lesional triglyceride (TG) and non-esterified fatty acids (NEFA) promoting SMC migration and proliferation in the recent literature. The lipoprotein lipase, an essential enzyme in the degradation of the TG is increased in atheromatous lesions and is released by the SMCs and the macrophages in an atheromatous plaque. Recent evidence suggests that the insulin resistance has adverse effects on both the endothelium and the platelets via the downregulation of the PI3K/AKT and IRS-1/AKT pathways ultimately promoting an imbalance of nitric oxide (NO) and reactive oxygen species (ROS). However, the role of insulin or insulin-resistance on SMCs is not well established [11].

The nitrergic pathway, intracellular guanosine monophosphate (cGMP) and ROS are considered to be the final common pathway linking all the other metabolic pathways in diabetes mellitus, namely the polyol, hexosamine, AGE and protein kinase

C (PKC) pathways [1, 29]. Many studies including the study by Pandolfi et al. had shown the upregulation of the intra-endothelial and intra-SMC nitric oxide synthetase (NOS) activity along with a concurrent fall in cGMP and rise in superoxide production in diabetic animals [16].

The renin-angiotensin pathway, hypertension and the paracrine factors may also affect the SMCs, though convincing evidences are still lacking. Similarly, no such factors can be implicated for the plaque instability [11].

From this discussion, it is evident that diabetes causes SMC dysfunction or diabetes is a state of altered function for SMCs. This SMC dysfunction is attributed to variable factors but hyperglycemia, insulin resistance and dyslipidemia all play roles in this. These factors are also keys to the vascular damage and subsequent atherogenesis. All these three factors promote oxidative damage to the endothelium bringing about a vasoconstrictive, pro-inflammatory and prothrombotic state all of which stimulate atherogenesis independently or in a combined manner. So, in brief, diabetes is a state of phenotypic modulation and dysfunction of the SMCs [30].

6.5 Significance of the Smooth Muscle Cell Changes in Diabetes

Once the morphological and functional changes in SMCs occur, consequently the imminent question that looms large is "Do these changes reflect mere research-related jargon or they have any diagnostic, prognostic or therapeutic value"? In simple words, what is the clinical relevance of these SMC changes in relation with diabetes which is necessarily twofold in clinical practice. The first one is prognostic and the second one is therapeutic. Currently, the diagnosis of diabetes-accelerated atherosclerosis is more clinical and comes from the end-organ-damage related signs and symptoms. The above-mentioned pathways are experimental studies either in cell culture or in animal models.

The involvement of the SMCs dictate the development, progression and the complications of the diabetes associated macrovascular disease. It is evident from the above-mentioned discussion that the SMCs undergo a series of phenotypic and functional changes in the diabetic setting. These changes may involve preferential vascular beds and can have regional preferences as well. The basic underlying change of the SMCs in diabetes is the proliferation and accumulation leading to the formation of the atheroma. Moreover, the SMCs undergo foam cell transformation in the early stage. The next step is progression of the atheroma into fibroatheroma which is brought about by the secretion of collagen by the SMCs. The complications can occur either in the form of plaque rupture or plaque vulnerability or stability of the plaque leading to luminal occlusion. Dissecting aneurysm and vasculopathy are also known complications of diabetic macrovascular disease. Although the exact mechanism of the plaque rupture is not well elucidated, it is probably caused by a thinner fibrous cap. The thinner fibrous cap can be formed by a lesser collagen

secretion by SMCs and this diminution of the collagen secretion could be because of less proliferation and/or accumulation of the SMCs or their death by apoptosis or a functional defect of the accumulated SMCs. The SMC apoptosis also promotes dissecting aneurysm and diabetic vasculopathy.

The therapeutic significance of the SMC involvement is again twofold. According to the previous concept, the endothelial cells are believed to be the key mediators in the diabetic vascular diseases. However, the recent concept proves the fact that SMCs play a major role in diabetic diseases independent of or dependent on the endothelial injury. This necessitates targeting the SMCs with or without the endothelial cell activation. As for example, the foam cells in early atherosclerosis are experimentally targeted as their presence and trafficking promotes the progression. As the foam cell population consists of both the macrophages and SMCs, they become the natural therapeutic targets [31]. SMC proliferation and migration along with the biosynthetic activity is also targeted for causing plaque regression [32]. Endoglin receptor modulator modulates the mural cell adhesion and their proliferation and is found to be beneficial in atherosclerosis [33, 34]. Secondly, the underlying mechanisms of the endothelial dysfunction have been postulated. The individual modulators of these postulated pathways are found to have beneficial effects (anti-proliferative and anti-migratory) on the SMCs in the animal models. Targeting these pathways in the human beings may prove to have therapeutic effects. Naturally, the antioxidants and free radical scavengers, anti-PKC agents, aldose reductase inhibitors are all effective [32]. The role of epigenetic modifiers and micro RNAs in this aspect are also being evaluated [35, 36].

6.6 Conclusion

In short, diabetes promotes a state of smooth muscle cell dysfunction and proliferation coexisting with its phenotypic changes. These changes probably occur as a result of the complex interaction of multiple biochemical parameters. This constitutes the effector mechanism of the formation, progression and the complications of the atherosclerotic plaque. A detailed knowledge of such underlying pathogenesis may help in the development of the newer targeted therapies in diabetes associated accelerated atherosclerosis.

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Chapter 7
Monocyte Factors in Pathogenesis of Vascular Lesions in Diabetes

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Abstract Atherosclerosis in patients with diabetes is initiated by activation of the endothelium by elevated glucose cholesterol and reactive oxygen species in blood. The resulting recruitment of monocytes, their differentiation into a pro-inflammatory phenotype and formation of foam cells in the sub endothelial space are the hallmark of early atherogenesis. This process is orchestrated by a range of cytokines, chemokines and chemoattractants, which influence all stages of the disease from monocyte adhesion to the endothelium to lipid uptake by monocyte derived macrophages. This chapter reviews the role of important cytokines associated with monocyte function in early atherogenesis as well as in diabetic vascular disease. Current approaches to modulate cytokine action to repress progression of atherosclerosis are also discussed.

Keywords Atherosclerosis • Monocytes • Type 2 diabetes • Cytokines • Chemoattractants • Cyclophilin A

Abbreviations

EC	Endothelial cells
LDL	Low density lipoproteins
DAMPs	Damage associated molecular patterns
VCAM-1	Vascular cell adhesion molecules
ICAM-1	Intercellular cell adhesion molecule-1
OxLDL	Oxidized LDL

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SR-A1	Scavenger receptor a1
CD 36	Cluster of differentiation 36
LOX-1	Lectin like oxidized LDL receptor
ILs	Interleukins
CCR2	C-C chemokine receptor type 2
CD62L	L Selectin
Tie-2	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1
MMP	Matrix metallo proteinase
C5a	Complement component 5a
CSF	Colony stimulating factors
TNF	Tumour necrosis factor
IFN	Interferons
ACAT-1	Acetyl-CoA acetyltransferase 1
TLR 2	Toll-like receptor 2
TGF	Transforming growth factors
PECAM-1	Platelet endothelial cell adhesion molecule
DCs	Dendritic cells
MCSF	Macrophage colony stimulating factor
PKC	Protein kinase C
CRP	C reactive protein
PAI-1	Plasminogen activator inhibitor-1
EGR-1	Early growth response protein 1
AP-1	Activator protein 1

7.1 Introduction

The innate immune system is a major contributor to initiation and propagation of atherosclerosis and monocytes are key players in this process. Monocytes represent 3–8 % of peripheral blood leukocytes in circulation [1]. They are mononuclear cells with a large bilobed kidney shaped nuclei. They are the main component of the innate immune system responsible for counteracting exogenous bacterial, viral and fungal infections by the process of phagocytosis. Monocytes are also involved in endogenous inflammatory processes. They have been implicated in various chronic inflammatory conditions such as rheumatoid arthritis, pulmonary fibrosis, cancer and atherosclerosis.

Chronic inflammatory processes in the vascular wall are initiated by recruitment of monocytes from circulation to the intima of the vascular wall. Monocytes adhere to endothelial cells (ECs) and subsequently migrate into the sub endothelial space in response to activation by chemokines. This process occurs in the presence of low density lipoproteins (LDL). As accumulation of lipids progresses, the lipids undergo oxidation and glycation. The ECs recognize these signals as damage associated molecular patterns (DAMPs) and initiate the body's defense system. The adhesion

molecules on ECs such as vascular cell adhesion molecules (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1) are upregulated facilitating monocyte adhesion to the EC. Adhesion is followed by transmigration of monocytes resulting in increased adhesion molecule expression, enhanced production and release of chemokines. Adhesion molecules and chemokines are important elements of monocyte transmigration, differentiation into macrophages and formation of foam cells. Hyperglycemia accelerates this process further by facilitating monocyte adhesion to EC cells, monocyte – macrophage differentiation and thus promotes atherogenesis and increases the risk of vascular disease. Increased oxidative stress in the vascular wall promotes modification of LDL. Oxidized LDL (OxLDL) has been found in human and mouse atheromas [2]. LDL oxidation in the artery wall is facilitated by free radicals such as superoxide, hydrogen peroxide and nitric oxide. At this stage, scavenger receptors are expressed by macrophages. Scavenger receptors such as SR-A1, CD 36 (also known as platelet glycoprotein 4) and lectin like oxidized LDL receptor (LOX-1) can bind to oxidized LDL and can increase foam cell formation [3]. Deficiency of SR-A1 and CD 36 can reduce foam cell formation in ApoE-/- mice. Mice deficient in SR-A1, CD 36 and ApoE have reduced signs of inflammation, macrophage apoptosis and secondary necrosis as well.

This chapter reviews the role of monocytes and macrophages and the cytokines that these cells secrete and/or express under chronic inflammatory conditions in the progression of atherosclerosis in diabetes mellitus. We have focused on the pivotal roles of cytokines and chemoattractants activated by monocytes in early atherogenesis and not in late atherosclerosis. In addition the chapter also provides insight into the molecules that need to be harnessed as drug targets to attenuate vascular inflammation and augment vascular function.

7.2 Blood Monocytes and Their Subsets

Blood monocytes are bone marrow derived leukocytes. They have the ability to phagocytose, produce cytokines and present antigens. Their identification was initially based on glass adherence and morphology [4] and cytochemical detection of monocyte specific esterase [5, 6]; the current standard approach is based on the cells physical properties such as light scatter.

Monocytes have a complex life cycle. Virchow and other pathologists of nineteenth century believed that macrophages were derived from mesenchymal tissue rather than blood cells. Radioisotype labeling and bone marrow cells established that circulating monocytes are the precursors for macrophages in all tissues. Landmark studies by Lewis and Lewis [7], Cohn and Benson [8], van Furth and Cohn [4], and Nichols et al. [9] threw light on mechanisms of monocyte development and differentiation. Later work revealed that monocytes are not homogeneous; there are at least two distinct subsets of mononuclear phagocytes [10–12].

When the monocyte migrates into tissues during inflammatory conditions, then these cells are termed macrophages. The monocytes transform into larger cells and

rapidly lose their monocyte characteristics. Circulating blood monocytes increase in number within minutes post exercise and in conditions of stress. Cell numbers return to baseline levels also rapidly. These circulating monocytes form a “marginal pool”. The adhesive properties of the marginal pool are distinct from monocytes in blood at resting conditions.

7.2.1 Cell Surface Markers of Monocytes/Macrophages

Several monoclonal antibodies against cell surface markers of monocytes and macrophages have been developed. In humans, CD14 and in mouse CD115, has been used as markers. There is a question of specificity of these markers as human B cells also express low levels of CD14; CD115 is downregulated in blood monocytes with inflammation [13, 14]. It has been suggested that, in addition to the use of cell surface markers such as CD14 and CD115, functional studies are needed to confirm the identity of cells as monocytes. CD16 staining can also be used to exclude dendritic cells in human blood.

7.2.2 Monocytes Subsets

Nomenclature of monocytes was established by International Union of Immunologic Societies and the World Health Organization. There are three types of monocytes in human blood:

1. The classical monocyte characterized by high level expression of CD14 cell surface receptor, (CD14++CD16-);
2. The non classical monocyte showing low level expression of CD14 and additional co expression of CD16 receptor, (CD14+CD16++) and
3. The intermediate monocyte with high level of CD14 and low level expression of CD16 (CD14++CD16+).

The classical monocytes represent 80–85% of total population of circulating blood monocytes. They are considered inflammatory mediators and are the predominant subpopulation identified in atherosclerotic plaques [15]. These monocytes express CCR2, CD62L and CD64. The non classical monocytes are also referred to as CD14+CD16-cells. This subtype depends on fractalkine for attraction and recruitment to endothelial cells. Fractalkine is expressed on activated endothelial cell surface and attracts monocytes from the circulation into the atherosclerotic plaque. The non classical monocytes have high expression of inflammatory cytokines.

A third type of human monocyte subpopulation identified as “intermediate” are CD14++CD16+cells. They express Tie-2, an angiopeotic receptor and are implicated in angiogenesis. This subset also expresses MHC Class II complexes. It is not

yet clear which monocytes subsets differentiate into various types of tissue macrophages and dendritic cells. Inside the intima, monocytes mature into DCs and macrophages. Macrophages comprise of two types: the classically activated which is proinflammatory in nature designated as M1 type and the anti inflammatory M2 type. The M1 macrophages promote inflammation and extracellular matrix degradation. M1 secretes IL-1β and induces MMP9 and TGF-β secretion stimulating fibroblast proliferation [16, 17]. The M2 macrophage promotes cell proliferation and tissue repair.

7.3 Monocyte Derived Cytokines in Atherosclerosis

Cytokines are a diverse group of low molecular weight proteins. They are grouped into different classes such as chemokines, interleukins (ILs), colony stimulating factors (CSF), tumour necrosis factor (TNF), interferons (IFN) and transforming growth factors (TGF). Cytokines are expressed in various forms during atherogenesis (Fig. 7.1). Most of the cells involved in atherosclerosis produce cytokines and can elicit a response. Cytokines secreted from monocytes are of prime importance as these are the sentinel signals which may provide information about vascular injury and inflammation. Under normal conditions, a small number of monocytes pass through the endothelial lining of the vascular wall to ensure maintenance of the

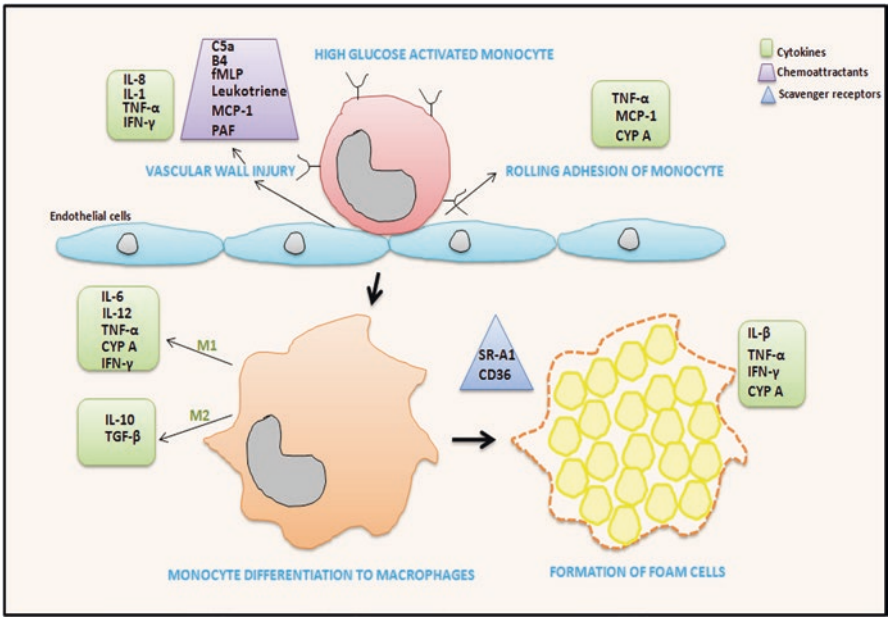

Fig. 7.1 Cytokines, chemokines and scavenger receptors expressed during early stages of atherosclerosis

resident macrophages. On injury to the vessel wall, cytokines such as IL1, IL8, TNFα, IFNγ and chemoattractants such as C5a, leukotriene B4, N formyl peptides (fMLP), monocyte chemoattractant protein (MCP-1) and platelet activating factor (PAF) mediate the adhesion of monocytes to endothelial cells and their subsequent transendothelial migration [18].

Monocytes prior to transmigration attach loosely to the vessel wall and roll over the endothelium; stick and spread over the surface of endothelial cells on the vascular wall and then migrate through junctions between tightly held endothelial cells. Interaction of various molecules is involved during this process. L Selectin on the surface of monocytes interact with ligands such as P-Selectin and E selectin on endothelial cells which are activated by TNF-α, an inflammatory mediator. Adhesion of monocytes to endothelium depends on the activation of β_2-Integrin which is facilitated by MCP-1 and their increased affinity for endothelial ligands such as ICAM-1, 2; β integrin, VLA-4 and LPS receptor CD14 on monocytes. Transendothelial migration involves the interaction of platelet endothelial cell adhesion molecule (PECAM-1) on monocytes with PECAM-1 on endothelial cells [19].

Inside the arterial wall, monocytes differentiate to macrophages or dendritic cells. Differentiation is facilitated by macrophage colony stimulating factor (MCSF), which responds to cytokine signals [20]. The M1 macrophage phenotype produces proinflammatory cytokines such as IL-6, IL-12 and TNF-α [20–23]. Alternatively activated M2 phenotype produces anti-inflammatory cytokines such as IL-10 and TGF-β which help in resolving inflammation. T – helper cytokines such as IFN-γ and Il-β are required for M1 differentiation, whereas Th2 cytokines such as IFN-γ and IL-β are necessary for M2 differentiation [20–22]. Studies have shown the inhibition of atherosclerotic progression by administration of Il-3 in Ldlr-/- model system as a result of M2 macrophage polarization [24, 25].

During foam cell formation, macrophages express pattern recognition receptors (PRRs) such as scavenger receptors (SRs), Toll like receptors (TLRs) and nucleotide binding oligomerisation domain (NOD) like receptors (NLR) [26]. The scavenger receptors play a critical role in foam cell formation. SRs such as CD36 and SR-A1 recognize modified LDL and aid uptake of these particles by macrophages and their conversion into lipid laden foam cells [20]. As there is no feedback regulation in this process uncontrolled uptake can occur. The cytokines, IFN-γ and TNF-α promote foam cell formation in vivo, whereas Il-1RA and Il-33 are inhibitory [20]. Formation of foam cells involves malfunctioning of a system that controls the uptake, intracellular metabolism and efflux of cholesterol by macrophages. Several cytokines are involved in this process via regulation of the expression and/or activity of key genes implicated in these processes. IFN-γ promotes modified LDL uptake by inducing expression of SR that binds phosphatidyl serine and oxidized lipoproteins. The cytokines induces expression of several SRs such as SR-A, CD 36 and SR-PSOX and decreased expression of ApoE, ABCA1 and ABCG1 [27, 28]. In contrast, TGF-β1, IL-10 and IL-33 inhibit macrophage foam cell formation. In ApoE-/- mice, IL-33 reduces foam cells by decreasing modified LDL uptake, reducing intracellular cholesterol esters and stimulating cholesterol efflux [29]. These changes result in reduced expression of genes involved in uptake and intracellular

storage of cholesterol esters such as SR A1, CD36, ADRP (adipocyte differentiation related protein) and ACAT-1 and increased expression of genes involved in intracellular trafficking and efflux of cholesterol [30].

A recent study by Bekkering et al. [31] suggests that macrophages derived from monocytes and exposed to modified forms of LDL including OxLDL are of a long term proinflammatory phenotype resulting from epigenetic reprogramming of histones. The long lasting proinflammatory phenotype are characterized by increased production of proinflammatory cytokines, chemokine augmented foam cell formation and increased production of MMPs. OxLDL induces cytokine and chemokine production in macrophages by stimulation of membrane bound CD36 and TLR2, TLR4 and TLR6 [32, 33]. OxLDL also promotes secretion of IL-8 and MCP-1 by increased acetylation of histones, H3 and H4 [34].

7.3.1 Monocyte Secreted Cytokines in Diabetes and Its Vascular Complications

Inflammation and activation of monocytes are understood to be important for enhancing insulin resistance in T2DM. Increases in inflammatory and oxidative stress markers are found in conjunction with the development of complications of diabetes. Vascular complications in patients with type 1 diabetes are associated with increase in plasma levels of CRP as well as in concentration of soluble vascular cell adhesion molecule 1 and nitrotyrosine [35]. Increase in inflammatory markers has been detected in apparently healthy individuals who later on develop type 2 diabetes [35]. Obesity and activation of adipose tissues may enhance the release of inflammatory factors and lead to the development of insulin resistance. Adipocytes on activation, release abnormal levels of bioactive molecules such as lipids, fatty acids, MCP-1 and various inflammatory cytokines such as CRP, PAI-1 and TNF-α. The release of these cytokines and other mediators results in the local recruitment of monocytes within adipose tissues. Differentiation of monocyte into macrophages results in enhanced release of inflammatory factors and chemokines locally within adipose tissues and the inflammatory responses spread to other tissues [36]. This hypothesis fits well with the observation that MCP-1, a proinflammatory chemokine mainly produced by macrophages are secreted by adipocytes as well.

In cardiovascular tissues, increased PKC and MAPK activity in the presence of insulin resistance [37, 38] leads to vascular complications. Under normal conditions, insulin interacts with insulin receptors to stimulate two main pathways: (i) a phosphoinositide-3 kinase (P13K) pathway that inhibits atherogenesis and has antiatherogenic effects and (ii) a MAPK activated pathway that promotes cell growth and enhances atherogenesis. In the setting of insulin resistance and diabetes, the increase in glucose and free fatty acids causes an increased release of inflammatory cytokines and altered regulation of PKC and MAPK activity. PKC inhibits P13K pathway resulting in progression of atherosclerosis through reduction in antiatherogenic nitric oxide production and impaired endothelium dependent vasodilation [39].

7.3.2 Major Cytokines in Diabetes Associated Vascular Disease

Major cytokines involved in vascular complications of T2D are IL-1β, TNF-α, IL-6, leptin and MCP-1.

IL-1β is a proinflammatory cytokine and an early mediator of inflammation [40]. A common signaling pathway has been suggested between glucose and IL-1 induced β-cell apoptosis [41, 42]. Elevated concentrations of both IL-6 and IL-β are associated with threefold increased risk of developing diabetes compared to control group [43, 44]. As atherosclerosis is a chronic inflammatory condition, IL-1β has been linked to this disease [45, 46]. Cholesterol crystals activate the NLRP3 inflammasome and generate IL-1β production. IL-β1 deficient mice have less atherosclerosis [47]. The Canakinumab Anti inflammatory Thrombosis Outcomes study (CANTOS), the first true test of the hypothesis that interrupting an inflammatory pathway involved in atherosclerosis will reduce cardiovascular events is designed to test the hypothesis that inhibitory IL-1β will reduce cardiovascular events. CANTOS trial enrolls more than 17,000 subjects [48]. CANTOS is an event driven trial which needs 1400 events for the study to reach an end point. The results are expected to be published in a year.

TNF-α, a major adipocyte cytokine, [49] can impair insulin action by interfering with insulin signaling [50–54]. TNF-α influences synthesis, secretion and activity of other cytokines. TNF-α in combination with other cytokines accelerates dysfunction and destruction of the β cells [42]. TNF-α, has been shown to increase leukocyte adhesion to endothelium and thus implicated in the pathogenesis of endothelial dysfunction [55].

IL-6 cytokines have the IL-5Rβ receptor belonging to the type 1 cytokine receptor family [56]. Other than monocytes and activated leukocytes the cells of the immune system, endothelial cells, skeletal and smooth muscle cells, adipocytes, islet β cells, hepatocytes, astrocytes and several other cell types also produce IL-6. IL-6 stimulates cell growth and inflammation. It also affects glucose homeostasis and metabolism both directly and indirectly by acting on various cells. IL-6 also increases plasma concentration of fibrinogen, PAI-1 and CRP [57]. It has been suggested that elevated levels of IL-6 may reduce insulin sensitivity by inhibiting GLUT4. Circulating levels of IL-6 are elevated years before the onset of type 2 diabetes. IL-6 mRNA is elevated in insulin resistant humans. Elevated levels of IL-6 predict future risk of type 2 diabetes development [58–60] even though CRP remains a stronger predictor. Association between IL-6 and progression to diabetes development may reflect an attempt to counter regulate low grade inflammation induced by other inflammatory mediators. IL-6 participates in the initiation and accelerates chronic inflammatory process and contributes to development of micro and macrovascular complications in diabetes mellitus [61]. Lowe et al. in 2014 studied the associations of CRP, fibrinogen and IL-6 with risk of major macrovascular events in their Action in diabetes and vascular diseases: Preterax and Diamicron modified release controlled evaluation (ADVANCE) study in a case-cohort study

(n = 3865) and demonstrated that of the three proteins only IL-6 was an independent predictor of macrovascular events [62].

Leptin is a member of the IL-6 cytokine family encoded by the obese gene (Ob) and mainly produced by adipocytes [63–67]. The co existence of insulin resistance and obesity in humans has suggested a correlation between leptin and insulin signaling [68–70]. In rodent islets, leptin induces β-cell proliferation and protects from free fatty acid induced β cell apoptosis [71–74]. Chronic exposure of human islets to leptin however, leads to β cell apoptosis by reducing levels of IL-1 receptor antagonist and by increasing IL-1β synthesis and secretion. Ob/ob leptin deficient mice are hyperglycemic and insulin resistant [75]. However, peripheral administration of leptin reverses hyperglycemia and hyperinsuliaemia before weight loss [75]. Leptin receptors are found on macrophages, foam cells, endothelium, platelets and VSMCs. Leptin affects each of these cell types and has a proatherogenic role in every step of atherogenesis. Circulating levels of leptin are shown as an independent predictor of cardiovascular morbidity and mortality. Several clinical studies demonstrate that hyperleptinemia predicts acute cardiovascular events, restenosis after coronary injury such as angioplasty and cerebral stroke, independent of traditional risk factors [76–78].

MCP-1 is a chemoattractant that attracts monocytes into vessel walls, promotes synthesis and release of proinflammatory cytokines which enhances the attachment of monocytes to the endothelium [79]. MCP-1 is also one of the key chemokines that recruits monocytes into adipose tissues. It activates the resident macrophages to secrete cytokines and chemokines to recruit additional monocytes and macrophages thus amplifying inflammation. The proinflammatory states that enhance innate immune signaling result in the activation of NFκB which activates transcription of genes encoding chemoattractant factors such as MCP-1 resulting in monocyte recruitment in the vessel walls during atherogenesis [40].

Cyclophilin A is a proinflammatory cytokine secreted by glucose activated monocytes. It is a ubiquitously distributed protein belonging to the immunophilin family [80]. In humans there are 16 family members of the cyclophilin family, the most abundant member being cyclophilin A which makes up 0.1–0.6% of the total cytosolic proteins. Cyclophilin has a beta barrel structure with two alpha helices and a beta sheet. Cyclophilin A was first recognized as the host cell receptor for the immunosuppressive drug cyclosporine A [81, 82]. It also possesses peptidyl prolyl cis trans isomerase activity playing a role in protein folding [80, 83]. The other cyclophilins are cyclophilin B, C and D. Cyclophilin B and C are localized in the endoplasmic reticulum (ER) where it maintains redox homeostasis. Depletion of these two cyclophilins leads to hyperoxidation of the ER. Cyclophilin D is localized in the mitochondria and interacts with Bax to promote mitochondrial pore formation, thus releasing pro apoptotic factors such as cytochrome C and apoptosis inducing factor (AIF).

Various studies have revealed that cyclophilin A can be secreted by cells in response to inflammatory stimuli. Secreted form of cyclophilin A is a chemoattractant for monocytes, neutrophils, eosinophils and T cells in vitro. Cyclophilin A functions as a chemokine in high glucose conditions promoting inflammation in the

presence of oxidized lipoproteins [84]. Cyclophilin A fulfils all the characteristics properties of a chemokine. It is of small molecular weight (17 Kda) and contains four cysteine residues [85]. It also has a known receptor, CD147 which has been implicated in several diseases [86].

Cyclophilin A can be secreted from cells in response to inflammatory stimuli such as hypoxia, infection, oxidative stress and high glucose [84, 87–90]. The secreted cyclophilin A acts as a autocrine/paracrine factor and participates in intercellular communication. Cyclophilin A in its extracellular form stimulates proinflammatory signals in endothelial cells and vascular smooth muscle cells. Extracellular cyclophilin A also has a chemotactic effect on leukocytes, monocytes and lymphocytes. It has a ubiquitous Ig like receptor CD147 which may be in part responsible for the chemotactic activity of cyclophilin A.

Cyclophilin A has a potential role in several human diseases (Fig. 7.2). There is a link between cyclophilin A expression and VSMC proliferation suggesting that VSMC derived cyclophilin A is important for recruitment of inflammatory cells [91, 92].

7.4 Cyclophilin A in Diabetic Vascular Disease

Monocytes and their interactions with the host microenvironment may secrete and shed proteins extracellularly into body fluids, particularly blood. It is thus possible to detect proteins relevant to monocyte endothelial interactions in blood. Cyclophilin A was first identified as a secretory protein of monocytes activated by high glucose using proteomic technologies [93]. Given its role as a inflammatory mediator of vascular tissue damage associated with inflammation and oxidative stress, plasma levels of cyclophilin A in normal healthy volunteers were compared with patients with type 2 diabetes with or without coronary artery disease [94]. The plasma cyclophilin A levels were increased in patients with diabetes and CAD suggesting a role for this protein in accelerating vascular disease in diabetes.

Cyclophilin A has been studied extensively in vascular disease such as atherosclerosis and coronary artery disease (CAD) as well as type 2 diabetes. Cyclophilin A is known to stimulate LDL uptake in the vessel wall by regulating expression of scavenger receptors [84]. Cyclophilin A increases EC activation and inflammation by increasing VCAM-1 expression [95]. Cyclophilin A decreases endotheial NO synthase (eNOS) expression through Kruppel like factor 2 (KLF2) transcriptional expression in ECs. Cyclophilin A is also a key determinant for tumor necrosis factor (TNF-α) induced apoptosis. Cyclophilin A enhances macrophage differentiation of monocytes into macrophages in high glucose conditions by regulating the expression of scavenger receptors such as CD36 on its surface owing to the chaperone activity of cyclophilin A. This accelerates accumulation of modified lipoproteins in macrophages subsequently forming foam cells. This triggers an inflammatory response increasing cytokine levels leading to secretion of the intracellular cyclophilin A into the circulation in its extracellular form [84]. Cyclophilin A has been shown to accelerate atherogenesis in diabetes by functioning as a cytokine and

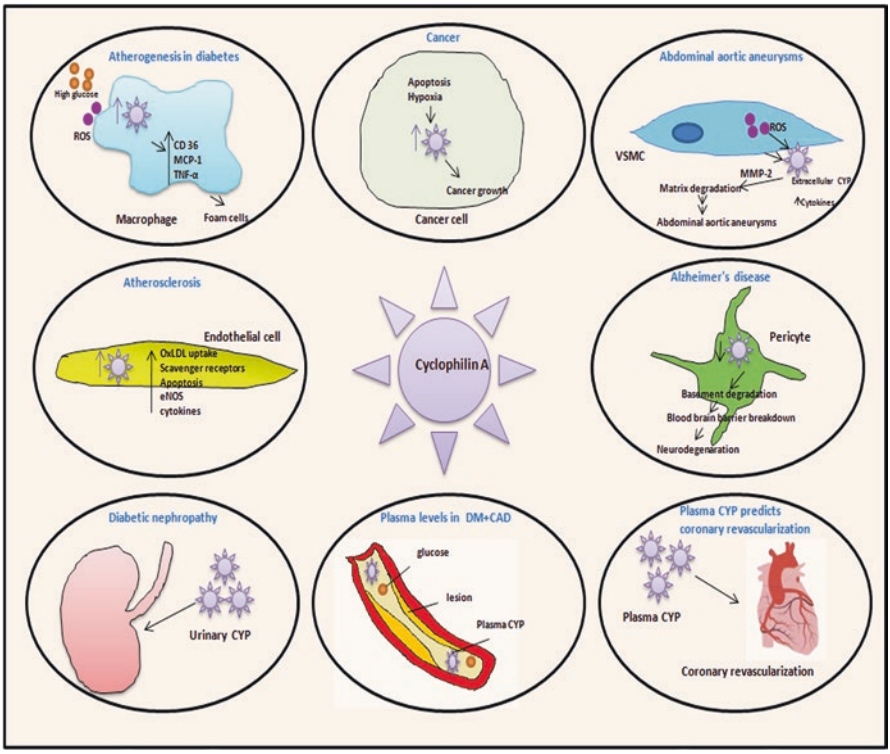

Fig. 7.2 Cyclophilin A in chronic inflammatory human diseases (starting from *left* to *right* in clockwise direction): extracellular and intracellular forms of cyclophilin A are overexpressed during atherogenesis in diabetes. Cyclophilin A is upregulated in various cancers and is a key determinant for malignant transformation and metastasis. Cyclophilin A involvement in abdominal aortic aneurysm has been demonstrated suggesting that extracellular cyclophilin A contributes to the recruitment of inflammatory cells in abdominal aortic aneurysms and that cyclophilin A induces ROS formation by a positive feedback loop. The activation of proinflammatory pathways in pericytes of the brain by cyclophilin A leads to MMP activation, consequently degrading basement membrane proteins and causing neurodegeneration. Cyclophilin A has been projected as a valuable marker for predicting coronary revascularization in patients with CAD and severity of acute coronary syndromes; predicting vascular inflammation in patients with diabetes associated CAD. Cyclophilin A is also increased in urine of patients with diabetic nephropathy and increases inflammatory processes during atherosclerosis

increasing adhesion and transmigration of monocytes and increasing levels of proinflammatory cytokines such as TNF-α and MCP-1. Thus, cyclophilin A gains clinical significance as a target for preventive strategy in vascular complications in patients with diabetes.

7.5 Advances and Shortcomings in Developing Monocyte Activated Cytokines

Hyperglycemia through various pathways causes damage to the vascular wall. The mechanisms include activation of protein kinase C isoforms, overactivation of hexosamine pathway, increased flux of glucose through the polyol pathway and increased formalities of advanced glycation end products. All these pathways lead to oxidative stress and increased permeability of the vascular wall.

Early atherogenesis involves the activation of endothelial cells, recruitment of monocytes and formation of foam cells. Monocyte derived macrophages play an important role in all these phases of atherogenesis. On exposure of macrophages to high glucose, inflammation is induced [96, 97]. A means to reduce inflammation is by blocking cytokine action. As described earlier in this chapter, various inflammatory mediators are secreted or over expressed in the presence of activated monocytes. Enhanced monocyte activation in diabetes induces activation of various cytokines and chemokines as well as monocyte derived secretory proteins relevant to the pathogenesis of atherosclerosis. These cytokines are interleukins, TNF-α, leptin, MCP-1, scavenger receptors and cyclophilin A. These cytokines are involved in the action of insulin to maintain an inflammatory response. These cytokines are also involved in insulin resistance and development of atherosclerosis.

Numerous pharmaceutical and non-pharmaceutical interventions which target cytokines and chemokines expressed during low grade chronic inflammation have been investigated. Insulin sensitizers of the thiazolidinedione class have been shown to exert an anti inflammatory effect in addition to their glucose lowering effects in patients with diabetes, reducing TNF-α plasma levels [98]. Rosiglitazone also reduces plasma MCP-1 in obese patients and patients with diabetes [99, 100]. However rosiglitazone does not reduce IL-6 levels in blood of patients [100]. The drug also significantly potentiates TNF-α induced production of Il-6 in epithelial cells. Anti-lipedemic agents such as statins also have a reducing effect on TNF-α, IL-8 and MCP-1 levels. Fenofibrate therapy decreases circulating levels of Il-6 and significantly increases plasma adiponectin levels and insulin sensitivity [101]. In patients with hypertension, Canderstan therapy, an angiotensin II receptor antagonist, significantly reduces plasma MCP-1 levels. Treatment with temocapril and candesartan significantly increases adiponectin levels as well as insulin sensitivity. Insulin can also be anti-inflammatory and therefore anti atherogenic, as it suppresses several proinflammatory transcription factors such as NFκB, Egr-1 and AP-1 and also suppresses plasma concentration of ICAM-1 and MCP-1 [102, 103]. Hyperinsulineamia can be seen as a compensatory effort to suppress inflammation and overcome insulin resistance [104]. Treatment of type 2 diabetes with insulin for 2 weeks results in reduction in MCP-1 levels in blood [105].

Metformin used as a first line therapy for managing type 2 diabetes has been shown to mediate its atheroprotective effects by activation of the AMPK pathway. The AMPK pathway can be activated by reactive oxygen species (ROS). As monocytes migrate and differentiate into foam cells, the levels of TNF-α, MCP-1 and

cyclophilin A substantially increases. Metformin at this stage activates AMPK, reduces cyclophilin A levels and counter balances the over production of ROS thus controlling redox activity (unpublished data). Arresting foam cell formation using AMPK activators such as metformin and inhibiting proinflammatory cytokine levels such as TNF-α, MCP-1 and cyclophilin A could thus be a preventive strategy for limiting progression of atherosclerosis in diabetes.

New anti-inflammatory therapies aimed at manipulating cytokine action are in the process of evaluation. The Cardiovascular Inflammation Reduction Trial (CIRT) was initiated to evaluate the efficiency of low doses of methotrexate for secondary prevention of myocardial infarction [106]. The CANTOS study mentioned earlier has also reported lowering of several inflammatory markers [48, 107, 108]. A monoclonal antibody (MLN 1202) that targets CCL2 and interaction with its receptor reduces CRP levels in blood [109].

Another approach is manipulating cytokine signaling using small molecule inhibitors that attenuate the action of pro inflammatory components or enhancing naturally occurring molecules to suppress inflammation. Molecules under development include inhibitors of monocyte secreted proteins such as cyclophilin A, non coding miRNA that suppress cytokine signaling, TNF-α antibody, antagonists for IL-1 receptor and inhibitors of AMPK and JNK pathways.

As the role of each of these molecules has not been extensively clarified and their undesirable effects are unknown, caution is warranted before their clinical use. We should keep in mind that inflammatory genes, proteins or pathways implicated in atherogenesis in diabetes also participate in the normal regulation of inflammation and energy homeostasis such as insulin signaling.

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Chapter 8
Endothelial Progenitor Cell Dysfunction in the Pathogenesis of Vascular Complications of Diabetes

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Abstract Diabetes mellitus, a metabolic disorder characterized by high blood glucose, is one of the main risk factors in the development of vascular complications affecting both large and small blood vessels. A major challenge is the discovery of new mediators and biomarkers for diabetes-related vascular complications. In this regard, accumulating evidence indicate that endothelial progenitor cells (EPCs), derived from the bone marrow and peripheral blood, are critical for the maintenance and regeneration of endothelial cells contributing to repair and restoration of vascular wall integrity. The studies reveal that the reduced number of circulating EPCs under diabetic conditions can predict cardiovascular outcomes, and EPC dysfunction could contribute to the pathogenesis of diabetes – associated vascular disease.

This chapter discusses the EPC dysfunction in relationship to vascular complications of diabetes, highlighting the pathophysiology of diabetic vascular complications, mechanisms leading to EPC dysfunction in diabetes and diabetic vascular complications, significance of EPCs in the pathogenesis of vascular complications of diabetes and potential therapeutic implications of EPCs in diabetes-associated vascular complications. In particular, to understand the EPC significance in diabetes, the effects of hyperglycaemia, insulin resistance, insulin like growth factor 1, nitric oxide, oxidative stress, PI3K/Akt signaling pathway, inflammation, and of altered microRNA expression on the EPC dysfunctionality have been considered.

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A comprehensive knowledge of EPC role in all diabetic complications may help to develop new research strategies to demonstrate and consolidate their clinical relevance so that they become diagnostic biomarkers and pharmacological targets to prevent and treat diabetes-related vascular complications. Increasing the number and functional capacity of EPCs by different approaches may favorably modify the risk for cardiovascular complications and survival for people suffering from diabetes.

Keywords Endothelial progenitor cells • Diabetes • Cardiovascular disease • Cerebrovascular disease • Vascular complications • Endothelial dysfunction • Hyperglycemia • Insulin resistance

8.1 Introduction

Diabetes mellitus represents a very serious issue in every public health system. Its worldwide prevalence is continuously increasing; recent statistics released by the International Diabetes Federation reveal that 1 in 11 adults suffer from diabetes (415 millions) and those numbers will increase to 1 in 10 adults (642 millions) by the year 2040 [1]. The global rise in diabetes occurs due to population growth and ageing, genetic susceptibility and to increasing trends towards an unhealthy diet, obesity, and sedentary lifestyle. The vascular complications of diabetes are among the most serious manifestations of the disease. Patients with type 2 diabetes (T2DM) represent about 85–95% of the people with diabetes in developed countries and an even higher percentage in developing countries [1]. The microvascular complications, like nephropathy, retinopathy or neuropathy, as well as the macrovascular ones – atherosclerotic disease in all its forms: ischaemic heart disease, cerebrovascular disease, or peripheral arterial disease (PAD) are usually irreversible and lead to a decrease in life expectancy and to a higher mortality rate in these patients.

Despite the progress made in the last few years, vascular complications due to diabetes mellitus still remain a huge problem, and identifying new mechanisms involved in their development, like dysfunction of endothelial progenitor cells (EPCs), could lead to new curative and preventive therapeutic options.

8.2 Pathophysiology of Diabetic Vascular Complications

8.2.1 Diabetes and Vascular Risk Factors

It is well known that diabetic patients are more frequently affected by cardiovascular disease (CVD) compared with those without diabetes. CVD increases the rate of all-cause death nearly threefold and the rate of cardiovascular death nearly fivefold

in subjects with diabetes [2]. Most of this excess risk is associated with an increased prevalence of well-known traditional risk factors such as hypertension, dyslipidaemia, obesity (generalised or visceral), and smoking in these subjects. Hypertension is more than twice as common in people with diabetes as in people with normal blood glucose levels [3]. Premenopausal women who have diabetes have an increased risk of heart disease because diabetes cancels out the protective effects of estrogen. Nevertheless, these established risk factors do not fully explain the excess risk for CVD associated with diabetes.

Therefore, other non-traditional risk factors may be important in people with diabetes: insulin resistance and hyperinsulinemia; postprandial hyperglycaemia and glucose variability; microalbuminuria; haematological and thrombogenic factors; inflammation assessed by high-sensitivity C-reactive protein; homocysteine and vitamins; genetics and epigenetics [4, 5].

Large clinical trials in type I diabetes mellitus (T1DM) and type II diabetes mellitus (T2DM) have demonstrated that hyperglycaemia plays an important role in the pathogenesis of microvascular complications [6]. Although diabetic patients with the most severe hyperglycaemia have the highest risk of microangiopathy, hyperglycaemia, however, is a necessary, but not sufficient, cause of clinically important microangiopathy. Hypertension, smoking, hypercholesterolaemia, dyslipidaemia, obesity and hyperhomocysteinaemia are additional major causes of microangiopathy. The risk of macroangiopathy does not appear to be strongly related to hyperglycaemia, but is related to general risk factors for atherothrombosis, such as age, smoking, hypertension, hypercholesterolaemia, dyslipidaemia, obesity and hyperhomocysteinaemia. Cardiovascular risk factors such as hypertension, dyslipidaemia, obesity, insulin resistance, hyperinsulinaemia and impaired fibrinolysis cluster in the metabolic syndrome [7]. All of the above-mentioned factors create a state of constant and progressive damage to the vascular wall, manifested by a low-grade inflammatory process and endothelial dysfunction [8].

8.2.2 Diabetes and Vascular Complications

8.2.2.1 Microvascular Complications

Diabetic Retinopathy This is one of the most important microvascular complications in diabetes mellitus and is a leading cause of visual impairment in working-age adults [9]. Development of diabetic retinopathy in patients with T2DM was found to be related to the severity of hyperglycemia, duration of diabetes, and presence of hypertension [10].

Retinopathy is classified as nonproliferative (background) or proliferative. The most common early clinically visible manifestations of diabetic retinopathy include microaneurysm formation and intraretinal hemorrhages. Microvascular damage leads to retinal capillary nonperfusion, cotton wool spots, increased number of hemorrhages, venous abnormalities, and intraretinal microvascular abnor-

malities. During this stage, increased vasopermeability can result in retinal thickening (edema) and/or exudates that may lead to a loss in central visual acuity. Proliferative retinopathy is characterized by the formation of new blood vessels on the surface of the retina and can lead to vitreous hemorrhage. White areas on the retina ("cotton wool spots") can be a sign of impending proliferative retinopathy. These new vessels then lead to traction retinal detachments and neovascular glaucoma, respectively. Vision can be lost in this stage as a result of capillary nonperfusion or edema in the macula, vitreous hemorrhage, and distortion or traction retinal detachment [11].

Diabetic Nephropathy It is one of the most common complications of diabetes mellitus. Among patients with T1DM, the incidence of diabetic nephropathy has decreased to 10–15% in more recent cohorts [12]. However, due to the increase in T2DM, the absolute prevalence of diabetic nephropathy has increased over the past two decades; in 2015, diabetic nephropathy was reported to be the cause of 43.9% of all cases of end-stage renal disease (ESRD) in the United States [13].

Diabetic nephropathy is characterized by an expanded mesangial volume, changes in the physical and biochemical properties of the glomerular basement membrane, and a decreased glomerular filtration rate. Diabetic nephropathy is a clinical syndrome characterized by the following: persistent albuminuria (>300 mg/day or >200 μg/min) that is confirmed on at least two occasions, 3–6 months apart; progressive decline in the glomerular filtration rate; elevated arterial blood pressure [14]. It is preceded by lower degrees of proteinuria, or "microalbuminuria" defined as albumin excretion of 30–299 mg/24 h. In the absence of an intervention, diabetic patients with microalbuminuria typically progress to proteinuria and overt diabetic nephropathy. This progression occurs in both T1DM and T2DM. As many as 7% of patients with T2DM may already have microalbuminuria at the time they are diagnosed with diabetes [15]. The evidence suggests that early treatment delays or prevents the onset of diabetic nephropathy or diabetic kidney disease.

The exact cause of diabetic nephropathy is unknown, but the main mechanisms are: hyperglycemia (causing hyperfiltration and renal injury), advanced glycation end-products (AGEs), and activation of cytokines. More recent research highlights the role of toll-like receptors, regulatory T-cells (Treg), and increased expression of transforming growth factor β (TGF-β) in the glomeruli [16]. TGF-β and vascular endothelial growth factor (VEGF) may contribute to the cellular hypertrophy and collagen synthesis and may induce the vascular changes observed in persons with diabetic nephropathy. Hyperglycemia also may activate protein kinase C (PKC), which may contribute to renal disease and other vascular complications of diabetes. Moreover, hypergycemia was shown to induce renal artery dysfunction in streptozotocin-induced diabetic mice [17]. This study has reported that the renal artery dysfunction is the result of the reduction of nitric oxide (NO) bioavailability, endothelial nitric oxide synthase (eNOS) expression, phospholipase C activity, and intracellular free calcium concentrations [17].

Diabetic Neuropathy It has become the most common complication of diabetes, affecting as many as 50% of patients with T1DM and T2DM [18]. In T1DM, distal polyneuropathy typically becomes symptomatic after many years of chronic prolonged hyperglycemia, whereas in T2DM, it may be apparent after only a few years of known poor glycemic control or even at diagnosis. Chronic sensori-motor distal symmetric polyneuropathy is the most common form of neuropathy in diabetes. Diabetic autonomic neuropathy also causes significant morbidity in patients with diabetes. Neurological dysfunction may occur in most organ systems and can manifest by gastroparesis, constipation, diarrhea, anhidrosis, bladder dysfunction, erectile dysfunction, exercise intolerance, resting tachycardia, silent ischemia, and even sudden cardiac death [19].

Development of symptoms depends on many factors, such as total hyperglycemic exposure and other risk factors such as elevated lipids, blood pressure, smoking, increased height, and high exposure to other potentially neurotoxic agents such as ethanol. Genetic factors may also play a role. Important contributing biochemical mechanisms in the development of the more common symmetrical forms of diabetic polyneuropathy likely include the polyol pathway, AGEs, and oxidative stress [20].

8.2.2.2 Macrovascular Complications

Atherosclerosis This is the central pathological mechanism in diabetic macrovascular disease. CVD is the primary cause of death in people with either T1DM or T2DM. T2DM is one of the components of metabolic syndrome which also includes abdominal obesity, hypertension, hyperlipidemia and increased coagulability; these factors act together to promote CVD.

Atherosclerosis results from chronic inflammation and injury to the arterial wall in the peripheral or coronary vascular system. The result of the process is the formation of a lipid-rich atherosclerotic lesion with a fibrous cap. The rupture of this lesion leads to acute vascular infarction [21]. Other mechanisms involved in macrovascular disease are: increased platelet adhesion and hypercoagulability, impaired NO generation, increased free radical formation in platelets and increased levels of plasminogen activator inhibitor type 1 (PAI-1) [22, 23].

Coronary Heart Disease Coronary heart disease (CHD) has been associated with diabetes in numerous studies beginning with the Framingham study [24]. Other studies have shown that the risk of myocardial infarction (MI) in people with diabetes is equivalent to the risk in nondiabetic patients with a history of previous MI [25]. These results have lead to the recommendations of the American Diabetes Association and American Heart Association that diabetes should be considered a coronary artery disease (CAD) risk equivalent rather than a risk factor [26].

Stroke and Cerebrovascular Disease Stroke and cerebrovascular disease have a higher incidence in patients with diabetes, the later being a strong independent predictor factor for these conditions [27]. Risk of stroke-related dementia and recurrence, as well as stroke-related mortality, is elevated in patients with diabetes [22].

Various subtypes of cerebrovascular diseases have been defined in T2DM. Lacunar strokes or the occlusion of the penetrating arteries that provide blood to the brain deep structures are the main subtypes of cerebrovascular disease in diabetic patients. It is considered that 28–43% of lacunar strokes are due to diabetes [28]. Ischemic stroke, caused by occlusion of the large cerebral vessels, and transient ischemic attacks are found in a smaller percentage compared to lacunar strokes and are mainly due to the strong association between diabetes mellitus and other cardiovascular risk factors [29]. Hemorrhagic stroke is also frequent in diabetic patients as several studies have assigned a relative risk for hemorrhagic stroke of 2.4 in diabetic patients [30].

Diabetes is an independent predictor of poor outcomes [31]. Various studies have highlighted the impact of hyperglycemia during the post-stroke phase. Apparently, hyperglycemia ≥155 mg/dL in patients with stroke, with or without diabetes, is associated with a higher risk of short-term mortality and a reduced chance of recovery [32].

Diabetes contributes significantly and increasingly to the burden of stroke [33]. In the INTERSTROKE case–control study, diabetes increased the rate of stroke by 35% when comparing the top to the bottom tertile, and has been associated with 5% of the population attributable risk for stroke [34]. The Emerging Risk Factors Collaboration analysed 698 782 people from 102 prospective studies, finding that diabetes was associated with a 2.27-fold increase in the risk of ischaemic stroke and 56% excess rate of haemorrhagic stroke [35]. Following stroke, diabetes attenuates cognitive recovery, limits functional outcome, and increases mortality. Diabetes increases the risk of recurrent stroke as well. In the Life Long After Cerebral ischemia (LiLAC) cohort study, diabetes increased the risk of recurrent fatal and non-fatal stroke more than two-fold [36].

Peripheral arterial disease Peripheral arterial disease (PAD) is another macrovascular complication in diabetic patients. Compared with patients without diabetes, patients with diabetes had a higher prevalence of PAD (26.3 vs. 15.3%) and intermittent claudication (5.1% vs. 2.1%) [37]. The rate of PAD in patients with diabetes also increases with age, as it does in non-diabetic persons. The PAD occurs earlier and is often more severe and diffuse [38]. In a multicentre cross-sectional study of patients older than 70 years with diabetes, 71% had PAD when detected by abnormal ankle–brachial index [39]. Diabetes increases the incidence of critical limb ischaemia (CLI) four-fold in patients with peripheral artery disease; moreover, in diabetic patients with CLI, 50% will develop CLI in the contralateral limb within 5 years [40].

Intermittent claudication occurs three times more often in men with diabetes and almost nine times more often in women with diabetes than in their counterparts without diabetes [41]. It is also important to note that diabetes is most strongly associated with femoral-popliteal and tibial PAD, whereas other risk factors (e.g. smoking and hypertension) are associated with more proximal disease in the aorto-ilio-femoral vessels [33].

The true prevalence of PAD in people with diabetes has been difficult to determine, as most patients are asymptomatic, many do not report their symptoms as pain perception may be blunted by the presence of peripheral neuropathy [42].

Given the inconsistencies of clinical findings in the diagnosis of PAD in the diabetic patient, the measurement of ankle-brachial pressure index (ABI) has emerged as the relatively simple, non-invasive and inexpensive diagnostic tool of choice. An ABI smaller than 0.9 is not only diagnostic of PAD in the asymptomatic patients, but it is also an independent marker of increased morbidity and mortality from CVD [43].

8.2.3 Molecular Basis of the Vascular Dysfunction in Diabetes and Diabetic Vascular Complications

A better understanding of the mechanisms underlying diabetic vascular disease is mandatory because it may provide novel approaches to prevent or delay the development of its complications. The common etiology link for the different types of diabetes-associated vascular diseases is chronic hyperglycemia that evokes pathologic responses in the vasculature, which finally cause constitutive NO inhibition, smooth muscle cell dysfunction, overproduction of vascular endothelial growth factor, chronic inflammation, hemodynamic dysregulation, impaired fibrinolytic ability and enhanced platelet aggregation [44].

8.2.3.1 Hyperglycemia, Oxidative Stress and Vascular Disease in Diabetes

Vascular dysfunction in diabetes is based upon endothelial and smooth muscle cells dysfunction which eventually leads to atherothrombosis. Micro- and macrovascular complications are mainly due to prolonged exposure to hyperglycemia and its frequent association with other risk factors and genetic susceptibility [45]. Interestingly, the endothelial, mesangial and retinal cells are equipped to handle high sugar levels when compared with other cell types [46]. The detrimental effects of glucose already occur with glycemic levels below the threshold for the diagnosis of diabetes; this is explained by the concept of 'glycemic continuum' across the spectrum of prediabetes, diabetes and cardiovascular risk [45, 47]. There is a strong relationship between dysglycemia, obesity-related insulin resistance and impaired insulin secretion that will determine functional and structural alterations of the vessel wall. Endothelial dysfunction occurs as a consequence of the imbalance between the accumulation of reactive oxygen species (ROS) and NO bioavailability, a decrease in the latter being a strong predictor of cardiovascular events [48]. The overproduction of ROS by the mitochondria is considered one of the key triggers of vascular complications in diabetes [49].

Schematically (Fig. 8.1), high concentrations of intracellular glucose determine [45]:

- PKC activation, followed by:
  - increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase levels [50], phosphorylate p66Shc at serine 36 [51], and oxidative stress and

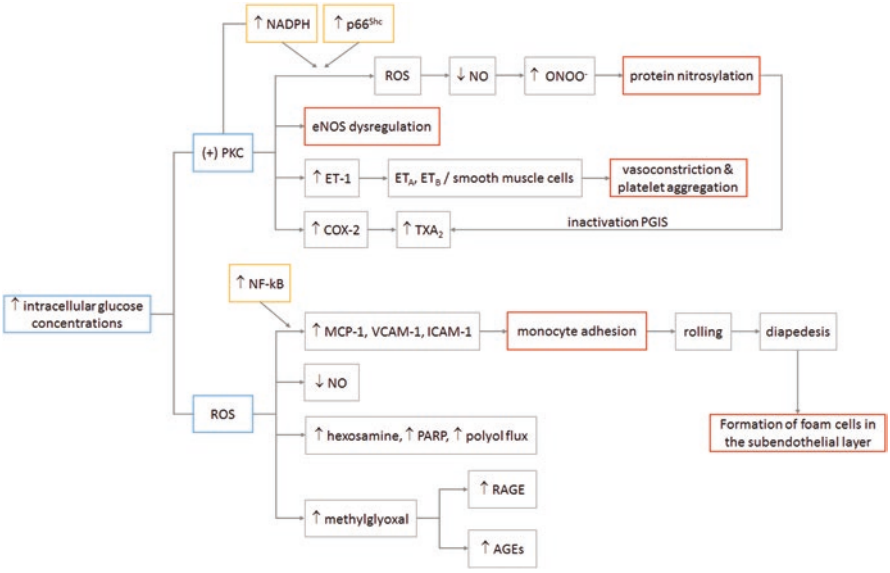

Fig. 8.1 Mechanisms of hyperglycemia-induced vascular damage

ROS generation; all of which quickly inactivate NO and facilitate peroxynitrite ($ONOO^-$) formation, a pro-oxidant compound responsible for protein nitrosylation;

- eNOS deregulation with decreased activity, further reduction of NO availability, and accumulation of free radicals [52]; furthermore, hyperglycemia reduces eNOS activity by blunting activatory phosphorylation at Ser1177;
- increased synthesis of ET-1, favouring vasoconstriction and platelet aggregation [53];
- increased synthesis of vasoconstrictors and prostanoids by up-regulation of cyclooxygenase-2 (COX-2) associated with increased thromboxane A_2 (TXA_2) synthesis and decreased prostacyclin (PGI_2) release [54];
- structural and functional changes in the vasculature: alterations in cellular permeability, inflammation, angiogenesis, cell growth, extracellular matrix expansion and apoptosis [53].

- Overproduction of ROS by mitochondria is involved in:
  - decreased NO bioavailability;
  - up-regulation of proinflammatory genes encoding for monocyte chemoattractant protein-1 (MCP-1), selectins, vascular cell adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule-1 (ICAM-1), via activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) subunit p65 signalling; these factors cause monocyte adhesion, rolling and diapedesis with foam cells formation in the sub-endothelial layer, thus accelerating the atherosclerotic process [55];

– increased synthesis of methylglyoxal (a glucose metabolite) leading to AGE synthesis, accumulation and ultimately to endothelial dysfunction [56]; generation of AGEs leads to cellular dysfunction by activation of AGEs receptors (RAGE); AGE-RAGE signalling activates ROS-sensitive biochemical pathways such as the pro-oxidant hexosamine flux [57];
– activation of the polyol pathway flux involved in vascular redox stress [49].

8.2.3.2 Insulin Resistance and Atherothrombosis

The main feature of T2DM, insulin resistance, often precedes its onset by many years. Insulin resistance is critically involved in vascular dysfunction in subjects with T2DM [58] and is strongly related with obesity, since the adipose tissue is the main source for inflammatory mediators and free fatty acids (FFAs). Increased levels of FFAs stimulate toll-like receptors (TLR) that cause, on one hand, the activation of NF-kB nuclear translocation, with subsequent up-regulation of inflammatory genes interleukin-6 (IL-6) and tumor necrosis factor (TNF-α), and, on the other hand, the activation of c-Jun amino-terminal kinase (JNK) and PKC, phosphorylation of insulin receptor substrate-1 (IRS-1), thus blunting its downstream targets phosphatidylinositol 3-kinase (PI3K) and Akt (a serine/threonine kinase also known as protein kinase B). These results in down-regulation of glucose transporter GLUT-4 and, hence, insulin resistance [45, 59]. In the vascular endothelium, a decrease in PI3K/Akt levels leads to increased FFA oxidation and subsequent ROS generation, with the aforementioned consequences: PKC activation, AGE synthesis, reduced PGI2 synthase activity and protein glycosylation; as a result, NO levels decrease dramatically and endothelial dysfunction ensues [60]. The blood coagulation system is also affected by insulin resistance, through alterations in IRS1/PI3K pathway leading to Ca^{2+} accumulation and increased platelet aggregation. Furthermore, insulin resistance facilitates atherothrombosis through increased cellular synthesis of PAI-1 and fibrinogen and reduced production of tissue plasminogen activator (tPA) [61].

The tight bond between insulin resistance and atherosclerosis is further established by the alterations in the lipid profile, such as high triglycerides, low HDL cholesterol, increased remnant lipoproteins, elevated apolipoprotein B (ApoB) as well as small and dense LDL cholesterol [62]. Accordingly, the experimental association of hyperlipemia with diabetes diminished the relaxation of the resistance arteries to bradykinin by an NO-dependent and an NO-independent mechanism (mediated via Ca^{2+} activated K^+ channels) [63]. Moreover, the simultaneous insult of hyperlipemia-hyperglycemia has been associated with the highest contractility of the resistance arteries to prostaglandin F2a and the highest circulating glucose and cholesterol levels; the activation of PKC pathway, the alteration of cyclooxigenase and the Ca^{2+} dependent K^+ channels generate the augmented contractility [64].

8.2.3.3 Micro RNA and Diabetic Vascular Disease

MicroRNAs (miRNAs) are a newly identified class of small non-coding ribonucleic acids (RNAs); they regulate gene expression at the post-transcriptional level. Alterations in miRNA expression occurring in T2DM play an important role in hyperglicemia-induced vascular damage pathogenesis [65]. Thus, in endothelial cells exposed to hyperglicemia, miR-320, miR-221, miR-503 are highly expressed, while miR-222 and miR-126 are submitted to down-regulation. The alterations in miRNA expression lead to decreased angiogenesis, generation of AGEs, decreased EPC proliferation, migration and homing, endothelial dysfunction and impaired vascular repair [45].

There is evidence that suggest that reduced miR-126 expression levels are partially responsible for impaired vascular repair capacities in diabetes; in contrast, restored expression of this miRNA promotes EPCs-related repair capacities and inhibits apoptosis [66].

8.2.3.4 Thrombosis and Coagulation

Both diabetes and insulin resistance are associated with a prothrombotic status, as a result of the alterations in clotting factors and platelet aggregation [67]. The most frequent alterations consist of: increased PAI-1 and fibrinogen, reduced tPA levels, increased expression of tissue factor (TF) with procoagulant activity and thrombin generation, platelet hyperreactivity, up-regulation of glycoproteins Ib and IIb/IIIa, increased levels of microparticles (MPs) released in the circulation [45]. Platelet hyperactivity and hyperaggregability in T2DM is induced by several factors including oxidative stress, abnormal intracellular Ca^{2+} homeostasis and hyperhomocysteinaemia. It has been showed that the endogenous production of ROS, Ca^{2+} mobilization and platelet aggregation are significantly greater in platelets from diabetic patients than in controls, even though they have been exposed to the same concentrations of homocysteine (Hcy), indicating that platelets from diabetic donors are more sensitive to plasma Hcy levels [68]. Besides, the exogenous oxidative stress, thrombin activation, and ageing lead to protein carbonyl formation in platelets from diabetic patients [69]. Moreover, it has been shown that MPs from patients with T2DM increase coagulation activity in endothelial cells. MPs carrying TF promote thrombus formation at the sites of injury, representing a novel and additional mechanism of coronary thrombosis in diabetes [70]. On the other hand, it has been reported that enoxaparin – a low molecular weight heparin, restores the altered vascular reactivity of resistance arteries in aged and aged-diabetic hamsters [71]. The author concludes that, these pharmacological effects supplement the anticoagulant properties of enoxaparin and may be of relevance for improving perfusion/circulation in the microvasculature of aged and of aged–diabetic persons [71].

8.2.3.5 Vascular Hyperglycemic Memory

The "hyperglycemic memory" concept derived from large observational studies, where adequate control of patients' glucose blood levels acquired years after disease onset, failed to result in a lower cardiovascular risk [72]. However, in patients with early-onset therapy, well-established benefits were obtained [73]. The persistence of hyperglycemic stress despite blood glucose normalization has been defined as "hyperglycemic memory" [45]. Transitory episodes of hyperglycemia activate NF-kB, with a lingering effect even after blood glucose level become optimal. Hyperglycemia induces endothelial dysfunction, vascular inflammation and apoptosis through Sirtuin 1 (SIRT1) downregulation, p53 and $p66^{Shc}$ activation, PKCβII activation, inhibition of eNOS activity, expression of inflammatory genes and mitochondrial ROS accumulation, thus perpetuating a vicious circle that maintains the vascular lesional status in patients with diabetes despite optimal glycemic control [74].

8.3 Endothelial Progenitor Cell Biology

8.3.1 Definition of Endothelial Progenitor Cells

EPCs are a heterogeneous population of cells that reside in the bone marrow (BM) in close association with hematopoietic stem cells (HSCs) and the stroma [75]. These cells can be found (circulate) in the peripheral and umbilical cord blood and have been first isolated using magnetic micro beads by Asahara et al. (1997) [76]. EPCs represent between 1 and 5% of the total BM cells and less than 0.0001–0.01% of peripheral circulating mononuclear cells [77]. EPCs are involved in the maintenance of endothelial regeneration, vascular repair and in angiogenesis processes [78].

8.3.2 Ontogeny of Endothelial Progenitor Cells

In circulation two categories of EPCs can be found: a population with hematopoietic origin, and another population named non-hematopoietic EPCs [79]. It is well known that hematopoietic EPCs arise from a progenitor cell of mesodermal origin, defined as hemangioblast [76, 80, 81]. This cell type is rare, slowly proliferating and is described as a precursor for hematopoietic cells (myeloid and lymphocytic lineages), and also for a part of EPCs [82, 83]. The angioblast (immature stage of EPCs) and primitive HSCs present common hematopoietic stem cell markers as: CD133, CD34, CD45 or Flk-1/KDR [80, 84–87] (Fig. 8.2). During the differentiation process the angioblasts start to express new cell surface markers (CD) and become primitive EPCs, an immature population of cells (Fig. 8.2). Some markers

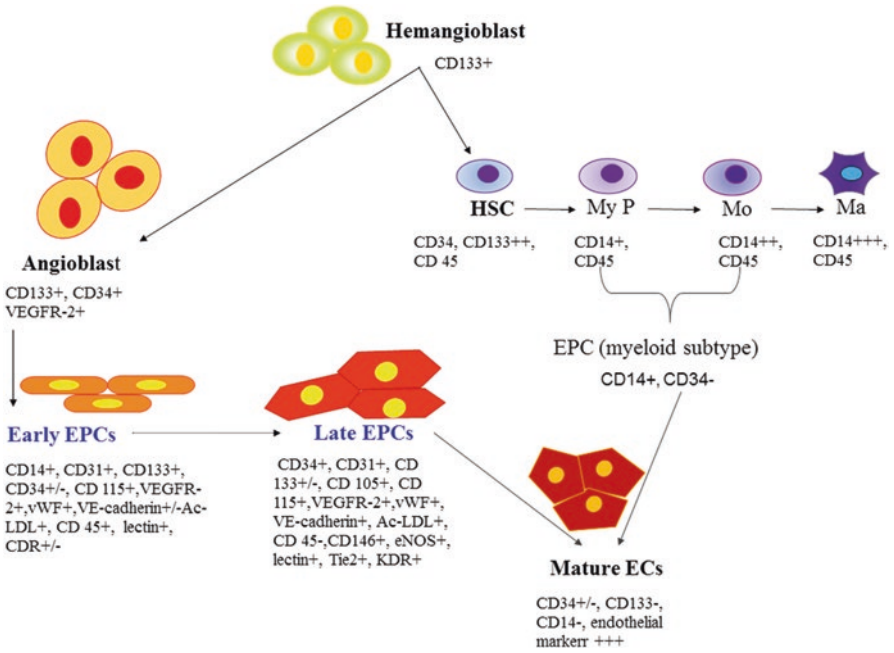

Fig. 8.2 The origin and differentiation of EPCs from hematopoietic and non hematopoietic stem cells: the profile of cell surface markers (+: positive cells, –: negative cells)

(CD14 and CD45) are common with myeloid lineage suggesting the hematopoietic origin of these EPCs [88]. In BM or in circulation, the hematopoietic EPCs begin to express specific markers for endothelial cells (ECs): vascular endothelial growth factor receptor 2 (VEGFR-2) and von Willebrand factor (vWf), in addition to CD133 and CD34 [80, 82, 85]. Regarding CD133, its expression is downregulated in non-hematopoietic cells and absent in mature ECs [85, 89, 90].

In vitro, **hematopoietic EPCs** generate the endothelial cell colony forming units (CFU-ECs) [91] with spindle shape and low proliferative capacity, named also "early endothelial colonies" or **early EPCs** (Table 8.1). These cells are able to incorporate acetylated LDL (AcLDL) and to bind specific lectins (BS-1 and ulex europaeus) which are usually considered endothelial specific [92, 93]. They are also characterized by the expression of vWf, VEGFR-2 and CD31 [88, 94]. However, early EPCs do not generate vascular tubes *in vitro*, but they can induce the angiogenesis indirectly by producing angiogenic factors and inflammatory cytokines/chemokines permitting new vessels to form and to extend [95–99]) (Table 8.1).

Another EPC subtype is known as **non-hematopoietic EPCs** or **late EPCs** or outgrowth endothelial cells (**OECs**), because in the culture they generate the endothelial colony forming cell (ECFC) that develop into monolayers with a typical "cobblestone" morphology [79, 85]. OECs have a higher proliferative potential and they easily form tube-like structures *in vitro* [79]. These cells are present in

Table 8.1 Differentiation of early compared with late EPCs (as two types of culture period-dependent cells)

	Early EPCs or hematopoietic EPCs	Ref.	Late EPCs or non-hematopoietic EPCs or (EOCs)	Ref.
In vitro features	Grown on fibronectin-coated surfaces	[76, 91, 98, 101]	Grown on collagen type I-coated surfaces	[97, 102]
	Appear in 3–5 days in culture		Appear after 2–3 weeks in culture	
	Are round cells surrounded by spindle-shaped cells		Are elongated cells in culture (3–5 weeks), and form a cobblestone-shaped monolayer	
	Proliferate slowly with a peak growth in culture at 2–3 weeks		Have a great proliferative potential	
			Can be cultured until 15 passages	
Angiogenic potential	Do not generate vascular tubes *in vitro*	[96, 97]	Generate tube-like structure *in vitro*	[79, 103]
	Secret angiogenic factors and induce angiogenesis by paracrine mechanism		Have vasculogenic and angiogenic potential, processes underlying the generation of new blood vessels	
			Form vascular networks de novo	
Endothelial properties	VEGFR-2, CD31, vWf, ability to bind AcLDL and lectins	[92, 93]	VEGFR-2, CD31, CD105, CD144, vWf, CD34, eNOS, Tie-2, VE-cadherin, ability to bind AcLDL and lectins	[100, 103]
Role and function	High cytokine release	[104, 105]	Low cytokine release	[104, 106]
	Phagocytic function		Incorporation and tube-forming capability	
			No phagocytic function	

peripheral and cord-blood and non-hematopoietic tissues [79]. Late EPCs do not express hematopoietic marker CD45 or the monocyte markers CD14 and CD115, but they express many EC antigens CD31, CD105, CD144, CD146, vWF, KDR, and UEA-1 [100] (Table 8.1 and Fig. 8.2). It has been also observed that, *in vivo,* these cells continue to differentiate and incorporate into the endothelium, and the expression of CD31 and vWF increases [91].

8.4 Endothelial Progenitor Cell Dysfunction, a Link Between Diabetes and Vascular Disruption

Cardiovascular risk factors induce endothelial injury. The occurring damages represent a balance between the degree of injury and the capacity of various complex mechanisms of repairing it. Diabetes mellitus is considered to be a clinical condition characterized by early and extended endothelial dysfunction. Hyperglycemia impairs vascular endothelial function and contributes to the vascular damage in diabetic patients [107]. Current studies suggest there is a negative correlation between the severity of diabetes and EPC count and function [108].

The complex pathophysiology of vascular damage in diabetes is not fully comprehended. Oxidative stress plays a crucial role in the pathogenesis of late diabetic complications. EPC dysfunction in diabetic patients has been correlated to oxidative stress and the generation of ROS [109]. Reduced extracellular superoxide dismutase (SOD) activity, the major antioxidant enzyme system of the vessel wall, has been associated with increased vascular oxidative stress and has been implicated in the endothelial dysfunction. In patients with CAD, SOD activity was substantially reduced [110].

NO is a biologically active unstable radical that is synthesized in vascular endothelial cells by eNOS. EPC mobilization from bone marrow to the peripheral blood and function requires NO [111]. Endothelial dysfunction is characterized by low biovailability of endothelium-derived NO, witch is itself an independent predictor of future cardiovascular events.

Chen et al. [112] have reported that prolonged exposure of early or late EPCs to high glucose concentrations reduces their number and proliferative ability, NO bioavailability, and the extent of phosphorylation of eNOS [112]. Exposure of EPC to high glucose concentrations has increased NADPH oxidase activity which results in increased O2- generation and reduced NO bioavailability because O2- inactivates NO and uncouples eNOS [113]. Therefore, decreased NO bioavailability is one of the determinants of vascular damage in diabetes.

On the other hand, ischemia induces neovascularization in diabetic patients. The oxygen deficit is considered the strongest stimulus for EPC mobilization from the bone marrow, through the up regulation of VEGF. It seems that EPC recruitment in regenerating tissues is mediated by a hypoxic gradient by Hypoxic Inducible Factor -1 (HIF-1) [114]. The expression of angiogenic factors, VEGF and HIF-1, has been reduced in the hearts of diabetic patients during acute coronary syndromes (ACS). In rats, myocardial infarct size has increased in hyperglycemic conditions and has been associated with a reduced expression of the HIF-1 gene [115]. Lambiase et al. (2004) have shown that modest coronary collateral vessels development, which is typical for diabetes, may be related to low levels of circulating EPCs [116]. Diabetic EPCs have not been able to stimulate vascularization, even becoming anti-angiogenic. Gill et al. [117] have reported that coronary artery bypass grafting is followed by a marked increase in circulating EPCs that peaks at 6–12 h, resembling very closely to VEGF increase effects [117].

Nondiabetic patients with PAD alone and patients with uncomplicated diabetes have had similar EPC reduction versus control subjects [118]. Patients with diabetes and PAD have had a further significant decrease in circulating EPC levels, especially in the presence of ischemic foot lesions. EPC levels have been strongly correlated with the ankle-brachial index, the most objective diagnostic and prognostic test for lower-extremity arterial disease.

In addition, hyperglycemia induces retinal ischemia and the release of angiogenic factors that stimulate the proliferation of microvessels, leading to proliferative retinopathy. EPCs may be involved in the development of proliferative retinopathy. This is a paradox as, in diabetic patients, the vascular ischemia may coexist with a condition of pathological neovascularization. Interestingly, the pericyte loss is an early and selective event leading to endothelial activation and proliferation in the retina, and $CD34^+$ progenitors of perivascular cells have been demonstrated in peripheral blood [119]. Thus, depletion of generic $CD34^+$ progenitor cells may be one cause of pericyte loss.

Another possible link between diabetes and EPC alterations is the effect of insulin resistance per se. It has been demonstrated that patients with metabolic syndrome have decreased levels of $CD34^+KDR^+$EPCs compared with patients without the syndrome [120].

Given the EPC effects revealed by ongoing clinical studies we may consider new pathways of understanding and treatment of diabetic complications.

8.5 Mechanisms Leading to Endothelial Progenitor Cell Dysfunction in Diabetes and Diabetic Vascular Complications

EPCs from humans and animals with T2DM have multiple functional defects *in vitro*, with biologically relevance *in vivo*, including decreased migration to chemotactic stimuli, reduced proliferative potential and differentiation, diminished ability to form vascular-like structures, which limit their regenerative capacity [121, 122].

In the following sections, we highlight the putative mechanisms by which metabolic features of diabetes impair EPC functions.

8.5.1 Effect of Hyperglycaemia

The abnormalities of glucose regulation are associated with changes in EPC biology, including reduced circulating EPC numbers, incorrect mobilization from bone marrow, decreased functional properties, lowered capacity to mediate endothelial repair, and altered differentiation propensity. These alterations of EPCs reduce their potential to generate vascular regenerative cells favouring the development of proinflammatory cells [123–125].

It has been shown that in both patients with T2DM or pre-diabetic states (meaning impaired fasting glucose and reduced glucose tolerance) and animal models of diabetes, the function and number of circulating EPCs are decreased compared to normoglycemic conditions and these are correlated with disease severity [118, 125–130]. EPCs have been negatively associated with glucose levels after a glucose challenge, in individuals with impaired glucose tolerance [131], and also with serum glucose and glycated haemoglobin A1c levels, in patients with T2DM [132].

The mechanisms by which hyperglycaemia influences EPC function involve the formation of AGEs and oxidative stress with augmentation of ROS production through the activation of NAPDH oxidase in mitochondrion, with role in EPC apoptosis [133]. Increased ROS generation could also stimulate the AGE production, which further triggers ROS production. These activate nuclear factor-kappa B (NF-B) and subsequently the target genes that encode inflammatory proteins inducing interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α). In parallel, NF-κB transcription factor activates p53 accelerating cell senescence and inducible NOS (iNOS) that further potentiates the ROS production [134–136]. Hyperglycaemia causes also endoplasmic reticulum (ER) stress and excessive autophagy which further facilitate EPC death and reduce their migration [137, 138]. Apart from that, high glucose concentrations influence the proliferative capacity of EPCs either via inhibition of Akt phosphorylation followed by NOS activation or via activation of C-JunN-terminal kinase (JNK) pathway [139–141]. It has been demonstrated that the exposure to high levels of glucose, *in vitro,* induces decreased early and late EPC number and activity by downregulation of eNOS expression and phosphorylation, suggesting that eNOS is an important target for high glucose adverse effects [112]. However, it is still unclear whether high glucose-associated eNOS damage causes oxidative stress or if oxidative stress associated with high glucose causes eNOS deactivation [142]. Hamed et al. (2009) showed that in patients with T2DM an inverse relationship between plasma glucose and reduced NO bioavailability in EPCs can be found, due to enhanced oxidative stress which damages the protein signaling pathways that lead to diminished NO generation [143]. The relationship between the NO signaling pathway and EPC dysfunction will be discussed in detail below. High glucose levels also induce EPC senescence by one of NF-κB target genes, p53, and by the activation of the p38 mitogen-activated protein kinase (MAPK) pathway [144] (Fig. 8.3).

A very recent study has shown that the main factors (AGE, oxidative stress) for EPC apoptosis and dysfunction induced by hyperglycaemia are also potent inducers for epigenetic changes in EPCs [145]. For example, ROS has been associated with a series of histone changes in the promoter and enhancer of superoxide dismutase (SOD) 2 gene in retinal endothelial cells isolated from diabetic rats with retinopathy [146]. In human microvascular endothelial cells, hyperglycaemia has induced the increase of H3K4mel expression and decreases of H3K9me2 and H3K9me3 levels on the of NF-κB promoter leading to NF-κB activation [147]. Moreover, the histone codes H3K9ac, H3K12ac, H3K4me2, and H3K4me3 suppress the eNOS transcription conducting to decreased NO [148].

Taken together, these studies demonstrate the obvious and complex influence of hyperglycaemia on impaired EPC levels and function.

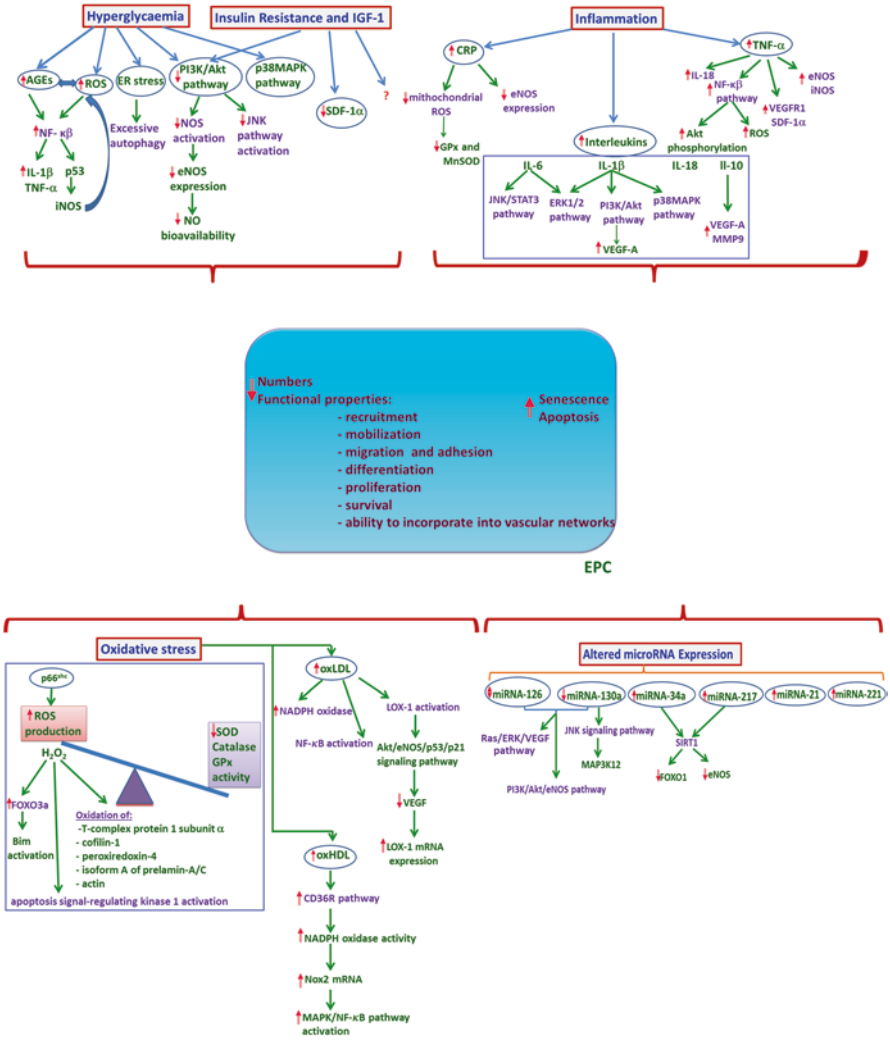

Fig. 8.3 The mechanisms by which diabetic hallmarks induce EPC dysfunction

8.5.2 Effect of Insulin Resistance and Insulin Like Growth Factor 1

Insulin resistance, a key feature of T2DM and the metabolic syndrome, results in a variety of metabolic and vascular phenomena such as dyslipidaemia, inflammation and a pro-thrombotic tendency, which eventually promote the development of atherosclerosis. Insulin resistance has been correlated with impaired downstream insulin signal transduction that reduces the glucose uptake in metabolic tissues [124].

The homoeostasis model assessment (HOMA) of insulin resistance (a method used to quantify insulin resistance and beta-cell function), has been found to be negatively correlated with EPCs, in patients with cardiovascular risk [120]. In addition, it has been shown that healthy men of South Asian descent, that are more insulin resistant than Caucasian peoples, present a reduced EPCs number and function [149]. Also, in insulin receptor (IR)-null mice, the number of circulating EPCs has been decreased [150]. Flow cytometric and cell culture analyses have revealed lower levels of circulating EPCs across the spectrum of insulin-resistant states [124]. Furthermore, the treatment with an insulin sensitizer, metformin, or thiazolidinediones, such as rosiglitazone, restored circulating EPC levels in diabetes [151–153]. The reduction of circulating EPC levels could be the result of a number of factors, such as defective mobilization, diminished proliferation and shortened survival into the circulation [94, 154].

However, the direct effect of insulin on the mobilization and differentiation of EPCs remains underexplored [155]. On this line, it has been shown that insulin resistance is closely associated with abnormalities in NO bioavailability and PI3K/Akt signaling, both playing an essential role in EPC mobilization from the bone marrow [94, 156–159]. Furthermore, in diabetic patients, EPCs have reduced clonogenicity and uncoupled eNOS mediated by ROS, which additionally contribute to augmented oxidative stress and impaired vascular repair [113] (Fig. 8.3). In one study on patients with poorly controlled T2DM, insulin significantly enhanced EPC mobilizationin subjects with the stromal cell-derived factor 1 (SDF-1)-3′-A/G allele, a polymorphism known to be correlated with increased EPC mobilization, suggesting that this peptide plays a role in this EPC function [160, 161].

The mechanism by which insulin stimulates the *in vitro* outgrowth of EPCs from patients with T2DM involved the insulin-like growth factor (IGF-I) receptor, the stimulation of MAPKs and extracellular-signal-regulated kinase (ERK1/2) signaling pathways, but not IR [162]. IGF-I has complementary activity to insulin, and low IGF-I levels are recognized as an independent risk factors for CVD [163]. Treatment with growth hormone, which increases circulating IGF-I levels induced, in middle-aged humans, both the enhancement of circulating EPC levels and their incorporation into tube-like structures, and eNOS expression followed by the improvement of EPC colony forming and migratory capacity [157]. *In vitro*, IGF-I stimulates via the IGF-I receptor the EPC differentiation, migratory capacity and ability to incorporate into vascular networks [157]. Furthermore, it has been demonstrated that haploinsufficiency of the IGF1-receptor increases endothelial repair and favorably modifies the angiogenic progenitor cell phenotype. This angiogenic trait accelerated the endothelial regeneration *in vivo*, and increased the tube formation ability and adhesion potential of progenitor cells *in vitro*, and in general enhanced vascular repair [164]. It should be noted that a study has shown that IGF-I increases the eNOS expression, phosphorylation and activity in a PI3K/Akt-dependent manner in EPCs [157] (Fig. 8.3).

The heterozygous mouse models for IR knockout (IRKO), although non-diabetic, have revealed the presence of endothelial dysfunction and reduced EPC number and function. The descendants of IRKO mice crossed with transgenic mice with Tie-2-driven human IR expression in endothelial cells (HIRECO), have presented restored insulin signaling in endothelial cells through IR, and improved blood pres-

sure, endothelial function, NO bioavailability, and vascular repair in the setting of global IR. This has not been related to glucoregulation or EPC abundance [165]. Insulin resistant, non-diabetic hemizygous mice for IRKO have presented a lower number of circulating EPCs in peripheral blood, but not in bone marrow and decreased EPC mobilization compared to wild-type mice [150]. Moreover, in IRKO mice, after arterial injury, the endothelial regeneration was delayed, but it has been restored after the transfusion of mononuclear cells or c-kit+bone marrow cells from wild-type mice [150].

All these studies demonstrate that both insulin and IGF-I significantly influence the EPC function, but more investigations are needed to understand their mode of action.

8.5.3 Nitric Oxide as a Key Factor of Endothelial Progenitor Cell Dysfunction

NO, a biologically active unstable free radical is synthesized from L-arginine in vascular endothelial cells by eNOS, an enzyme which is constitutively expressed in these cells. NO bioavailability depends on the balance between the rate of its generation and its inactivation, particularly by ROS [166, 167]. Moreover, NO and eNOS play an important role in mobilization of EPCs from bone marrow stem cell niches to the peripheral circulation [11, 168, 169]. NO bioavailability in sites of active vascularization seems to be crucial for EPC biology and function. The administration of endogenous NOS inhibitors, such as asymmetric dimethylarginine (ADMA), induces decreased EPC mobilization, differentiation, and proliferation in patients with CVD, suggesting the essential role of this enzyme in EPC function regulation [170].

Impaired NO bioavailability, the hallmark of endothelial dysfunction, is one of the contributing factors to the vascular damage in T2DM. NO bioavailability may be diminished either due to the lower overall systemic fraction of L-arginine that is converted to NO, or due to the reduction of essential eNOS cofactor and (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4) [171, 172]. Reduced NO concentration contributes to defective migratory activity in diabetic EPCs. It has been demonstrated that EPCs isolated from diabetic patients have an impaired migration to stimulation with SDF-1 due to defective cell deformability, and the NO treatment improves deformability and normalizes the migration of these diabetic cells [173]. The EPC dysfunction in T2DM has been reported to be restored through NO-dependent mechanisms by various ways: (i) treatment with a NO donor drug which normalized their migration [173]; (ii) treating wounds with SDF-1α which reestablished their homing [140]; (iii) inactivation of NADPH oxidase which improved their reendothelialization capacity, *in vivo* [174]; (iv) preservation of the NO bioavailability with SOD which restored EPC proliferation [169]. Furthermore, since it has been demonstrated that prostacyclin (PGI_2), an vasorelaxant prostanoid, has a direct effect on EPC functions and number in an autocrine or paracrine manner through an NO-

dependent mechanism [175–177], it has been considered that PGI_2 may have a substantial therapeutic role in diabetes as well [142].

8.5.4 PI3K/Akt Signaling Pathway and Endothelial Progenitor Cell Dysfunction

The phosphatidylinositol triphosphate kinase/protein kinase B (PI3K/Akt) pathway has been suggested to be involved in the regulation of EPC recruitment, mobilization, and proliferation [178]. Well-known activators of the PI3K/Akt pathway such as hydroxymethylglutaryl-coenzyme A reductase inhibitors (statins), erythropoietin, estrogens, and VEGF, are able to increase circulating EPC levels, proliferation and migration [156, 179]. Pharmacological inhibition of PI3K and the overexpression of a dominant-negative Akt construct have been shown to abolish EPC proliferation and differentiation induced by statin and VEGF, *in vitro* and *in vivo* [156]. Moreover, Akt is an upstream enzyme of the eNOS signaling pathway which, as we mentioned above, is essential for EPC mobilization. Thus, perturbations in the PI3K/Akt/eNOS/NO signaling pathway or in one of its members may result in EPC dysfunction [168].

8.5.5 Oxidative Stress Impairs the Function of Endothelial Progenitor Cells

Oxidative stress is defined as an imbalance between ROS production and antioxidant defences. ROS generation is promoted by the $p66^{shc}$, an adaptor protein [180, 181], while the antioxidant protection is provided by catalase, SOD, and glutathione peroxidase (GPx), which scavenge the excess of oxygen-free radicals and reduce ROS action. Previous reports have shown that the oxidative stress has a pivotal damaging effect on EPC functions [155, 182]. Thus, enhanced superoxide generation reduces the EPC levels and impairs EPC function [113]. Dysregulations of $p66^{shc}$ expression and SOD activity have been detected in AGEs-stimulated late EPCs, changes that are mediated by high mobility group box-1 (HMGB-1), a nonchromosomal nuclear protein [183, 184]. However, in the early-stage of diabetic EPCs, increased levels of ROS are not found, owing to the enhanced expression of antioxidant enzymes such as catalase [185].

Additionally to the indirect effects of ROS on EPCs it has been suggested that ROS exert direct effects on EPCs. Hydrogen peroxide (H_2O_2) induces in EPCs the increase of Forkhead box O3 (FOXO3a) protein expression, in a dose-dependent manner, and thereafter the activation of pro-apoptotic protein, Bim, that leads to the following effects: decreased viability, increased apoptosis, and the impairment of tube formation [186]. Also, H_2O_2 stimulates EPC apoptosis by the activation of apoptosis signal-regulating kinase 1 (ASK1), due to the oxidation of sulfhydryl groups of multiple anti-oxidant proteins such as glutaredoxin and thioredoxin [187,

188]. Moreover, H_2O_2 produces the oxidation of important EPC proteins such as the T-complex protein 1 subunit α, cofilin-1, peroxiredoxin-4, isoform A of prelamin--A/C, and actin [189].

Under diabetic conditions, enhanced oxidative stress induces the excessive generation of oxidized low density lipoprotein (oxLDL) [142]. It has been shown that the oxLDL reduces the number of viable EPCs in culture and induces the dysfunction of cultured EPCs isolated from healthy subjects [190–192]. These effects are mediated by NADPH oxidase, NF-κB activation, or LOX-1 activation that subsequently inhibit the Akt/eNOS pathway [190, 192]. In T2DM patients, elevated levels of circulating oxLDL contribute to cardiovascular symptoms [193]. OxLDL accelerates the EPC senescence by the activation of the Akt/p53/p21 signaling pathway [144, 190] and inhibits VEGF-mediated differentiation via LOX-1 receptors, increasing the LOX-1 mRNA expression [194].

High-density lipoproteins (HDL), particles with antioxidant and anti-inflammatory properties, have a positive impact on EPC physiology [195, 196]. In T2DM patients, the HDL particles are dysfunctional, and the serum levels of oxidized HDL (oxHDL) and myeloperoxidase (MPO) enzyme have been found to be elevated as well [197, 198]. The administration of reconstituted HDL to T2DM patients has improved circulating EPC functions [199], while the treatment with HDL of cultured EPCs has induced the intensifications in their proliferation, migration, adhesion, and tube formation and also protected them from apoptosis [200]. In addition, HDL protects EPCs from the deleterious effects of ox-LDL. On the other hand, high concentrations of HDL (>400 μg/ml) seem to induce EPC senescence and to decrease their tube formation ability via the activation of Rho kinase that inhibits the Akt and p38 MAPK signaling pathways [201]. Conversely, ox-HDL stimulates EPC apoptosis in a dose-dependent manner, via the CD36 pathway. Interaction of ox-HDL with CD36 also enhances the NADPH oxidase activity, upregulates Nox2 mRNA (NADPH oxidase subunit), and activates the MAPK/NF-κB pathway [202].

Other data have revealed that ROS induce the impairment of EPC function in diabetes, but the mechanisms that explain this phenomenon have not yet been studied by these authors [155, 203]. One of the mechanisms of diabetes-induced oxidative stress action has been recently investigated by Wu et al. (2016). This study has indicated that HMGB-1 has a significantly involvement via a positive feedback loop including the AGE/ROS/HMGB-1 pathway [203].

Regarding the antioxidant protection, it has been shown that EPCs from healthy humans contain high intracellular expression levels of manganese SOD (MnSOD) [204, 205], while EPCs from T2DM patients have increased SOD activity that neutralizes the high levels of superoxide anions [142]. Moreover, it has been reported that the antioxidant therapy with SOD in diabetic mice has reduced oxidative stress and improved EPC levels and differentiation capacity [206]. The treatment with SOD of glucose-stressed EPCs has restored their proliferation through an NO-dependent mechanism suggesting that the interaction between NO and superoxide anions has an important role in the development of EPC dysfunction and subsequently in CVD development in T2DM patients [169]. The augmentation of SOD expression in human EPCs by shear stress can accelerate the neutralization of superoxide anions, preventing the peroxynitrite formation, and thus increasing NO bio-

availability in EPCs [207]. Likewise, the MnSOD overexpression effectively reversed the diabetic EPC dysfunction including tube formation, migration, while the transplantation with MnSOD-overexpressed diabetic EPCs improved *in vivo* wound healing ability [208]

8.5.6 Inflammation and Endothelial Progenitor Cells Dysfunction

Inflammation affects both EPC number and function, and EPCs react in two different ways to an inflammatory environment [208]: (1) at low concentrations of inflammatory cytokines, the number and function of EPCs are positively regulated, meaning that the increased number of circulating EPCs adheres and is recruited to the injured area; (2) at high concentrations of inflammatory cytokines, in a severe and chronic inflammatory environment such as diabetes, EPC functions (mobilization, adhesive capacity and proliferation) are impaired and the EPC number is reduced, leading to deficiency in angiogenesis. Subclinical inflammation has been shown to be a powerful predictor of cardiovascular events and T2DM [155]. In these conditions, the systemic inflammation is characterized by elevated levels of C-reactive protein (CRP), TNF-α, and many cytokines, such as interleukins (ILs): IL-1, IL-6, IL-10 and IL-18 [209, 210]. The interaction of these factors with different receptors results in the increase of oxidative stress and activation of NF-κB in EPCs, which lead to their dysfunction (Fig. 8.3).

CRP has been reported to have the following effects, mediated through receptors for AGE, on EPCs: (i) significantly disturbs migration, adhesion and proliferation; (ii) reduces eNOS expression, increases apoptosis and necrosis [211, 212]. In addition, CRP increases mitochondrial ROS production, modulating the expression of anti-oxidant enzymes, such as GPx and MnSOD [212]. There was no association found between plasma levels of CRP and EPCs [213]. Regarding the effect of ILs, it has been shown that IL-1β: (i) induces murine EPC viability, proliferation, and migration both *in vivo* and *in vitro*, via ERK1/2 pathway activation [214]; (ii) increases mRNA and protein levels of VEGF-A in EPCs, via the PI3K/Akt signaling pathway [215]; (iii) reduces the number and proliferation of pig EPCs, and also EPC migration, adhesion, and angiogenesis, through p38 MAPK pathway activation [216]. Also, IL-18 reduces the ability of EPCs from healthy individuals to differentiate into mature endothelial cells [217] while IL-6 increases EPC migration, proliferation, and differentiation in cell culture, by activating both the JNK/STAT3 pathway and the ERK1/2 pathway [218]. Moreover, IL-10 alone has no effect on EPC migration and differentiation, although it did augmente significantly the expressions of VEGF and matrix metallopeptidase-9 (MMP-9) and potentiated the negative effects of TNF-α on EPCs [219].

TNF-α serum levels are higher in diabetes and have been associated with various complications of this disease [220, 221]. It has been shown that TNF-α influences

the EPC function by different ways: (i) induces IL-18 expression that has negative effects on EPC differentiation; (ii) decreases Akt phosphorylation mediated by insulin and increases apoptosis through NF-κB pathway activation [222]; (iii) inhibits migration and proliferation in a dose and time-dependent manner; (iv) mediates downexpressions of VEGFR-1 and SDF-1 as well as of the iNOS and eNOS [223]. On the contrary, in another study it has been reported that TNF-α enhances EPC migration, adhesion, and tube formation [219].

Regarding the effect of NF-κB, it has been indicated that its overexpression: (i) improves EPC adherence to the endothelium by increasing the expressions of E-selectin and P-selectin glycoprotein ligand-1 [224]; (ii) does not impair the migration or vasculogenesis, in murine embryonic EPCs. In addition, simultaneous stimulation with TNF-α and NF-κB of EPCs isolated from insulin resistant ZO rats induces apoptosis via caspase-3 [222]. The activation of NF-κB can mediate the damage induced by Benzo[a]pyrene, an environmental toxin, on EPCs by increasing ROS production, thus impairing their migration, proliferation, and vasculogenesis [225].

8.5.7 Altered Micro RNA Expression and Dysfunctionality of Endothelial Progenitor Cells

The small noncoding molecules, microRNAs (miRNAs), are key regulators of diverse cellular processes, and their expression reflects the disease pathology [226]. The miRNAs in the body fluid seem promising to be used as biomarkers to monitor diabetes onset, and their number has been found to play a significant physiological role in tissues where diabetes complications occur.

Regarding the involvement of miRNAs in diabetic EPC dysfunctions, there are several data sustaining this aspect. For example, it has been shown that in T2DM, the miRNA-126 expression has been downregulated in EPCs, inhibited EPC proliferation/migration ability, and induced apoptosis, leading to diabetes-mediated CVD [227]. The altered expressions of miRNA-126 as well as of miRNA-130a have been involved in EPC dysfunction through extracellular signal–regulated kinase, Ras/ERK/VEGF, and the PI3K/Akt/eNOS signaling pathway [227, 228]. In addition, dysregulated miR-130a has impaired EPC function by directly targeting MAP3K12, a newly identified target gene of the JNK signaling pathway [141]. Alternatively, in T1DM patients the expression of miR-126 in EPCs has increased compared to control subjects [229]. In primary cultured EPCs from diabetic patients, an increased expression of miR-21 has been detected compared to that from control individuals, and it was suggested that elevated levels of muR21 protect EPCs from apoptosis via the regulation of downstream target DAXX [230]. Moreover, the overexpression of miR-34a in EPCs results in an increase in EPC senescence with impaired angiogenesis and SIRT1 expression [231] (Fig. 8.3). Also, augmented

levels of miR-34a and miR-217 have induced the downregulation of some important targets of SIRT1, such as FOXO1 and eNOS, thereby leading to premature endothelial cell senescence and apoptosis [231, 232]. More recently, it was demonstrated that in T1DM patients with diabetic retinopathy, the miR-221 expression in EPCs has been significantly higher than in T1DM patients without diabetic retinopathy and control subjects [229]. Thus, it was hypothesized that when retinal damage is widespread with chronic hypoxia and nonperfusion, the EPCs would respond by increases of miR-221 expression and specific chemokines, a process not activated in earlier stages in noncomplicated diabetic patients.

The identification of miRNAs as diabetic biomarkers and pathogenic factors would not only contribute to the detection of early complications and progressive changes of diabetes, but also would provide targets for strategic therapeutic approaches in diabetes mellitus.

8.6 Significance of Endothelial Progenitor Cells in the Pathogenesis of Vascular Complications of Diabetes

Several studies have revealed the innate complex mechanisms underlying changes that occur in the vasculature during diabetes and lead to the cardiovascular risk associated with macrovascular and microvascular complications of diabetes [233]. It is well known that EPCs play an essential role in endothelial repair, angiogeneosis, neovascularization and attenuation of vascular dysfunction. Therefore, alterations in EPC number and functions are considered markers of cardiovascular risk in the general population and in diabetic patients, as well as a cause of diabetic vascular complications [120, 234, 235].

8.6.1 Endothelial Progenitor Cell Dysfunction and Macrovascular Complications in Diabetes

The linkage between diabetes mellitus and macrovascular disease has been very well established in many scientific studies [236]. It has been reported that diabetic patients have a two to fourfold increased risk of developing CAD and PAD compared with non-diabetic individuals [22]. Also, the severity of macrovascular complications in diabetes has been attributed to a profoundly impaired collateralization of vascular ischemic beds [237]. In addition, EPCs have been found to be involved into the mechanisms that delay ischemia-induced neovascularization in diabetes. In animal models of diabetic vasculopathy, it has been shown that diabetic EPCs are not able to promote vascularization, becoming antiangiogenic [238, 239], while the administration of EPCs from control animals has reduced defective

collateralization. Consequently, a referenced study has established that EPCs play an important role in the vascularization and also, in healing of diabetic wounds [240]. Additionally, it has been demonstrated that the EPC reduction in diabetes is strongly correlated with the severity of both carotid and lower-limb atherosclerosis, suggesting that EPC number can be a valuable marker of atherosclerotic involvement [115]. In agreement with these findings, other studies have indicated that the lower circulating EPC number reflects the evolution of atherosclerotic disease both in animal models [241–243] and in patients [244]. These papers have used for EPC analyzing and quantification the flow cytometry technique. Furthermore, it has been reported that the determination of EPC number, using flow cytometry, is sufficiently reproducible to be used in the clinical practice, providing additional information over the classical risk factor analysis. This EPC measuring reflects not only vascular function and atherosclerotic changes, but also the endogenous vasculoregenerative potential [120, 245, 246]. The $CD34^+$ KDR^+ EPC count has been showed to predict the cardiovascular events independently of risk factors and hard indexes, such as left ventricular ejection fraction [244, 245, 247].

These findings have indicated that both decreased levels and dysfunction of EPCs play a significant role in enhanced cardiovascular risk and diabetes-related complications.

8.6.1.1 Endothelial Progenitor Cells and Diabetic Coronary Artery Disease

It is well known that diabetic patients die from CVD, diabetes representing the major cause of death among this population and contributing to a shortening of average life span by 5–10 years in these patients [248]. Diabetes increases the risk of future MI more than any other risk factors, and the consequences of MI are greater in these patients compared to the patients without diabetes mellitus [236].

It has been shown that EPCs isolated from the peripheral blood (PB-EPCs) of subjects with cardiovascular risk factors and previously diagnosed *diabetic* CAD, have altered phenotypes [247, 249], while in patients with known CAD, these cells have exhibited a reduced migratory capacity and weak proliferative response [250]. Additionally, lower levels of EPCs have been found in patients with severe atherosclerosis or diabetes-related vasculopathy [251, 252], and it was concluded that the circulating EPC levels predict cardiovascular events in patients with CAD [245, 253].

Most importantly, due to the EPC heterogeneity and the variable changes in the EPC phenotype at different stages of CAD and diabetes development, there are some limitations in establishing the predictive value of the number and functionality of EPCs in cardiovascular risk calculation [233].

Moreover, modulating EPC levels in T2DM with known CAD using different drugs is still under study. Regarding this aspect, it was found that valsartan, an angiotensin-2 receptor blocker, in high doses, has a positive influence on bone marrow-derived EPCs phenotyped as $CD14^+$ $CD309^+$ and $CD14^+$ $CD309^+$ $Tie2^+$ in T2DM patients with known asymptomatic CAD [254]. Additionally, strong evi-

dence has been provided to support that statins (atorvastatin and pravastatin) have a favourable *in vitro* effect on functional parameters of EPCs derived from diabetic patients with acute ST segment elevation MI (STEMI) [248]. These data indicate that treatment with statins may be beneficial for EPC-driven vascular repair after an acute MI (AMI) and may improve the cardiovascular outcome of diabetic patients.

8.6.1.2 Endothelial Progenitor Cells and Diabetic Peripheral Arterial Disease

PAD is a common vascular complication in the diabetic population, diabetes increasing the risk of developing PAD at least two-fold [255, 256]. Patients suffering from both diabetes and PAD present poor lower extremity function and are at risk of developing critical limb ischaemia and ulceration, potentially requiring limb amputation [257, 258]. Moreover, these patients respond poorly to the treatment of PAD and exhibit a higher mortality [245, 246].

Regarding EPC involvement in this pathology, it has been shown that patients with PAD alone and patients with uncomplicated diabetes had similar EPC decrease versus control subjects, while patients with PAD and diabetes had a more significantly reduction in circulating EPC levels, mainly in the presence of ischemic foot lesions [115]. EPC levels are strongly correlated with the ankle brachial index, the most objective diagnostic and prognostic test for lower extremity arterial disease [118]. A recent study has demonstrated that ankle-brachial index is the determinant of EPC population state in disease-affected groups, and EPCs could predict the prevalence and severity of symptomatic PAD [259]. Moreover, EPCs isolated from diabetic patients with PAD have exhibited impaired proliferation and adhesion capacity to mature endothelium [260], while EPCs isolated from diabetic mice had suppressed EPC mobilization following hindlimb ischaemia [261–265]. In ischaemic tissue the existence of an inverse relationship was proven between diabetes duration and EPC number [266]. Furthermore, it has been reported that the administration of: (i) non-diabetic EPCs into diabetic hindlimbs, following ischaemia, have accelerated the blood flow restoration [238]; (ii) vitamin B1 analogue, benfotiamine or statins, have prevented the diabetes-induced reduction in circulating EPCs in mice subjected to limb ischaemia [265, 267]; (iii) insulin and G-CSF (granulocyte colony stimulating factor) have partially restored the deficient EPC mobilization in diabetic rats after ischaemia/reperfusion injury [268].

8.6.1.3 Endothelial Progenitor Cells and Diabetic Cerebrovascular Disease

In diabetic patients, ischemic cerebral damage is exacerbated, and the outcome is poor, but the responsible mechanisms are not well known. Likewise, there is less information regarding the correlation of circulating EPCs with cerebral vascular density (as an index of angiogenesis) and ischemic injury [269]. Information on

ischemic stroke in diabetic animal models is also lacking. In a study using *db/db* mice as a T2DM animal model for *in vivo* ischemic stroke it has been shown that impaired circulating EPC number, reduced EPC production/function, and increased generation of microparticles (MPs) might be the mechanisms responsible for increased ischemic damage [269]. Moreover, these data suggest that circulating EPCs and MPs could be used as predictive biomarkers for ischemic stroke complications in diabetes and might be thus targeted, offering new therapeutic possibilities for diabetes and ischemic stroke. In another study it has been reported that EPC transplantation alone had a modest effect on stroke recovery in diabetic mice in terms of angiogenesis, neurogenesis, axonal remodeling, and neurological behavior. These phenomena may be explained by the fact that only a small number of transplanted cells survived and successfully homed to the ischemic brain in these diabetic animals [270]. Recently, the same group has reported that EPC transplantation combined with p38 mitogen-activated protein kinase inhibitor administration into *db/db* diabetic mice, after ischemic stroke induction, have accelerated recovery, by increasing levels of proangiogenic and neurotrophic factors [271].

As a result, EPC dysfunction is perhaps a promising target for diabetes treatment strategies. Indeed, the improvement of EPC number and functionality seems to reduce cardiovascular risk and diabetes-related macrovascular complications, but the mechanisms underlying these outcomes are not fully clear, requiring more investigations.

8.6.2 Endothelial Progenitor Cell Dysfunction and Microvascular Complications in Diabetes

Patients with diabetes mellitus are at high risk for the development of microvascular complications and major adverse cardiovascular events. The EPC dysfunction related to the three manifestations of microvascular disease in diabetes: retinopathy, nephropathy, and neuropathy, will be discussed in further detail below.

8.6.2.1 Endothelial Progenitor Cells and Diabetic Retinopathy

Diabetic retinopathy represents an important cause of visual deficiency in the Western world [9]. In the United States this disease has been responsible for ~8% of cases of legal blindness and ~12% of all new cases of blindness in each year in the last decade of the twentieth century [236]. The majority of T1DM patients and more than 60% of patients with T2DM develop background retinopathy. The severity of hyperglycemia, duration of diabetes mellitus, insulin resistance and additionally, hypertension, dyslipidemia, inflammation and smoking are important factors that contribute to the development of microvascular disease [272, 273].

The role of EPC in the development of diabetic retinopathy is controversial [145]. EPC number has been reported as either decreased, increased or unchanged in diabetic patients with severe retinopathy when compared to diabetic patients with or without mild retinopathy, or to healthy subjects [229, 274–277]. Additionally, there are studies showing that in patients with nonproliferative diabetic retinopathy the circulating EPC number is reduced [127] compared to proliferative diabetic retinopathy patients which have increased EPC levels [278]. In T1DM and T2DM patients with diabetic retinopathy, it was found that although the EPC number is increased, their functions, such as migration, mobilization and homing, are often impaired [277, 279]. Intravitreal delivery of cartilage oligomeric matrix protein-angiopoietin 1 (COMP-Ang1) recovers the endothelial integrity and ameliorates the vascular leakage by promoting incorporation of endothelial colony-forming cells into retinal vasculature [280] in diabetic mice, and this way reverses diabetic retinopathy. Moreover, it has been demonstrated in culture studies that the early EPC (eEPCs) are responsible for 'provisional repair', first homing at the lesion and attracting the $CD34^+$ cells, and later on attracting late outgrowth endothelial progenitor cells (late EPCs) [281]. In nonproliferative diabetic retinopathy, eEPCs are dysfunctional and they can not recruit late EPCs into the retina to repair the acellular capillaries, while in proliferative diabetic retinopathy the eEPCs take a proinflammatory phenotype and recruit too many late EPCs leading to pathological neovascularization. Correcting these dysfunctions may allow the use of a diabetic patient's own EPCs to repair their injured retinal and systemic vasculature, in both the early and intermediate phase of vasodegeneration, to enhance vessel repair, reverse ischemia, and prevent progression to the late stages of diabetic retinopathy [281]. Thus, for durable repair and sustained correction of retinal ischemia the use of these expanded in vitro cells (eEPCs and late EPCs) has been proposed as being better than the use of the freshly isolated ones [282–284]. Nevertheless, more rigorous investigations are needed to solve this problem.

8.6.2.2 Endothelial Progenitor Cells and Diabetic Nephropathy

Diabetic nephropathy is found at a rate of ~7% of patients already diagnosed with T2DM. It occurs in less than 12% of patients with T1DM at 7 years after the diagnosis has been made, and in ~25% of patients with T2DM at 10 years after diagnosis [236]. Diabetic nephropathy is characterized in the early stages by hyperperfusion and hyperfiltration, due to the endothelial cell damage and abnormal angiogenesis, and in the late stages by the development of glomeruli fibrosis that results in renal failure. However, the exact mechanisms of nephropathy are not fully elucidated. At the present time, it has been reported that AGEs, oxidative stress, and the activation of the renin-angiotensin-aldosterone system (RAAS) are involved in these changes partially through the activation of TGF-1 signaling and increased VEGF expression in the kidney [285–287]. The negative correlation between EPC number and microalbuminuria or albumin excretion rate reported in both T1DM and T2DM patients, has suggested that EPCs have a protective effect in the structure and function of

glomeruli [179, 288]. The involvement of dysfunctional EPC has been described in both endothelial damage and microcirculatory impairment that occurs in the early pathogenetic events in diabetic nephropathy and also in defective glomerular repair and renal disease progression in diabetes [115]. Moreover, it has been suggested that EPCs, being pluripotent, have the ability to transdifferentiate into different phenotypes. Due to the kidney-derived hormone, erythropoietin, that has a major role in the regulation of EPC mobilization and differentiation, the relations between EPCs and renal function are more complicated [179]. In diabetes, the oxygen-erythropoietin feedback that depends on the hypoxia-sensing system, hypoxia-inducible factor 1-alpha (HIF-1α), is dysregulated. The erythropoietin response is affected by microangiopathy and progressive tubulointerstitial fibrosis which increase the latency of the erythropoietin system, and by ROS production and hyperglycemia which themselves stabilize HIF-1α [289]. It has been demonstrated that HIF-1α downregulation had a negative impact on EPC mobilization in diabetes [268]. Another factor that has complicated the relationship between EPCs and renal function is represented by ADMA. This endogenous NO inhibitor that is accumulated in patients with chronic kidney disease (CKD) [290] and diabetes [291], is also a potent inhibitor of EPC mobilization and function [170]. Thus, the disrupted erythropoietin system and an excess of ADMA in CKD seem to inhibit EPC mobilization, differentiation, and homing, while EPC alterations that occur in diabetes impair the renal microvasculature. Due to this vicious circle, diabetic nephropathy can be associated with a deficiency of EPCs rather than with CKD in general, which would represent an additional risk for CVD and death [268].

It has been recently suggested that for treating diabetic nephropathy the endothelial colony-forming cells (ECFCs) could be a promising and complimentary therapeutic target [145]. Another promising idea is to apply ECFC with higher level of NO or angiopoietin 1 (Ang1) that will be favorable for stabilizing capillaries by reversing ‘uncoupled VEGF with NO’ balancing ‘Ang1/Ang2 competition’ and ‘rendering Ang1/VEGF’. Alternatively, induced pluripotent stem cells (iPSC)-based ECFCs would be one of the major strategies for diabetic microvascular abnormality treatment. In this direction it has been disclosed that the endothelial progenitors generated from human iPSCs derived from cord blood have a greater capacity for homing and long term incorporation into injured retinal vessels [292, 293]. To improve endothelial function and protect vessel from retinopathy as well as nephropathy, ECFC administration has been proposed in the early stage of diabetes for better efficacy [145].

8.6.2.3 Endothelial Progenitor Cells and Diabetic Neuropathy

The development of diabetic neuropathy is associated with vascular and nonvascular abnormalities. The neuropathy is characterized by basement membrane thickening, pericyte loss, reduced capillary blood flow to C fibers, resulting in attenuated nerve perfusion and attendant endoneurial hypoxia, axonal thickening and eventual loss of neurons [294]. There are two major types of clinical manifestations: (1)

chronic, symmetrical, length-dependent sensorimotor polyneuropathy, that is associated with the severity and duration of hyperglycemia [295]; (2) asymmetrical polyneuropathies that develops at more unpredictable times during the development of diabetes [296].

In the experimental diabetic neuropathy, the reduction of vasa nervorum is an obvious characteristic of peripheral nerves, and decreased blood supply to peripheral nerves can accelerate disease progression [297]. It was hypothesized that EPCs may have a crucial role in the homeostasis of the nutritive microvasculature, their dysfunction contributing to the acceleration of disease. Due to the ability of these cells to differentiate also toward the neural phenotype [298], it is possible that the imbalance of immature circulating cells in diabetes influences this chronic complication, downregulating both endothelial and neuronal progenitors [268]. To support this hypothesis it has been reported that the EPC intramuscular administration can reverse the impairment of sciatic nerve conduction velocity and nerve blood flow in diabetic rats [299]. Chavez et al. (2005) have demonstrated that the EPC dysregulation in diabetic neuropathy may be attributed to a defective HIF-1α activation [300]. Other groups have shown that diabetic neuropathy can by delayed by the administration of some EPC-modulating agents, such as erythropoietin and statins [301]. Consequently, the EPC alterations have contributed to the pathogenesis of diabetic neuropathy, but future studies are needed to elucidate the involved mechanisms.

Taken together, these findings indicate that, although very important, the role of EPCs in the pathogenesis of diabetic microvascular diseases is still uncertain and future investigations are necessary to reveal the EPC mysterious nature for therapeutic applications.

8.7 Potential Therapeutic Implications of Endothelial Progenitor Cells in Diabetes-Associated Vascular Complications

8.7.1 Prognostic Value of Endothelial Progenitor Cells

In the recent years many studies have focused on an attempt to define the role of EPCs in identifying patients with increased cardiovascular risk. Clinical studies have demonstrated a correlation between the levels of circulating EPCs and the increasing cardiovascular risk profile [250, 302]. Thus, the adjuvant potential of EPCs as a cardiovascular risk biomarker has been proposed, based on the inverse link between EPC number, their migratory/proliferative potential and risk factors for CVD. Thereby, it has been demonstrated that the number of circulating EPCs and their migratory activity are reduced in the presence of classic cardiovascular risk factors such as smoking [94, 303–305], hypertension [306–308], hypercholesterolemia [250, 309], obesity [310, 311], T1DM and T2DM [115, 121, 128, 235] (Fig. 8.4). These effects could be possibly explained by three different mechanisms,

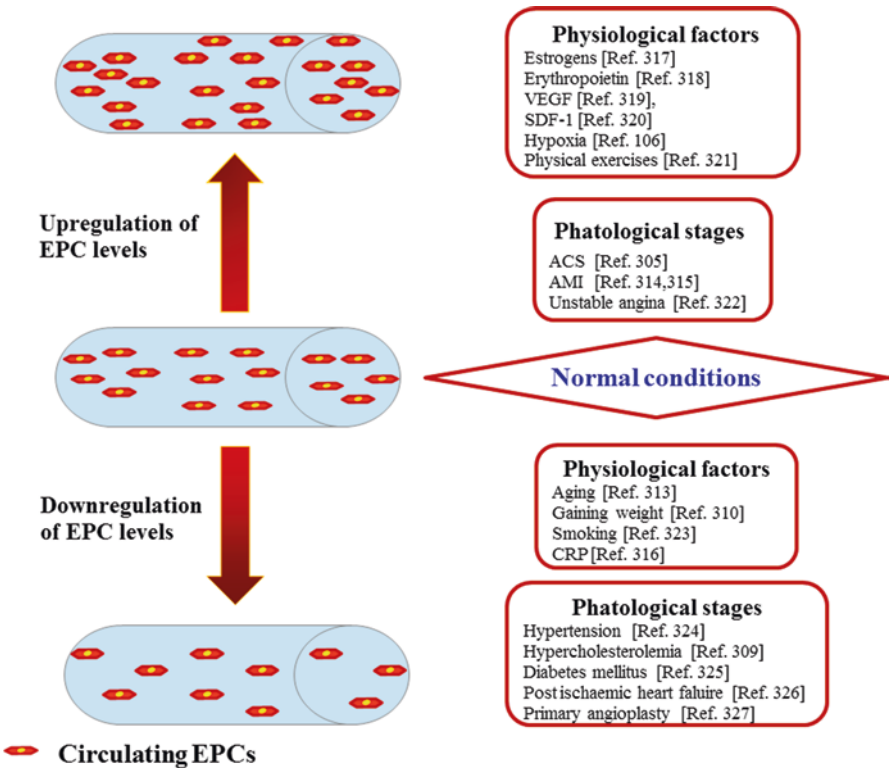

Fig. 8.4 The influence of physiological and pathological factors on EPC number and function

either separate or in combination: (a) an impaired mobilization of EPCs from the bone marrow, (b) an increased uptake of EPCs at sites of vascular injury to induce the endothelial repair; and (c) a decreased half-life of circulating EPCs by accelerated senescence and apoptosis of the remaining cells [94, 312]. In this way the reduction in mobilization, homing, and differentiation/survival of EPCs may limit their ability to repair injured tissues. The endothelial dysfunction and alteration have also determined the higher tissue request for EPCs and their increased turnover [305]. On the other hand, with ageing there is a decrease in the production of EPCs in BM [313].

In contrast, some pathologies such as ACS and acute myocardial infarction (AMI) cause hypoxia and vascular injury determining increased levels of inflammatory and hematopoietic cytokines, which induced a rapid mobilization of EPCs in the circulation [314, 315] (Fig. 8.4). Also, it is well known that physical exercises, hypoxia and some chemokines and growth factors (VEGF, SDF-1, angiogenin and colony-stimulating factor-CSF) increase EPC number and improve their function [106, 315, 316] (Fig. 8.4).

Table 8.2 Effect of drug therapy on EPC number and function

Medication	Response
Antihypertensive medication	
Angiotensin II receptor blockers	
Candesartan – [Ref. 328]	↑ EPC number in hypertensive patients
Telmisartan – [Ref. 329, 330]	↑ EPC proliferative activity *in vitro*; ↑ EPC number in normotensive patients with CAD
Irbesartan – [Ref. 241]	↑ EPC number in hypertensive-hypercholesterolemic animal model
Irbesartan – [Ref. 244]	↑ EPC number in patients with hypertension and dyslipidemia
Angiotensin converting enzyme inhibitors	
Ramipril – [Ref. 331]	↑ EPC number and EPC migration, proliferation, adhesion abilities in patients with stable CAD
Enalapril – [Ref. 332]	↑ EPC number in hypertensive patients
Zofenopril – [Ref. 332]	↑ EPC number in hypertensive patients
Calcium channel blockers	
Nifedipine – [Ref. 333]	↑ EPC number and function in stage I hypertensive patients
Barnidipine – [Ref. 334]	↑ EPC number in mild essential hypertension patients
Nitrates	
Nitroglycerin – [Ref. 335]	↑ EPC number *in vitro*
Cholesterol lowering medication	
Statins	
Atorvastatin – [Ref. 250, 336, 337]	↑ EPC number and migration in patients after cardiac surgery and in patients with ischemic cardiomyopathy
Rosuvastatin – [Ref. 338]	↑ EPC number in patients with chronic heart failure
Pravastatin – [Ref. 339]	↑ EPC number in patients with essential hypertension
Simvastatin – [Ref. 340]	↑ EPC adhesion *in vitro*
Valsartan – [Ref. 341]	↓ EPC senescence in chronic smokers
Rosiglitazone – [Ref.153]	↑ EPC number and migratory activity in patients with T2DM
Ramipril – [Ref. 342]	↑ EPC number and EPC proliferation, migration, adhesion, vasculogenesis capacity *in vitro*
Anti-diabetic medications	
Insulin – [Ref. 162]	↑ EPC number and clonogenic properties *in vitro*
Metformin – [Ref. 152]	↑ EPC number in patients with T2DM
Pioglitazone – [Ref. 343]	↑ EPC number in patients with T2DM
Metformin + Pioglitazone [Ref. 158, 344]	↑ EPC number and EPC migration in patients with T2DM and CAD

8.7.2 *Pharmacological Manipulation of Endothelial Progenitor Cells*

Besides their role as diagnostic and prognostic biomarkers, EPCs may be important targets in the CVD therapy. Thereby, many cardiovascular pharmacotherapies have

been used to improve the number and function of EPCs in patients with cardiovascular risk (Table 8.2).

8.8 Conclusions

In diabetes mellitus, the hyperglycemia has profound detrimental effects on the vascular endothelial cells, due to their anatomical location in the blood vessel, leading to the emergence of endothelial dysfunction. The vascular complications, particularly macrovascular (*coronary artery disease, peripheral arterial disease, cerebrovascular disease)* and microvascular *(retinopathy, nephropathy, neuropathy)*, are principal causes of disability and death in patients suffering from diabetes mellitus.

Accumulating data evoke that the mechanisms which are involved in the pathogenesis of vascular complications in diabetes have a well-defined role in the mobilization and function of EPCs. Thus, hyperglycaemia, insulin resistance, insulin like growth factor 1, nitric oxide, oxidative stress, PI3K/Akt signaling pathway, inflammation, and altered microRNA expression can contribute to decreasing of circulating EPC levels and to EPC dysfunctionality in diabetes. Many studies have shown that, in patients with diabetes and CVD, the number of EPCs from peripheral blood is reduced and EPC function is impaired. On the other hand, the alterations in EPC number and function may have a relevant role in the development of diabetes-related vascular complications.

A better understanding of the mechanisms leading to impairment of EPC mobilization and function in diabetes can further help in identifying the targets to prevent or reduce the risk of disease progression towards vascular complications.

It is currently hoped that addressing EPCs as targets for diagnostic and therapy in diabetes will favourably modify the risk for cardiovascular complications and survival. The drug therapy on EPC number and function can enhance the protection against vascular complications during diabetes. Therefore, EPCs could represent a diagnostic biomarker and pharmacological target to conduct the preventive or therapeutic interventions in diabetes. Nevertheless, further studies need to elucidate the exact role of EPCs in the pathogenesis of vascular complications in diabetes and their potential therapeutic implications.

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Chapter 9
Pathogenetic Mechanisms in Diabetic Retinopathy: From Molecules to Cells to Tissues

Saumik Biswas and Subrata Chakrabarti

Abstract Diabetic retinopathy is a debilitating ocular condition that occurs as a chronic microvascular complication of diabetes. The presence of distinct clinical features categorizes diabetic retinopathy into different severity stages (mild to very severe), where vision loss is eminent in the advanced stages of diabetic retinopathy. Further, each stage of diabetic retinopathy is associated with unique pathological features at the cellular level such as basement membrane thickening, pericyte and endothelial cell dysfunction/loss, breakdown of the blood-retinal barrier, retinal capillary non-perfusion, and retinal neovascularization. These cellular alterations are the end products of various biochemical and molecular pathway abnormalities: polyol pathway, protein kinase C activation, hexosamine pathway, advanced glycation end-products formation, retinal renin-angiotensin system, and neural-and-immuno-inflammatory mechanisms. Although there are several metabolic pathway alterations in a hyperglycemic environment, the heightened production of reactive oxygen species may interconnect the foregoing pathways. Nevertheless, recent advances in genetic technology have identified that a significant number of epigenetic alterations participate in the development and progression of diabetic retinopathy: DNA methylation, histone modifications, and non-coding RNAs. This chapter will first provide the reader with sufficient background on the clinical and pathological features of diabetic retinopathy and then provide significant insight into the current known pathogenic mechanisms implicated in the progression of diabetic retinopathy.

Keywords Diabetic retinopathy • Retinal microvascular abnormalities • Metabolic pathway abnormalities • Reactive oxygen species • Epigenetics • DNA methylation • Histone modifications • Non-coding RNAs

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Abbreviations

α-MSH	Alpha-melanocyte-stimulating hormone
ACE	Angiotensin-converting enzyme
AGE	Advanced glycation end-product
ANRIL	Antisense non-coding RNA in the *INK4* locus
AP-1	Activator protein-1
Ang-2	Angiopoietin-2
ATP	Adenosine triphosphate
BRB	Blood-retinal barrier
BM	Basement membrane
$–CH_3$	Methyl group
CH_3CO	Acetyl group
CNP	Capillary non-perfusion
COX-2	Cyclooxygenase-2
DAG	Diacylglycerol
DCCT-EDIC	Diabetes Control and Complications-Epidemiology of Diabetes Interventions and Complications Trial
DGCR8	DiGeorge syndrome critical region 8
Dll4	Delta-like ligand 4
DM	Diabetes mellitus
DME	Diabetic macular edema
DMNTs-	DNA methyltransferases
DNA	Deoxyribonucleic acid
DR	Diabetic retinopathy
EndMT	Endothelial-to-mesenchymal transition
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
ETC	Electron transport chain
EZH2	Enhancer of zeste homolog 2
$FADH_2$	Fully reduced form of flavin adenine dinucleotide
FasL	Fas Ligand
FN	Fibronectin
FOXO1	Forkhead box protein O1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAT	Glutamine:fructose-6-phosphate amidotransferase
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
HDMCs	Histone demethylases
HIF-1α	Hypoxia-inducible factor-1α
HMGB1	High-mobility group box-1
HMTs	Histone methyltransferases
HSP	Hexosamine pathway
IL-6	Interleukin-6

IL-1β	Interleukin-1β
iNOS	Inducible nitric oxide synthase
JAK-STAT	Janus kinase/signal transducers and activators of transcription
LSD1	Lysine-specific demethylase 1
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1
MCP-1	Monocyte chemoattractant protein-1
MIF	Macrophage migration inhibitory factor
NPDR	Non-proliferative diabetic retinopathy
PEDF	Pigment epithelium derived factor
PDR	Proliferative diabetic retinopathy
PDGF-BB-PDGFRß-	platelet-derived growth factor-BB-platelet-derived growth factor receptor subunit B pathway
PDGF-BB	platelet-derived growth factor-BB
PI3K	Phosphatidylinositol-3 kinase
MAPK	Mitogen-activated protein kinase
miRNAs	micro RNAs
MMP-9	Matrix metalloproteinase-9
mRNA	messenger RNA
NADH	Reduced form of nicotinamide adenine dinucleotide
NAPDH	Reduced form of nicotinamide adenine dinucleotide phosphate
NAD^+	Oxidized form of nicotinamide adenine dinucleotide
ncRNAs	Non-coding RNAs
sncRNAs	Small non-coding RNAs
lncRNAs	Long non-coding RNAs
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
NV	Neovascularization
PAI-1	Plasminogen activator inhibitor-1
PARP	Poly (ADP-ribose) polymerase
PGE2	Prostaglandin E2
PKC	Protein kinase C
PLGF	Placental growth factor
PRC2	Polycomb repressive complex 2
RAGE	Receptor for AGEs
RAS	Renin-angiotensin system
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNA pol II	RNA polymerase II
ROS	Reactive oxygen species
SAA3	Serum amyloid antigen three
SDF-1	Stromal derived growth factor
SHP-1	Src homology-2-domain-containing phosphatase-1

SIRT1	Sirtuin (silent mating type information regulation 2 homolog) 1
SOD2	Manganese superoxide dismutase gene
TCA	Tricarboxylic acid
TET	Ten-eleven translocase
TGF-β1	Transforming growth factor-beta1
TNF-α	Tumor necrosis factor- alpha
UKPDS	United Kingdom Prospective Diabetes Study
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau
VIP	Vasoactive intestinal peptide
3′ UTR	3′ untranslated regions

9.1 Introduction

With nearly 642 million people projected to live with diabetes in the year 2040, the risk for developing diabetes-related complications will drastically increase [1]. Diabetes mellitus (DM) is a chronic degenerative metabolic disease that is characterized by sustained hyperglycemia. Hyperglycemia correlates with a number of DM-related complications and is one of the preeminent factors for causing vascular damage in the human body [2–4]. The majority of diabetic complications can be viewed as either microvascular disease (small vascular injury) or macrovascular disease (large vessel injury) [4, 5]. Diabetic retinopathy (DR) remains the most prevalent chronic microvascular complication of DM [6, 7]. This debilitating ocular condition is also the leading cause of blindness in the working-age population in industrialized countries [7, 8]. The relationship between DR and diabetes has been reported in several studies with the majority of type 1 diabetic patients and over 60% of patients with type 2 DM developing evidence of DR within 20 years of diagnosis [9–14]. With the incidence of visual impairment due to DR strongly related to the duration of diabetes, retinopathy remains asymptomatic until the pathology significantly progresses [14, 15]. In this chapter, we will first highlight both clinical and pathological features of DR and then discuss our current understanding of the mechanisms involved in the pathogenesis of DR in diabetes.

9.1.1 Clinical Features

To impede the progression of non-vision threatening DR to vision-threatening DR, distinct clinical features must be noted in the early stages of DR in order to implement appropriate treatments plans.

9.1.1.1 Non-proliferative DR

The earliest stage of disease progression in DR is known as non-proliferative DR (NPDR). Although hyperglycemia-induced damage to endothelial cells and capillary pericytes in the retinal microvasculature are associated with the preclinical stages of DR, the loss of these cells underlies a number of clinical features in NPDR. These clinical features are characterized by microvascular abnormalities that consist of micro aneurysms, intra retinal hemorrhages (dot and blot), increased retinal vascular permeability, nerve fiber layer infarcts (cotton wool spots), greater presence of intra retinal lipid deposits (hard exudates), and venous beading [16–19]. NPDR can be categorized into mild, moderate, severe, or very severe stages based upon the absence or presence of the aforementioned clinical features (Fig. 9.1). In the natural course of DR, the severity of retinal vascular occlusion increases, which in turn leads to impaired perfusion and retinal ischemia [19, 20]. The sequelae of increasing ischemia include various venous abnormalities and considerable retinal vascular leakage that is markedly distinguished by the increased presence of hard

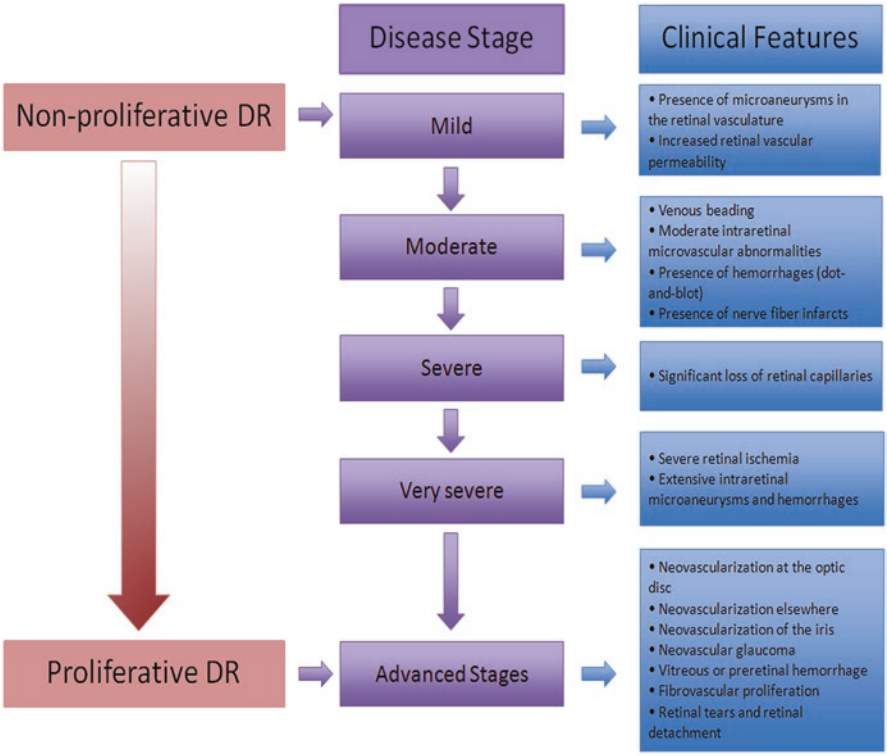

Fig. 9.1 Respective clinical features of the various stages in diabetic retinopathy. Diabetic macular edema, not depicted, can occur at any point during DR progression, which is characterized by retinal thickening or hard intra retinal lipid exudates near the macula

lipid exudates and retinal hemorrhages [20]. Once the progression of these features surpasses clinically defined thresholds, severe NPDR is diagnosed. During this stage, the risk of progression to proliferative diabetic retinopathy heightens. Among the severe NPDR patients, nearly 50% will develop proliferative diabetic retinopathy (PDR) within 1 year and 15% will develop high-risk PDR [21–23]. Whereas, 75% of patients classified with very severe NPDR are at risk of developing PDR within 1 year and 45% will become high-risk PDR during this period [22, 23].

9.1.1.2 Proliferative DR

Once diabetic retinopathy advances to the proliferative stage, visual loss becomes imminent if left untreated. In order to compensate for the sustained retinal ischemia, one of the distinguishing clinical hallmarks of PDR is the presence of neovascularization. The formation of abnormal vessels in the retinal circulation may occur through both endothelial cell migration and proliferation on or near the optic disc (neovascularization of the disk) or elsewhere in the retina (neovascularization elsewhere), on the iris (neovascularization of the iris), or into the vitreous cavity of the eye [24, 25]. Due to the fragility of the new vessels, the vessels become more susceptible to bleeding, leakage, fibrosis, and contraction, which can result in vitreous hemorrhaging, retinal tears, and retinal detachment—crippling ocular complications that inevitably lead to vision loss [26–29]. Further, neovascularization of the iris, also known as rubeosis iridis, and neovascularization of the anterior chamber angle can lead to neovascular glaucoma, a painful ocular disease that usually necessitates enucleation of the affected eye [30].

9.1.1.3 Diabetic Macular Edema

Diabetic macular edema (DME) represents a common vision-threatening complication of DR that is defined as retinal thickening in the macular area [31–34]. Although DME has three severity levels, DME can occur at any point during DR progression and promotes the breakdown of the blood-retinal barrier via microaneurysms and hyperpermeability of capillaries—causing lipids and plasma to be leaked into the macula [31–33]. The increased presence of hard lipid exudates in close proximity or at the center of the macula is associated with clinically significant macular edema [34].

9.2 Pathological Features of DR

There are five distinct vascular lesions underlying the DR response: dysfunctional pericytes and endothelial cells, basement membrane thickening, retinal capillary non-perfusion, retinal neovascularization, and breakdown of the blood retinal barrier. Each vascular disorder associated with DR is initiated by the microangiopathic

properties of the diabetic process, which mainly occurs through numerous growth factors that are altered by the changing ocular environment [35]. In this section, we will discuss the pathological features of DR in detail as the presence of one or more of these vascular disorders will help us understand the pathogenic mechanisms associated with DR.

9.2.1 Dysfunctional Endothelial Cells and Pericytes

One of the earliest pathological features that occur in DR are alterations in the microvasculature, which consist of modifications in cellular structure [35, 36]. Two essential cell types in the microvasculature are pericytes and endothelial cells, and the interaction between these cells is pivotal in the proper regulation of retinal hemodynamics and vascular function [31, 36]. With endothelial cells comprising the endothelium, which is the thin monolayer covering found in the interior surface of all blood vessels, retinal endothelial cells must ensure that proper nutritional requirements and protection of the ocular tissues, critical to vision are met [37, 38]. The general structure of the endothelium in the retinal microvasculature consists of adjoining endothelial cells that are linked by adherens junctions and tight junctions, which constitute much of the blood-retinal barrier (BRB) [39–41]. An essential prerequisite in the development of diabetic retinopathy is the loss of endothelium integrity caused by chronic hyperglycemic exposure. Following endothelial cell damage, the interendothelial junctions are unable to maintain the precise permeable properties that necessitate proper BRB function [42]. Therefore, the presence of dysregulated endothelial cell-to-cell junctions in the BRB allows for the extravasation of plasma constituents into the retina. Moreover, diabetic animal models have demonstrated that the apoptosis of retinal endothelial cells is enhanced by the activation of the Fas/Fas ligand (FasL) pathway upon leukocyte adhesion to the vascular endothelium [181].

In the context of maintaining vascular homeostasis, pericytes are important multifunctional cells that serve to stabilize blood vessels, form the BRB, regulate blood flow, and are involved in angiogenesis, endothelial proliferation, and leukocyte recruitment [43–45]. Pericytes are situated on the abluminal surface of blood capillaries and are morphologically characterized as cells that possess finger-like projections, which extend along the capillary wall and wrap around endothelial cells [44, 46, 47]. While there are several intricate signalling pathways involved in the interaction between pericytes, astrocytes, and endothelial cells, the intercellular communication between endothelial cells and pericytes appears to determine the presence of pericytes on retinal microvessels [48]. One prominent signal transduction pathway utilized between pericytes and endothelial cells is the platelet-derived growth factor-BB-platelet-derived growth factor receptor subunit B pathway (PDGF-BB-PDGFRß) [48, 49]. During angiogenic or hypoxic stress, endothelial cells secrete PDGF-BB, which binds to the pericyte-specific PDGFRß with a strong affinity [50, 51]. Upon binding, the receptor is dimerized, autophosphorylated, and activated, which then further initiates the downstream cascade of PDGF-BB signal-

ling, leading to pericyte survival, migration, proliferation, attachment, as well as leukocyte trafficking [52, 53]. In the case of diabetic retinopathy, both *in vitro* and *in vivo* studies have shown that hyperglycemic stress induces dysfunctional PDGF-BB-PDGFRß signalling, consequently leading to pericyte apoptosis and failure of proper pericyte recruitment [53–55]. The inability to replace damaged retinal pericytes will ultimately lead to aberrant retinal vascular morphologies, increased development of microaneurysms, endothelial cell hyperplasia, and blood-retinal barrier breakdown [56, 57]. Nevertheless, the loss of pericytes coupled with endothelial cell apoptosis contributes to the formation of acellular, nonperfused capillaries, which are tubes of basement membranes devoid of cell nuclei [181].

9.2.2 Basement Membrane Thickening

The vascular basement membrane (BM) is a thin extracellular sheet-like structure, comprised of numerous components (including types IV and V collagen, laminin, fibronectin (FN), nidogen, heparan and chondroitin sulfate proteoglycans), that exists between pericytes and endothelial cells [58]. The methodical arrangement of the BM components and molecular interactions between them manages cell survival and provides both a selective permeability barrier and physical support for cell attachment [59–62]. Early induction of hyperglycemia can provoke BM thickening in retinal capillaries through accelerated synthesis and decreased degradation of BM components, which can contribute to the occlusion of capillaries [62, 63]. More specifically, hyperglycemic conditions heighten the mRNA expression of FN, laminin (subunits beta-1 and gamma-1), and types IV (alpha-1 and alpha-2), and V collagen in the retinal BM of both diabetic animals and patients, which can be detected long before the onset of morphological lesions due to DR [63–66]. Further, any alterations in the vascular BM structure or its components may have detrimental effects on its ability to prevent vascular permeability, consequently leading to the development of macular edema [67, 68]. Since the careful balance between synthesis and degradation of BM components to sustain proper BM turnover is disrupted in DR, an understanding of the mechanisms perpetuating BM thickening and accumulation of BM components is essential and will be later described in this chapter. The underlying mechanisms possibly include increases in protein kinase C (PKC) activity, polyol pathway flux, inflammation, advanced glycation end-product (AGE) accumulation, endothelin activity, and growth factor activity [69–76].

9.2.3 Breakdown of the Blood-Retinal Barrier

The preservation of the blood-retinal barrier (BRB) is a mandatory requirement for proper vision. Compromised BRB integrity can result in numerous ocular pathologies that can have irreparable damage to one's visual perception; therefore,

elucidation of the BRB structure is required. The BRB consists of both an inner and outer retinal barrier that serves to maintain a specialized environment for the neural retina [77]. In the inner BRB, retinal capillary endothelial cells form the inner lining of microvessels and are accompanied by pericytes, astrocytes, and glial cells (Müller cells) (shown in Fig. 9.2) [78]. These endothelial cells are linked together via junctional complexes that facilitate the transport of highly selective molecules between the circulating blood and the neural retina through either transcellular or paracellular routes [79]. Retinal pericytes and astrocytes also interact with the endothelial cells to provide vascular integrity [80]. On the contrary, the outer BRB is comprised of retinal pigment epithelial cells that are connected by tight junctions; the primary role of the outer BRB is to sustain homeostasis in the outer retina [81]. During DR, however, hyperglycemic conditions result in both structural and functional alterations to the barrier, which subsequently leads to both inner and outer BRB breakdown. Following BRB damage, large amounts of plasma protein begin to extravasate

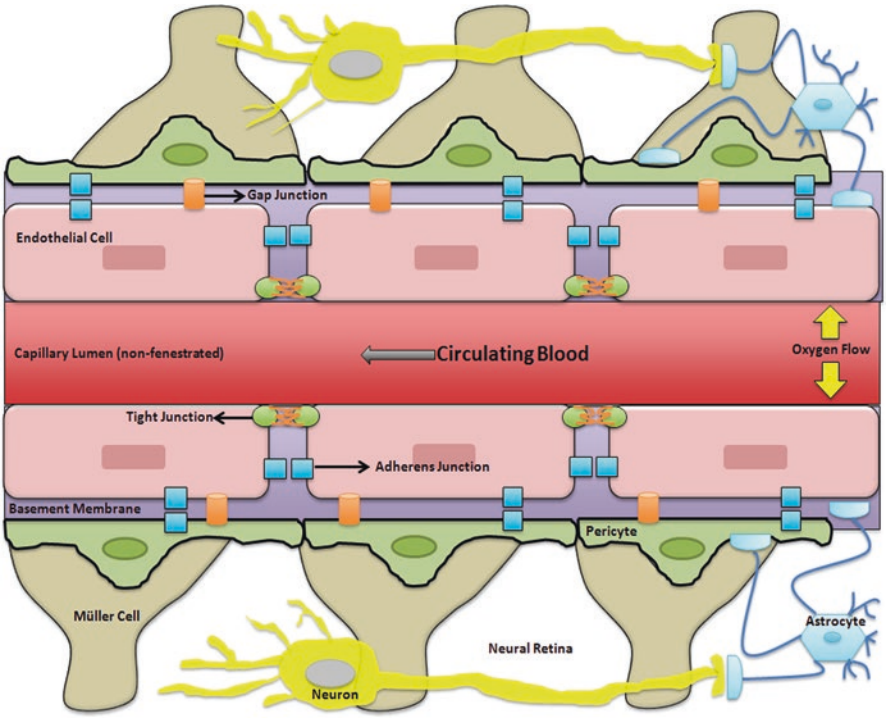

Fig. 9.2 (**a**) **An illustration depicting a stable inner blood-retinal barrier in a healthy patient**. The integrity of the endothelium is maintained by the presence of functional adherens junctions and tight junctions. Gap junctions authorize the passage of small molecules, and are predominantly located between pericytes and endothelial cells. Furthermore, Müller cells provide mechanistic support to the neural retina and also sustain balance of the extracellular environment in the retina. While, retinal astrocytes are involved in neuronal signaling and assist in managing the barrier properties of endothelial cells

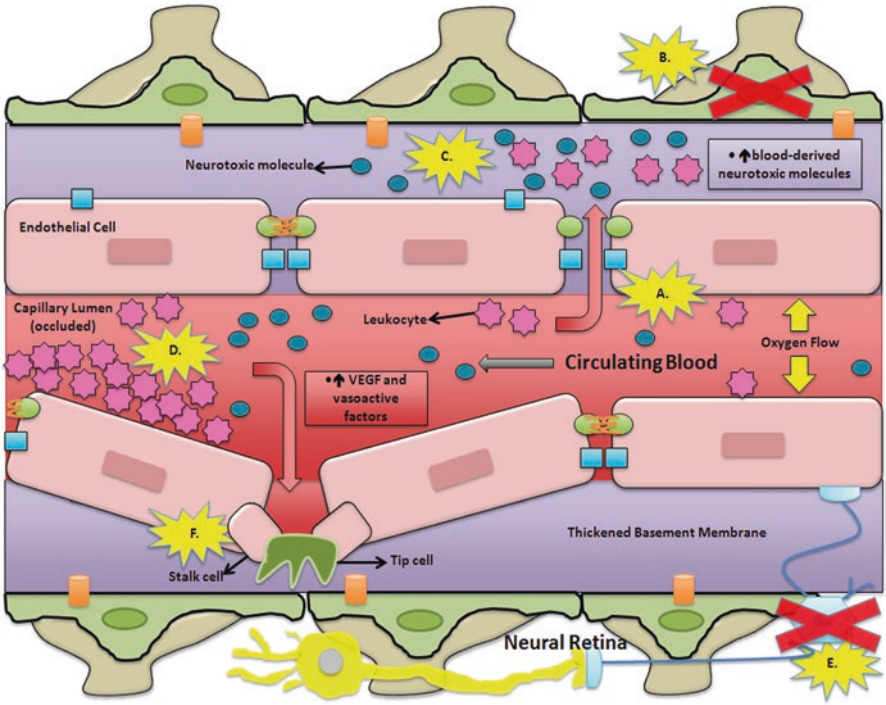

Fig. 9.2 (**b**) **An illustration depicting an unstable inner blood-retinal barrier in a patient with advanced proliferative diabetic retinopathy**. Chronic hyperglycemia compromises BRB integrity through numerous factors, which are depicted by *letters* in this figure: (*A*) Endothelial dysfunction, (*B*) Pericyte degeneration/apoptosis, (*C*) Basement membrane thickening, (*D*) Retinal capillary non-perfusion, (*E*) Neural inflammation and dysfunctional astrocytes, and (*F*) Retinal neovascularization

into the neural interstitium, producing high oncotic pressures that will eventually contribute to macular edema [82]. As a result of chronic hyperglycemia, several known factors are implicated in BRB disruption: dysfunctional endothelial cells, pericytes, Müller cells, and astrocytes, increased levels of VEGF, hypoxia, oxygen-free radicals, inflammatory mediators, advanced glycated end products, and protein kinase C activity [83].

9.2.4 Retinal Capillary Non-perfusion

Satisfying the high metabolic demands of the retina requires the maintenance of adequate tissue perfusion, which ultimately preserves retinal function. The cessation of blood flow to certain areas of the retina is known as capillary non-perfusion (CNP) and this phenomenon is associated with occluded vessels, a consequence of glucose-induced retinal vascular damage [84]. Chronic retinal ischemia is

manifested as large areas of CNP, which is the underlying cause of retinal neovascularization [85]. In the severe stages of non-proliferative DR (NPDR), the considerable presence of hypoxic regions resulting from retinal microvascular abnormalities can stimulate the retinal endothelial cells to release proinflammatory cytokines [86]. The subsequent release of cytokines perpetuates retinal hypoxia by recruiting and activating leukocytes, which adhere to the vascular endothelium—contributing to retinal capillary impedance [87, 88]. In the case of chronic retinal hypoxia, the heightened activation of several abnormal biochemical pathways induces the expression of numerous vasoactive factors [89]. These factors are instrumental in capillary dropout and the development of retinal neovascularization—a distinctive clinical feature of proliferative DR (PDR) [90]. Although the exact mechanisms of how retinal ischemia elevates the expression of vasoactive factors still require further elucidation, studies within the past decade have revealed that the activation of specific transcription factors increase a variety of vasoactive mediators implicated in the progression of DR [91–96].

9.2.5 Retinal Neovascularization

Angiogenesis is a critical physiological process in growth, development, and wound repair that induces the neogenesis of blood vessels from pre-existing vessels. However, in the case of DR, pathological retinal angiogenesis (retinal neovascularization) is a detrimental complication to vision. As observed in the mid-to late-1900s, retinal neovascularization (NV) transpires parallel to areas of CNP supporting the notion that vasoactive factors released from ischemic tissues are pivotal in the development of pathological angiogenesis [97–99]. The discovery of hypoxia-related transcription factors and their role in mediating angiogenesis has shed more insight into the complicated pathogenesis of DR. The hypoxia-inducible factor (HIF)-1α protein is one such transcription factor that is significantly accumulated in the presence of low oxygen levels and subsequently upregulates numerous hypoxia-regulated gene products [100, 101]. Under normoxia, the tumor suppressor protein, von Hippel-Lindau (VHL) binds to HIF-1α, targeting it for degradation through the ubiquitin-proteasome pathway [102, 103]. In contrast, hypoxic conditions prevent HIF-1α and VHL interaction, which subsequently results in HIF-1α to cumulate, dimerise with HIF-1β, and translocate into the nucleus where it binds to the hypoxia-response elements in the promoters of vasoactive genes [104]. Following the activation of transcription, multiple pro-angiogenic factors are then upregulated including vascular endothelial growth factor (VEGF), placental growth factor (PLGF), stromal derived growth factor (SDF-1), platelet-derived growth factor (PDGF-B), and their receptors, and angiopoietin-2 (Ang-2) [105, 106]. In particular, VEGF not only stimulates the development of endothelial cells, but it also induces both the disassembly of endothelial cell-to-cell junctions, which drives vascular permeability, and the sprouting of new vessels in combination with Ang-2 [106, 107]. Before sprouting vessels develop, specific subsets of endothelial cells differentiate into either tip or stalk cells [108]. The sprouting process is controlled through the antagonistic actions of delta-like

ligand 4 (Dll4) and Jagged1 ligands in the hypoxia-induced Notch signalling pathway [109–112]. As Notch-Dll4 and VEGF-induced signalling increases, the specialized endothelial tip cells direct the sprout vessel growth along a specific VEGF gradient, comprised of VEGF-A that is detected by VEGF receptor-2 expressed on the filopodia of these cells [113]. Although tip cells do not proliferate, the proliferative activity of stalk cells is driven by the availability of VEGF, Ang-2, and additional growth factors [114]. Together, the interaction between tip and stalk cells and the surrounding pro-angiogenic factors stimulate the growth of new blood vessels in the retina. It is important to note that within the retina, several cell types can produce VEGF: endothelial cells, pericytes, Müller cells, astrocytes, and retinal pigment epithelial cells [148]. To further emphasize the role of VEGF in DR, current treatment of DME using intravitreal injections of anti-VEGF agents have met with success.

9.3 Biochemical and Molecular Mechanisms Involved in the Pathogenesis of DR

Hyperglycemic insult gives rise to a diverse number of biochemical pathways that are implicated in the pathogenesis of DR. As our knowledge over the years has significantly developed in regard to the molecular mechanisms attributed to DR, more theories begin to emerge and therefore expand and enrich our knowledge of preexisting DR mechanisms. Currently, as shown in Fig. 9.3, several mechanisms/pathways that have an involvement in hyperglycemia-induced DR progression have been proposed: polyol pathway, protein kinase C pathway, hexosamine pathway, advanced glycation end-products formation, retinal renin-angiotensin system, and inflammatory mechanisms that include neural-and-immuno-inflammatory responses.

9.3.1 Polyol Pathway

Under normal glucose concentrations in non-diabetic patients, glucose metabolism utilizing the polyol pathway comprises only a small portion of total glucose use [115]. However, the elevation of intracellular glucose concentrations under diabetic conditions activates increased glucose flux through this pathway [116, 117]. Aldose reductase, the initial and NAPDH-dependent enzyme present in the polyol pathway, plays a critical role in the reduction of glucose to sorbitol [115–117]. Further metabolization of sorbitol is completed by sorbitol dehydrogenase, using NAD^+ as a cofactor, which allows for the formation of fructose [118]. Hyperglycemic conditions serve as a catalyst for enhancing aldose reductase activity, subsequently leading to sorbitol agglomeration [117, 118]. Although the polyol pathway and its exact mechanism in DR pathogenesis still remains inconclusive, several hypotheses have been reported that can ultimately commence and augment cellular damage mechanisms

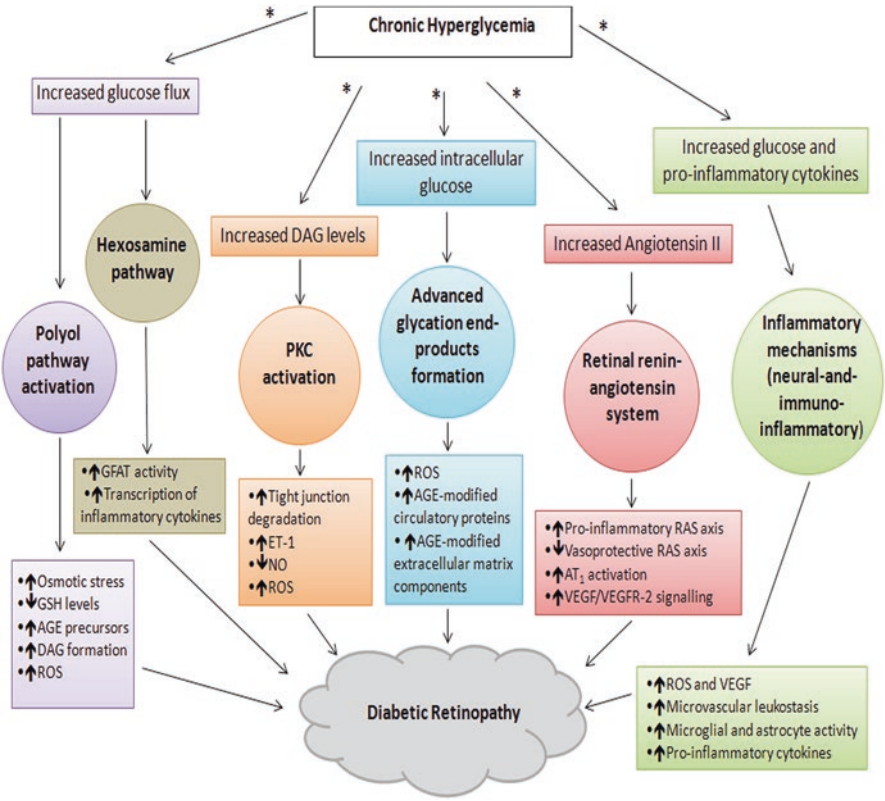

Fig. 9.3 Chronic hyperglycemia in DR gives rise to abnormalities in diverse biochemical pathways, which ultimately contribute to and advance DR pathogenesis. The application of a large initial stimulus, such as increased glucose levels, can activate a chain of reactions that incorporate unique pathways: polyol, hexosamine, neural -and-immuno-inflammatory, retinal renin-angiotensin, advanced glycation end-products, and protein kinase C. The "*" in this figure signifies that reactive oxygen species (ROS) may regulate these pathways to some extent (please see Fig. 9.4)

after the activation of the polyol pathway: changes in intracellular tonicity (osmotic stress) via the accumulation of sorbitol and fructose, development of advanced glycation end-product precursors (methylglyoxal, fructose-3-phosphate, and 3-deoxyglucosone), decreased Na^+/K^+ ATPase activity, diminished cellular anti-oxidant defense mechanisms as a consequence of reduced glutathione levels, protein kinase C (PKC) activation by elevated diacylglycerol (DAG) formation, and increased generation of reactive oxidant species (ROS) through the hyperglycemic activations of poly (ADP-ribose) polymerase and NADH oxidase [119–125].

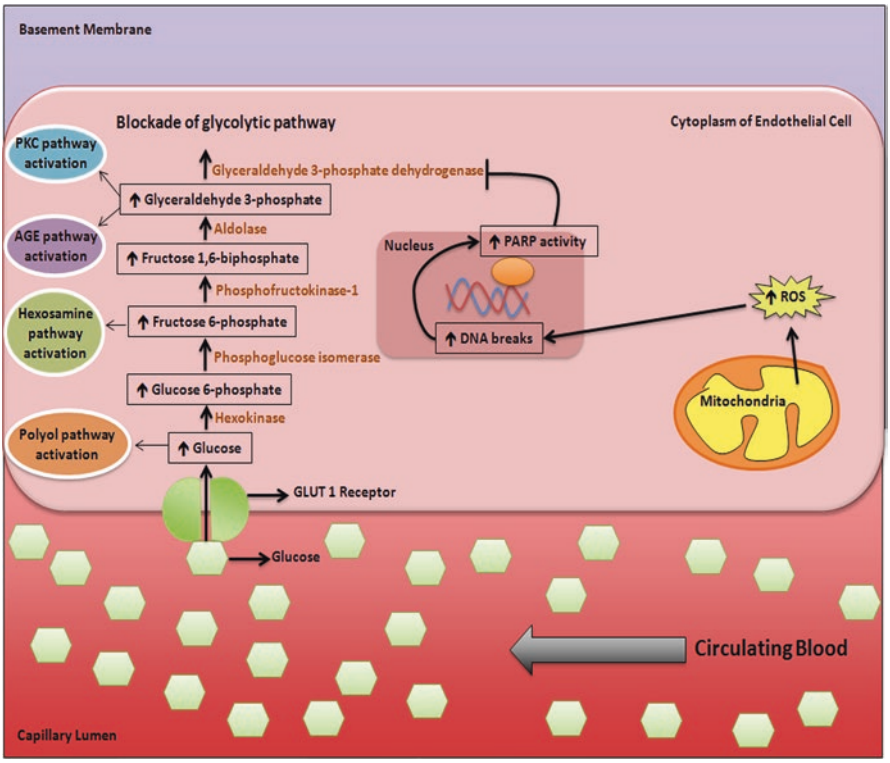

Fig. 9.4 The figure illustrates the effects of reactive oxygen species (ROS) on the retinal endothelial cell and the association of ROS with other biochemical pathways implicated in the pathogenesis of diabetic retinopathy. To summarize, the presence of high glucose stimulates the increased production of mitochondrial ROS, which induces breaks in DNA strands and activates poly (ADP-ribose) polymerase (PARP). As a consequence of PARP activation, glyceraldehyde 3-phosphate dehydrogenase activity is significantly reduced due to its interactions with PARP. Dysfunctional glyceraldehyde 3-phosphate dehydrogenase results in the accumulation of glycolytic metabolites upstream of this enzyme, which ultimately activates several biochemical pathways. Note, the endothelial junctional complexes, the retinal renin-angiotensin system, and the neural -and-immuno-inflammatory mechanisms are not shown in this figure. Furthermore, there are additional mechanisms of ROS generation that can subsequently contribute to cellular dysfunction. This figure only illustrates one of the ROS mechanisms in retinal endothelial cells

9.3.2 Protein Kinase C (PKC) Pathway

As hyperglycemia elevates DAG levels via the *de novo* pathway, the subsequent elevation of this intracellular messenger activates several isoforms in the PKC family that consists of serine/threonine kinases. More specifically, both *in vitro* and *in vivo* experiments have shown that the hyperglycemic activation of PKC-β can mediate VEGF-A levels and increase vascular permeability by phosphorylating endothelial cell tight junction proteins. These tight junction proteins are then targeted for ubiquitin-mediated

protein degradation—contributing to blood-retinal barrier breakdown [126, 127]. PKC-β is also involved in altering nitric oxide (NO) production, endothelial nitric oxide synthase (eNOS) expression, and endothelin-1 (ET-1) that consequently supports abnormal retinal hemodynamics [128, 129]. While, on the other hand, hyperglycemic activation of PKC-δ and Src homology-2-domain-containing phosphatase-1 (SHP-1) has been reported to induce retinal pericyte apoptosis through the nuclear factor-kappa B (NF-κB) signalling pathway [130]. The combined effects of hyperglycemic stimulus and PKC-δ activation can additionally provoke increased ROS generation, which will have detrimental consequences on retinal function [128–131]. Furthermore, the link between PKC activation and increased mitogen-activated protein kinase (MAPK) activity has been established— suggesting that the interplay between several PKC isoforms and MAPK activity can lead to subsequent phosphorylation of numerous transcription factors that heighten the expression of multiple stress-related genes associated with DR pathogenesis [132].

9.3.3 Hexosamine Pathway

During intracellular glucose metabolism, the redirection of fructose-6-phosphate from the glycolytic pathway to the hexosamine pathway (HSP) can ultimately induce increased transcription of pro-inflammatory cytokines, insulin desensitization, and oxidative stress— all of which are prominent features contributing to retinal neuronal apoptosis [133–136]. Glutamine:fructose-6-phosphate amidotransferase (GFAT) is the first and rate-limiting enzyme present in the HSP that catalyzes the conversion of fructose-6-phosphate and glutamine to glucosamine-6-phosphate and glutamate, respectively—preparing their entry into the HSP [137]. After a series of conversions, the major HSP end product is uridine diphosphate-N-acetylglucosamine, which allosterically inhibits GFAT and serves as an important substrate for O-linked N-acetylglucosamine transferase to facilitate the process of O-linked glycosylation on the serine and threonine residues of nucleocytoplasmic proteins [137–140]. In the context of diabetes, hyperglycemia elevates both HSP flux and GFAT activity, which leads to post-translational over modifications and subsequently alters the expression of numerous genes implicated in DR pathogenesis. After all, O-linked glycosylation can target transcription factors, signalling molecules, cofactors, and even RNA polymerase II [141–143]. Although O-linked glycosylation may compete with phosphorylation for the specific serine and threonine sites on a protein, hyperglycemia-induced HSP activation has shown to elevate O-linked glycosylation and reduce serine and threonine phosphorylation of the transcription factor Sp1 [144]. The successive glycosylation of Sp1 additionally increases the transcription of transforming growth factor-beta1 (TGF-β1) and plasminogen activator inhibitor-1 (PAI-1)—assisting DR development [144, 145].

9.3.4 Advanced Glycation End-Products (AGEs) Formation

The perpetual exposure of hyperglycemia to the retina can cause the formation and build-up of advanced glycation end-products (AGEs), which participate in endothelial dysfunction, chronic inflammation, BRB breakdown and retinal neuronal degeneration through a variety of mechanisms [146, 147]. The formation of AGEs is accomplished in a series of sequential chemical reactions that initially begins with the non-enzymatic interaction between carbonyl groups of intracellular glucose molecules (and other reducing sugars) and the free amino groups of intracellular proteins [148]. As a result of this interaction, an unstable compound known as a Schiff base is formed and then by molecular rearrangement, a more stable compound (an Amadori product) is constructed that later metabolizes to AGEs (an irreversible compound) [148–150]. Not only can the generation of AGEs modify and alter the function of intracellular proteins, but AGE precursors can also diffuse out of a cell and modify extracellular matrix components and their matrix receptors such as integrins, and circulating plasma proteins that greatly contribute to retinal microvascular leukostasis [151–155]. The receptor for AGEs (RAGE) is ubiquitously expressed on multiple cell types and the activation of RAGE, upon the binding of AGE precursors, activates and enhances pro-inflammatory and pro-oxidant signal cascades involving MAPK, NF-κB, activator protein-1 (AP-1), Janus kinase/signal transducers and activators of transcription (JAK-STAT), phosphatidylinositol-3 kinase (PI3K), PKC, VEGF, and tumor necrosis factor- alpha (TNF-α) [156–161].

9.3.5 Retinal Renin-Angiotensin System

The systemic renin-angiotensin system (RAS) is responsible for regulating blood pressure and maintaining the balance of electrolytes. Within the last 30 years, research has revealed that local RAS exists in various tissues (including the retina) that are independent from the systemic RAS and play a role in sustaining local equilibrium [162]. In the eye, the localization of RAS components are found to be predominantly expressed on retinal microvessels (endothelial cells and pericytes), various glial cells (Müller cells, astrocytes, and microglia), neurons (ganglion cells), and in other structures, such as the choroid and ciliary epithelium [163]. In addition to their cellular localization, distinct local RAS components have been implicated in ocular pathogenesis. For example, studies have reported that DR patients have elevated plasma and intraocular concentrations of prorenin, renin, angiotensin II, and angiotensin-converting enzyme (ACE)—these levels additionally correlate with DR severity [164–167]. Furthermore, *in vivo* experiments have reported that DR may have an association with local RAS imbalances through the activation of the pro-inflammatory RAS axis (comprised of ACE, renin, the renin receptor, and angiotensin II receptor type I) that promotes vasoconstriction and a subsequent reduction in the vasoprotective axis (comprised of ACE2, angiotensin-(1–7), and

Mas) of RAS [168]. Although the retinal RAS signalling mechanisms in DR still require further elucidation, several studies have demonstrated that the activation of angiotensin II receptor type I by angiotensin II facilitates the upregulation of VEGF/VEGFR-2 signalling [169–171]. This upregulation induces vascular permeability and retinal neovascularization that assists in BRB breakdown [169–171].

9.3.6 Inflammatory Mechanisms

Although acute inflammation is generally the body's natural way of protecting tissues from physiological stress or pathological stimuli, chronic inflammation, however, can mediate tissue destruction, enhance fibrosis, and drive angiogenesis. The persistence of low-grade inflammation is a prominent feature of DR that is mediated by several inflammatory mechanisms that can facilitate retinal vascular damage and neovascularization. Nevertheless, insight into the mechanisms regulating immune-and-neural-inflammation in DR pathogenesis is integral.

9.3.6.1 Immuno-Inflammatory Response

The immuno-inflammatory mechanisms involved in the ocular microenvironment remain ambiguous, as recent findings only allude to the complex interplay between the eye and the inflammatory system [172]. With the eye being an immune-privileged tissue, the presence of the inner and outer BRB allow the eye to create its own specialized microenvironment to suppress active inflammation and strictly regulate the activity of resident intraocular immune cells [172, 173]. The unique composition of the ocular immune microenvironment includes various immunosuppressive factors such as TGF-β2, alpha-melanocyte-stimulating hormone (α-MSH), neuropeptides, macrophage migration inhibitory factor (MIF), and vasoactive intestinal peptide (VIP), which restrict the actions of immune-competent cells (macrophages, microglial cells, dendritic cells, T-cells, and monocytes) [172–176]. During early NPDR stages, the chronic activation of pattern recognition receptors, such as RAGE and toll-like receptors expressed on immune-competent cells, can lead to the production of abnormal pro-inflammatory cytokines, upregulation of adhesion molecules, activation of other ocular resident immune cells, and increased retinal microvascular leukostasis [177]. More specifically, DR increases the production of several key ligands (includes high-mobility group box-1 (HMGB1) and AGEs) that greatly enhance RAGE activation in the retina [178]. Following activation, RAGE can stimulate MAPK and p38 signalling which in turn can trigger NF-κB transcription—contributing to pro-inflammatory cytokine production and ROS generation [179]. Heightened levels of inflammatory cytokines can then recruit additional leukocytes (i.e., monocyte chemoattractant protein-1 (MCP-1)) and stimulate VEGF-A activation, which up regulates the expression of adhesion molecules [180, 181]. Intercellular adhesion molecule-1 is one of the upregulated adhesion molecules expressed on the

surface of retinal endothelial cells that facilitates the binding of leukocytes to the vascular endothelium [181]. Upon binding, leukocytes generate ROS and additional inflammatory cytokines that promote retinal vascular permeability—consequently jeopardizing BRB integrity [182]. After all, maintenance of BRB integrity is critical for the preservation of the intraocular anti-inflammatory immune environment.

9.3.6.2 Neural Inflammation

Neural inflammation plays a critical role in DR pathogenesis. The neural retina is separated from retinal blood supply by the inner and outer BRBs and is an extremely delicate nerve tissue that is mainly comprised of Müller cells, astrocytes, microglia, and retinal ganglion cells [183]. Macroglial cells, which include Müller cells and astrocytes, have critical functions in maintaining normal retinal physiology: ensuring appropriate neuronal functioning by contributing metabolic and physical support forming and maintaining BRB integrity, maintaining homeostasis in the extracellular environment, and regulating retinal blood flow, metabolic waste product removal, local immune responses, and retinal glucose metabolism [184]. In addition to macroglial cells providing a local immune response, microglial cells also play an important role in initiating and mediating appropriate intraocular immune responses. Microglial cells are mononuclear phagocytes that are derived from the bone marrow and represent the retinal innate immune cells as they use cytotoxic and phagocytic mechanisms to eliminate foreign materials in the eye and lack specificity and memory [185, 186]. On the other hand, retinal ganglion cells are involved in transmitting visual information to pertinent brain centers that constructs our vision [187]. DR ultimately alters the morphology and function of neural retinal components. For example, in the early stages of DR, Müller cells can become extremely responsive to the hyperglycemia-induced retinal changes and significantly contribute to the inflammatory environment by releasing VEGF and decreasing anti-angiogenic factors (such as pigment epithelium derived factor (PEDF)) to promote retinal vascular permeability [188, 189]. Müller cells have also shown to induce RAGE activation, interleukin-1β (IL-1β), interleukin-6 (IL-6), nitric oxide (NO), prostaglandin E2 (PGE2), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) levels by numerous signalling pathways (including MAPK/NF-κB signalling) when exposed to high glucose *in vitro*—suggesting its roles in driving angiogenesis and furthering inflammation implicated in DR [188–192]. In regard to astrocytes, *in vivo* experiments have reported that astrocyte densities are significantly decreased in the retinas of diabetic rats and astrocytes can potentially excrete various inflammatory mediators (including TGF-α, COX-2, PGE2, and IL-1β) during hyperglycemic stress [193–197]. DR can also alter microglial morphology and induce microglial hyperactivity by RAGE activation—adding to the pro-inflammatory environment by releasing key inflammatory mediators [198–200]. Moreover, chronic hyperglycemia and accumulating levels of neurotoxic metabolites in the neuronal environment can induce apoptosis in retinal neurons by compromising the function of retinal ganglion cells [201].

9.4 Interconnection of the Pathways

Although chronic hyperglycemia can provoke the involvement of multiple abnormal biochemical pathways, research has revealed that these pathways are interconnected by mitochondrial-derived ROS [151, 152]. In aerobic cellular respiration, NADH and FADH2 from the tricarboxylic acid (TCA) cycle are generated to donate electrons to specific complexes in the electron transport chain (ETC)—NADH provides electrons to complex I, while FADH2 supplies electrons to complex II [202]. The electrons from both of these complexes are then shuttled through coenzyme Q, complex III, cytochrome-C, and finally complex IV, where oxygen is reduced to water [203]. The transfer of electrons through these complexes generates energy that is utilized to build an electrochemical proton gradient across the inner mitochondrial membrane [204]. Energy acquired from the electrochemical gradient regulates the generation of ATP through ATP synthase [204]. In the case of diabetes, however, elevated intracellular glucose levels lead to increased glucose oxidation in the TCA cycle; therefore, producing a substantial amount of electron donors that are then transported to the ETC [152]. Following the increase of electron donors, the voltage across the proton gradient rises until a critical threshold is attained, which subsequently impedes the electron transfer properties of complex III [205, 206] As a result of the complex III blockade, superoxides are constructed at an elevated rate; since, coenzyme Q donates the accumulated electrons to molecular oxygen [207]. The build-up of mitochondrial-derived superoxides then creates breaks in the DNA strands that signals the activation for PARP [208, 209]. Consequently, activated PARP inhibits the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a critical enzyme present in the cytosol and in the nucleus where it has a role in glycolysis and DNA repair, respectively [209–211]. The reduced capability of cytosolic GAPDH prevents glycolysis from completing, causing an aggregation of upstream glycolytic metabolites such as glucose, fructose-6 phosphate, fructose 1,6-biphosphate, and glyceraldehyde 3-phosphate [152]. Depending on the biochemical pathway and its respective glycolytic metabolite, the metabolites are subjected to additional modifications by pathway-specific enzymes that ultimately activate the major biochemical pathways previously mentioned in Chap. 3 (shown in Fig. 9.4).

9.5 Transcriptional Regulations

Despite the documented alterations of biochemical pathways and their associated genes in DR progression, the genetic variants explaining the considerable interindividual variations in DR susceptibility remain unknown [212]. Recent advances in genetic technology, however, have allowed genome-wide association studies to recognize the complicated interplay between genes and environmental factors in DR development. Nevertheless, the emergence of epigenetics may be a new paradigm for explaining the modifications involved in the transcriptional regulation of genes associated with DR pathogenesis.

9.5.1 Epigenetics

Despite improving glycemic control, the early exposure of hyperglycemia can still be implicated in late complications and disease progression [213–215]. This phenomenon is known as "metabolic memory" or "legacy effect" and was documented in the Diabetes Control and Complications-Epidemiology of Diabetes Interventions and Complications Trial (DCCT-EDIC), and the United Kingdom Prospective Diabetes Study (UKPDS), respectively [213–215]. When diabetic patients are transiently exposed to hyperglycemia, metabolic abnormalities such as ROS generation, PKC activation, increased activation of the hexosamine pathway, AGEs formation, and RAGE activation can affect epigenetic mechanisms by altering the expression of target-specific genes in cells without modifying the DNA sequence [216, 217]. These heritable epigenetic alterations can still facilitate DR pathogenesis despite the achievement of normoglycemia as epigenetic modifications can sustain constant activation of pro-inflammatory genes [218, 219]. The involvement of epigenetic changes in diabetes, cancer, and heart disease stresses the importance of understanding how epigenetic modifications lead to the manifestation of these diseases [219–221]. Currently, major epigenetic modifications are carried out by three select mechanisms: DNA methylation, histone modifications, and the activity of non-coding RNAs [222, 223].

9.5.1.1 DNA Methylation

Although the epigenetic modifications of DNA remain elusive, studies have reported that DNA methylation is typically associated with the silencing of gene transcription through the covalent addition of a methyl group on the fifth position of cytosine residues within CpG dinucleotide clusters (CpG Islands) [224, 225]. Moreover, the DNA methylation reaction involves the interaction between two enzymes that oppose each other: DNA methyltransferases (DMNTs) that create and sustain methylated DNA patterns, and DNA demethylases that remove methyl groups, such as the ten-eleven translocase (TET) enzyme [226]. In the context of diabetes, increases in DMNT have been reported with DMNT1, a maintenance enzyme among the three major types of DMNT, activity heightened in retinal capillary cells [227]. Further, new research has shown that TET2 is activated in diabetes and its activation demonstrates increased binding at the promoter region of matrix metalloproteinase-9 (MMP-9)—establishing a hypomethylated state in the MMP-9 promoter [228]. Due to the novelty of DNA methylation, the exact mechanisms of how the DNA methylation machinery assists DR pathogenesis remains unclear (Fig. 9.5).

9.5.1.2 Histone Modifications

Histone modifications are one of the better-characterized epigenetic mechanisms in DR. The nucleosome is the fundamental subunit that allows for the composition of chromatin, a tightly packaged structure that contributes to the final structure of

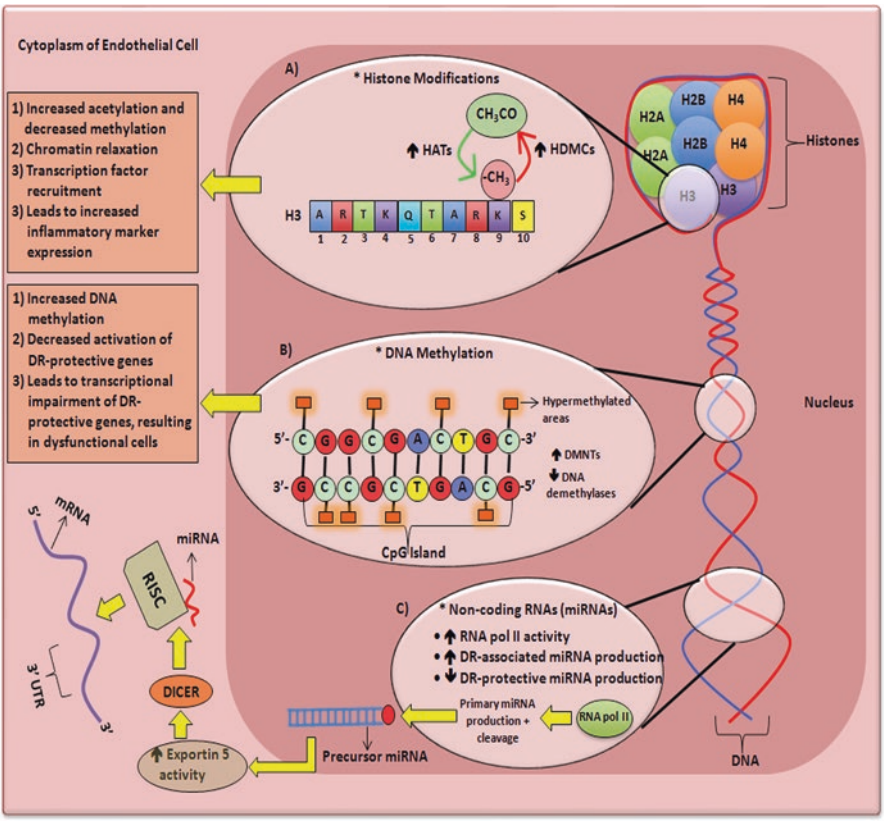

Fig. 9.5 The figure above depicts the general epigenetic modifications that can take place in a diabetic individual: histone modifications, DNA methylation, and modifications by non-coding RNAs. Two prominent histone modifications are methylation and acetylation of specific amino acid residues in the N-terminal tails of histones. Although the complete amino acid chain of histone H3 is not shown in the example above, decreased methylation of particular lysine residues (lysine 9 only shown in the diagram) allows for an increase in acetylation at these lysine positions—subsequently contributing to chromatin relaxation that allows for the recruitment of inflammatory transcription factors (*A*). As for DNA methylation, there are significant alterations in global DNA methylation patterns in diabetic individuals. Hypermethylation in the CpG islands of DR-protective genes can repress its activation, which promotes impairment of transcription, resulting in dysfunctional cells (*B*). Furthermore, it has been reported that several DR-protective miRNAs, a class of small non-coding RNAs, are downregulated in diabetic individuals, while DR-associated miRNAs are upregulated in diabetes. A decrease in DR-protective miRNAs promotes the expression of several inflammatory markers implicated in DR (*C*). The mechanisms of long non-coding RNAs, another type of non-coding RNAs, are not depicted in this figure, however the '*' near each epigenetic modification illustrates that long non-coding RNAs can play a role in these areas. Abbreviations: CH3CO acetyl group, −CH3 methyl group, HAT histone acetyltransferases, HDMC histone demethylases, DMNT DNA methyltransferases, RNA pol II RNA polymerase II, and 3′ UTR 3′ untranslated region

eukaryotic chromosomes. Individual nucleosomes consist of an octamer of histones, two molecules of each histone protein (H2A, H2B, H3, and H4), encased by approximately 147 base pairs of DNA [229, 230]. Nucleosomes are prone to rapid adjustments from external stimuli [229–231]. More specifically, the core histones possess N-terminal tails where a large number of post-translational modifications can occur by targeting the amino acid residues in this area [231, 232]. In this section, we will focus on the most common covalent modifications associated with gene expression and transcription in DR: histone acetylation and histone methylation [233].

Histone Methylation

In order for histone methylation to occur, histone methyltransferases (HMTs) are required to facilitate the transfer of methyl groups to amino acid residues including arginine and lysine residing in the N-terminal tails of histones [233, 234]. While, on the other hand, histone demethylases (HDMCs) possess the capacity to remove methyl groups from this area [235]. It is important to note that select amino acid residues (such as lysine) can be methylated using one, two, or three methyl groups [218, 235]. Depending on the type of stimulus present, the over-modifications performed by either HMTs or HDMCs on these amino acid residues will dictate chromatin accessibility to transcriptional factors that subsequently regulates the expression of specific genes and their respective translated products [229]. For example, studies have reported that the methylation of specific lysine residues, such as lysines 9 and 27 in histone H3 and lysine 20 in histone H4 are associated with suppressed gene activity [234–237]. In contrast, methylation of lysines 4, 36, and 79 in histone H3 are associated with active gene regulation [234–237]. Moreover, epigenetic-related *in vitro* and *in vivo* experiments have demonstrated that diabetic conditions elevate the activity of lysine-specific demethylase 1 (LSD1), a type of HDMC, that hypomethylates lysine 9 in histone H3 at the promoter region of the MMP-9 gene [238]. Following hypomethylation of lysine 9, this amino acid residue is then subjected to increased acetylation that promotes NF-κB transcription—contributing to elevated MMP-9 activity that evokes retinal mitochondrial damage and apoptosis in retinal capillary cells [238]. Other hyperglycemic studies have also reported decreased activity of the manganese superoxide dismutase gene (SOD2) by increased methylation of lysine 20 in histone H4, which subsequently increases retinal oxidative stress [239, 240]. Furthermore, a multimeric complex known as polycomb repressive complex 2 (PRC2) plays a critical role in epigenetic regulation as it is associated with gene suppression including microRNAs (miRNAs) by trimethylating lysine 27 of histone H3 [241, 242]. Previous work completed by our laboratory studied the role between PRC2 and miR-200b in DR, but we will discuss the detailed findings in the non-coding RNAs section.

Histone Acetylation

In addition to methylation, histones can also be acetylated. The acetylation process involves the interactions between histone acetyltransferases (HATs) and histone deacetylases (HDACs) that facilitates either the addition or removal of acetyl groups to lysine residues, respectively [243, 244]. More specifically, the acetylation of lysines 9, 14, 18, and 56 in histone H3 and lysines 5, 8, 13, and 16 in histone H4 are speculated to have a role in chromatin relaxation that augments transcription factor recruitment—subsequently contributing to gene activation [218, 245]. The direct modification of regulatory proteins and transcription factors can also occur by HATs and HDACs [245]. To further demonstrate the importance of HATs in DR, previous *in vitro* and *in vivo* work from our laboratory demonstrated that p300, a transcriptional coactivator that is also a HAT, was markedly expressed in a diabetic environment and its increase in activity led to the overexpression of ET-1, VEGF, and FN—molecules that are upregulated in DR [246, 247]. On the other hand, sirtuin (silent mating type information regulation 2 homolog) 1 (SIRT1) is an important NAD-dependent deacetylase that is implicated in a dynamic range of cellular processes [248–250]. Due to its unique localization in both the nucleus and cytoplasm, SIRT1 is categorized as a type III HDAC [250]. *In vitro* and *in vivo* results from our laboratory demonstrated that chronic hyperglycemia reduces SIRT1 activity, which consequently drives ROS formation mediated by the acetylation of forkhead box protein O1 (FOXO1) through increased p300 activity [251].

9.5.1.3 Non-coding RNAs

Before the sequencing of the human genome project was completed, scientists originally speculated that the genome would contain around 50,000–100,000 protein-coding genes [252, 253]. However, with the recent advances in high-throughput sequencing technologies, nearly 20,000 genes, or about 2% of the transcribed genome, codes for proteins [253, 254]. These findings revealed that a considerable portion of the human genome transcribe for non-coding RNAs (ncRNAs), which incorporate both small and long ncRNAs [255]. Due to the breadth of information that can now be found on ncRNAs, we will provide insight into some of the known functions of distinguished ncRNAs and their implications in the pathogenesis of DR.

Small Non-coding RNAs

Although there are several classes of small non-coding RNAs (sncRNAs), microRNAs (miRNAs) are an extensively studied class in the context of disease, which will be the primary focus in this section. Being a class of small, endogenous, single-stranded, non-coding RNA molecules, active miRNAs typically range from 20 to 25 nucleotides in length and post-transcriptionally regulate gene expression [256–258]. The initial synthesis of primary miRNA molecules transpires in the nucleus where

they are synthesized by RNA polymerase II and are several kilobases long [258, 259]. Similarly, within the nucleus, RNA polymerase III enzymes, Drosha and DiGeorge syndrome critical region 8 (DGCR8), further processes these primary miRNA molecules into precursor miRNAs that are roughly 70 to 100 nucleotides in length and hairpin-shaped [258–263]. Following processing, exportin 5, a nuclear export factor, facilitates the transport of precursor miRNAs into the cytoplasm [262, 263]. Once reaching the cytoplasm, another RNA polymerase III enzyme, DICER, modifies the precursor miRNAs into their active miRNA forms, where they are then incorporated into the RNA-induced silencing complex (RISC) with the help of argonaute proteins [261–263]. The formation of RISC then binds to the targeted mRNA by interacting with its 3′ untranslated regions (UTR) [258–263]. As a result of this binding, the targeted mRNA is subjected to either repressed translation or degradation [258–263]. Earlier reports from our laboratory identified alterations of several miRNAs in hyperglycemia-induced endothelial cells and in numerous tissues affected by chronic diabetes: miR-1, miR-133a, miR-146a, miR-195, miR-200b, and miR-320 [38, 264–270]. Moreover, our recent findings report a novel regulation mechanism between PRC2 and miRNAs through histone methylation in diabetic complications. For instance, human retinal microvascular endothelial cells exposed to hyperglycemia and diabetic mice and rat retinas demonstrated heightened PRC2 activity, which in turn regulated the repression of miR-200b through tri-methylation of lysine 27 in histone H3 [268, 269]. Subsequently, reduced miR-200b levels promoted increased VEGF levels—contributing to vascular permeability and neovascularization [268, 269]. In addition to these findings, previous studies from our laboratory reported that miR-146a and miR-200b play an integral role in preventing glucose-induced endothelial-to-mesenchymal transition (EndMT), a pathogenic mechanism implicated in diabetic complications that induces basement membrane thickening by accelerating the production and deposition of extracellular matrix proteins [38, 266].

Long Non-coding RNAs

Long non-coding RNAs (lncRNAs) are distinguished from sncRNAs and mRNAs based on their transcript length being greater than 200 nucleotides and possessing no protein-coding potential, respectively [271]. LncRNAs are commonly classified into several groups according to their genomic localization: sense, generally transcribed from the sense strand of genes encoding proteins; antisense, transcribed from the antisense strand of genes encoding proteins; bidirectional, transcribed in the opposite direction of the protein-coding transcript, which is within 1000 base pairs of the promoter; enhancer, transcribed from enhancer regions of the DNA; intronic, transcribed from intron regions of the protein-coding gene; or intergenic lncRNAs, transcribed from regions located between two genes encoding proteins [271, 272]. A majority of lncRNAs are localized to the nucleus and nearly 15% of lncRNAs have been reported to be present in the cytoplasm [273]. Nevertheless, due to conflicting reports, defining one localization point that characterizes all lncRNAs is difficult, as each lncRNA may possess unique structural features that allow it to

be expressed in either the nucleus, cytoplasm, or both [274, 275]. Although the definitive mechanisms for lncRNAs currently remain cryptic, emerging studies are beginning to give us insight into some of the processes that lncRNAs are involved in: epigenetic regulation (such as genomic loci imprinting), chromosome conformation changes, allosteric regulation of enzymatic activity, and cell-cycle control [275–278]. More specifically, nuclear lncRNAs can induce histone modifications or DNA methylation to activate or repress gene expression by recruiting chromatin-remodeling complexes to specific loci in the genome, removal of transcription factors as well as proteins from the chromatin, and regulating transcription by functioning as a transcriptional coactivator [274–278]. Cytoplasmic lncRNAs are capable of secluding miRNAs from proteins to indirectly enhance protein expression [274–278]. In the context of DR, many aberrant lncRNAs have been identified that are expressed in diabetic retinas; however, these lncRNAs have not been comprehensively characterized [275]. Therefore, defining the specific roles of each lncRNA becomes critical to understanding the interplay between lncRNAs and the pathogenesis of DR. Currently, a prominent lncRNA that is upregulated in DR and associated with increased inflammatory cytokine production is metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) [279, 280]. As a matter of fact, both *in vitro* and *in vivo* results from our laboratory have identified that hyperglycemia induces an upregulation of MALAT1 in endothelial cells, which in turn regulates increased expression of inflammatory mediators IL-6 and TNF-α through serum amyloid antigen three (SAA3) activation [280]. In addition to MALAT1, preliminary *in vitro* and *in vivo* experiments from our laboratory revealed that another lncRNA, antisense non-coding RNA in the *INK4* locus (ANRIL), is upregulated in human retinal endothelial cells under hyperglycemic conditions [281]. High glucose exposure also elevated direct ANRIL binding to p300 and another component of PRC2, enhancer of zeste homolog 2 (EZH2) [281]. Furthermore, we speculate that VEGF regulation may involve ANRIL-mediated control of PRC2 components p300 and miR-200b, since silencing of ANRIL prevented the hyperglycemia-induced VEGF expression [281].

9.6 Conclusion

With the risk for acquiring diabetes-related complications increasing globally, DR remains a prevalent vision threatening complication of DM. The asymptomatic characteristic of DR makes it a troubling condition, where it becomes absolutely critical for diabetic patients to undergo early ocular screening and receive immediate treatments for retinopathy to reduce the risk of vision loss. In order to successfully impede the progression of DR using pharmaceutical management and if necessary, ocular surgery, a thorough understanding of the complex pathogenic mechanisms implicated in DR progression is fundamental. In this chapter, we have provided insight into the current knowledge regarding the clinical features, pathological characteristics, biochemical and molecular mechanisms, and epigenetic alterations

involved in DR. Although current DR treatment modalities focus on targeting various inhibitors that arise from abnormalities in the molecular and biochemical pathways, recent advances in genomic technology have made it clear that the root of the problem may lie within the genome. Accordingly, future treatment strategies should take into account the regulatory gene network in its entirety rather than focusing on a single regulatory interaction. Shifting the current therapeutic approach to a more gene-based approach may take time, but this shift in focus will ultimately ameliorate DR as gene therapy can account for the large interindividual variations in DR susceptibility. Nevertheless, further research is warranted into the identification and characterization of the genetic components involved in DR pathogenesis.

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Chapter 10
Perspectives on the Vascular Pathogenesis of Diabetic Neuropathy

Anita Mahadevan and Shankar S. Krishna

Abstract Diabetic neuropathy is one of the common manifestations following hyperglycemic state. It manifests as varied clinical syndromes with overlapping symptomatology. Essential pathology is axonal with predominant small myelinated fiber involvement with secondary myelin changes. Two pathogenetic mechanisms are believed to be operational, with metabolic factors resulting in distal symmetric polyneuropathy and ischemic factors causing focal neuropathy, but, with significant overlap. Disease is more likely multifactorial with systemic hypertension, obesity, nutritional and trophic factors contributing to the clinical manifestations and pathogenic evolution. Animal models have provided leads to our understanding of the pathomechanisms and identified therapeutic targets that are effective in animals but not in humans, making the models ineffective to extrapolate the observations to human subjects. Effective therapeutic modality to establish control of hyperglycemic state is central to prevention of progression in diabetic neuropathy. Enhancing the perfusion of the nervous system to alter the clinical progression of the disease remains essentially hypothetical. Specific and individualized therapeutic strategy to arrest progression and alter the course of the disease needs to be identified. Study of varied clinical forms of neuropathic complications appears to be mainstay in gaining insight into the pathogenesis in human system.

Keywords Diabetes • Neuropathy • Pathogenesis • Pathology • Endothelium • Basement membrane • Vascular disease

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10.1 Introduction

10.1.1 Epidemiology

Diabetes has been considered the epidemic of the century. Its incidence has doubled in the last decade and has been attributed to the high prevalence of the metabolic syndrome with obesity and hypertension. The International Diabetes Federation, in 2013, estimated that, globally, 382 million people are affected by the disease and its prevalence is expected to increase to 592 million by the year 2035, and the majority will be from Asia due to changes in diet and lifestyle [1]. The major contribution to mortality and morbidity in diabetes arises from vascular dysfunction. Diabetes-associated vascular alterations induce anatomic, structural, and functional changes in both micro- and macrovasculature [2]. Diabetic microvascular (involving small caliber arterioles and capillaries) and macrovascular (involving large vessels, including arteries and veins) complications differ in the organs affected.

Macrovascular complications cause atherosclerotic changes in coronary artery, cerebral and peripheral vasculature, leading to ischemic heart disease, cerebrovascular stroke, cognitive dysfunction and peripheral vascular disease, in one third to one half of people with diabetes [3]. "Microvascular disease" associated with diabetes, affects the microvasculature throughout the body, resulting in retinopathy, nephropathy, and neuropathy(the "triopathy") that lead to blindness, renal failure, and neuropathies with non-healing ulcers and auto-amputation of the fingers in both upper and lower limbs. As microvascular disease is unrelated to atherosclerosis, it is not predictable by the association with hyperlipidemia. This review focuses on the diabetes related peripheral neuropathy and the central role of vascular pathology in its evolution and sequelae.

10.1.2 Clinical Aspects of Diabetic Neuropathy

Approximately one half of people with diabetes have some form of peripheral neuropathy (PN) [4]. Autonomic nervous system involvement is also common leading to cardiovascular autonomic dysfunction, and loss of vasomotor tone. Foot ulcers, a common cause of morbidity, is a consequence of involvement of sensory nerves and impaired peripheral vascular function. Similar to diabetic retinopathy, the risk factors for PN include poor glycemic control (i.e., elevated glycated hemoglobin levels and impaired glucose tolerance), age, duration of diabetes, tobacco use, dyslipidemia, and hypertension [5, 6]. Other independent risk factors for PN include increased height, presence of cardiovascular disease (CVD), severe ketoacidosis, and microalbuminuria [7].

Unlike diabetic retinopathy, the pathogenesis of PN appears to be related to both vascular and nonvascular metabolic mechanisms [8–10]. Diabetes mellitus is identified as the most common cause of peripheral neuropathy and an estimated 60–70%

of diabetics have some degree of neuropathy that contributes to high morbidity and mortality [11, 12].With the increasing worldwide prevalence of diabetes, peripheral neuropathy (PN) will be an important clinical malady.

The clinical symptoms of PN are dictated by the predilection for particular nerve fiber types (sensory, motor, autonomic, or a combination) and the type of stimulus that activates each form of nociception (e.g., thermal or mechanical). Cutaneous sensation is sub served by different types of nerve fibers – the unmyelinated C-fibers, the thinly myelinated A-δ fibers, and the myelinated A-β fiber. C and A-δ fibers sub serve thermal and pain sensation, whereas A-β fibers are responsible for mechanical sensation of vibration and proprioception. In diabetes, the small diameter fibers are the first to be affected, followed by large myelinated fibers with continued hyperglycemia. Early involvement of small myelinated and unmyelinated somato sensory fibers in diabetic neuropathy can be detected by intradermal nerve fiber density reduction in cutaneous biopsies. The reduction correlates with elevation of warm thresholds and is the major manifestation of type 2 diabetes. The extent of skin denervation increases with the duration of diabetes [5].

Sensory involvement produces negative or positive symptoms that maybe diffuse or focal; insidious in onset with a glove-and-stocking type distribution in the distal extremities. Motor neuropathy causes distal, proximal, or focal weakness that may be associated with sensory involvement (sensorimotor neuropathy). Autonomic neuropathy may involve the cardiovascular, gastrointestinal, and genitourinary systems and the cutaneous sweat glands. Sensorimotor and sensory neuropathies are more frequent than autonomic neuropathy.

There are two classification systems for diabetic neuropathy – the Thomas system [13] and the symmetrical-versus-asymmetrical system [14] (Table 10.1). Distal symmetrical sensorimotor versus asymmetric forms reflect differing pathogenetic mechanisms causing neuropathy the metabolic alterations implicated in the former and ischemic pathophysiology in the latter. Thomas et al. proposed a staging system for diabetic neuropathies based on signs, symptoms and electrophysiological abnormalities of neuropathic involvement [15] (Table 10.2).

Type 1 and 2 diabetes differ in the frequency and pathogenesis of neuropathy. In type 1 DM, distal polyneuropathy becomes symptomatic after many years of hyperglycemia, whereas in type 2 DM, it may become apparent after only a few years of poor glycemic control or maybe the presenting symptom of diabetes. Severe form of autonomic neuropathy is encountered only in type I diabetic patients whereas a milder autonomic involvement can be observed in both Type I and Type II DM patients. The main cause of morbidity and morality results from micro- and macrovascular complications and alterations of endothelial function is central to the development. Macroangiopathy however is more frequent in type 2 diabetes as other traditional risk factors of atherosclerosis, like, hypertension, dyslipidemia, or obesity and the metabolic syndrome coexist.

Table 10.1 Classification schemes for diabetic neuropathy [13, 14]

Thomas system	Symmetric-asymmetric system
Hyperglycemic neuropathy Generalized symmetrical polyneuropathies	**Relatively fixed deficits**
	Distal sensory polyneuropathy (DSPN)
Sensory neuropathy	Variants:
	Acute, severe DSPN in early onset diabetes
Sensorimotor neuropathy	Pseudosyringomyelic neuropathy
Autonomic neuropathy	Pseudotabetic neuropathy
Focal and multifocal neuropathies	Autonomic neuropathy
Superimposed chronic inflammatory demyelinating polyneuropathy	**Episodic symptoms**
	Diabetic neuropathic cachexia (DNC)
	Hyperglycemic neuropathy
	Treatment-induced diabetic neuropathy
	Asymmetric neuropathy/Focal and multifocal diabetic neuropathy
	Median neuropathy of the wrist (carpal tunnel syndrome)
	Single or multiple limb mononeuropathies
	Truncal neuropathy (Thoracic radiculoneuropathy)
	Lumbosacral/brachial radiculoplexus neuropathy

Table 10.2 Staging system of diabetic neuropathy [15]

NO	No objective evidence of neuropathy
N1	Asymptomatic polyneuropathy
	N1a – No symptoms or signs but tests[a] abnormal
	N1b – No symptoms, neuropathic impairment on neurologic exam plus tests[a] abnormal
N2	Symptomatic neuropathy
	N2a – symptoms, signs and tests abnormal
	N2b: N2a plus significant ankle dorsiflexor weakness
N3	Disabling diabetic polyneuropathy

[a]Tests include nerve conductions, QST, or autonomic test

10.2 Pathogenesis

Neuropathy, initially begins with sensory loss due to reduction in thermal and vibratory sensation and progresses to involve pain and autonomic fibers [16].

The factors leading to the development of diabetic neuropathy are not completely defined. Current understanding is that, it is a multifactorial process influenced by duration of hyperglycemia, elevated lipids, blood pressure, smoking, increased height, and exposure to other neurotoxic agents such as ethanol [17–20]. Genetic

factors also appear to play a role as not all patients with long standing diabetes develop neuropathic complications [21].

Two basic pathogenetic mechanisms have been postulated to explain the occurrence of peripheral nerve involvement in diabetes; metabolic pathway and microvascular (ischemic) injury.

10.2.1 Metabolic Pathway

The metabolic pathway produces the distal symmetric form of diabetic polyneuropathy. The role of hyperglycemia is central to the metabolic pathway and its importance is confirmed by the evidence of reduction in incidence of PN by strict glycemic control. Further as glucose transport into endothelial cells of microvasculature and neurons do not require insulin and hence accumulate glucose, making them susceptible to hyperglycemia mediated damage.

Longstanding hyperglycemia, induces metabolic and structural derangements, such as the production of advanced glycation end products (AGE), activation of signaling cascades (such as protein kinase C [PKC]), production of reactive oxygen species (ROS), and stimulation of renin-angiotensin system that damage the components of the peripheral nerve. Understanding of these pathways has been translated into the development of novel therapeutic approaches to neuropathies and these are briefly outlined below.

10.2.1.1 Polyol Pathway

With increasing duration of hyperglycemia, intracellular glucose accumulates within the nerve, saturates the normal glycolytic pathway and the excess glucose is then shunted into the polyol pathway where the enzymes aldose reductase and sorbitol dehydrogenase convert glucoseinto sorbitol and fructose [22]. Sorbitoltends to accumulate within the nerve, as it is relatively impermeable. Being hypertonic, it results in accumulation of water within endoneurium, thereby inducing hypoxia. Accumulation of sorbitol also lowers the levels of myoinositol within the nerve. This decreases membrane Na+/K+−ATPase activity, impairing axonal transport, and causes abnormal propagation of action potential, producing positive sensory symptoms. This was the rationale for using aldose reductase inhibitors to improve nerve conduction [23].

10.2.1.2 Advanced Glycation End Products (AGE)

Are produced when the excess glucose via non-enzymatic reaction complexes with intracellular components including proteins, nucleotides, and lipids. Non enzymatic glycosylation causes covalent linkage of glucose to lysine residues in tissue components to form a Schiff-base intermediate. In the peripheral nerve, AGE accumulation cross links with cytoskeletal proteins such as axonal neurofilament, and myelin

sheaths. End result of this is an insoluble and irreversible advanced glycation end product, which is measureable. Accumulation of these end products is toxic to cellular metabolism and disrupts fast axonal transport producing dying-back pattern of nerve damage [24].The peripheral nervous system cytoskeleton is found to be more vulnerable than that of the CNS to non-enzymatic glycosylation. AGEs also interfere with repair and the axonal regenerative sprouts fail to survive due to metabolic failure of neuron and Schwann cells and deleterious effect on extracellular matrix of the endoneurium. Ischemic effects from microvascular changes also contribute to the failure of regeneration.

Although these metabolic alterations have been considered as the main pathogenesis for symmetric neuropathy in diabetes, mechanistic structural characteristics unique to the peripheral nerve also contribute to pathogenesis. For instance, in peripheral nervous system, the extremely long axons originating from the distant neuronal cell body are vulnerable in the most distal end to any reduction in nutritional or metabolic insults. In addition, there is a superimposed element of ischemia as the sparse vascular supply with impaired autoregulation causes hypoxic damage in the nerve. These dual influences result in distal predominant nerve fiber degeneration.

10.2.2 Vascular Pathology

Pathomorphologic studies of nerve biopsies have contributed tremendously to provide insights into the pathogenesis of neuropathy by identifying alterations in vessels and the interstitium that cannot be determined from clinical or electrophysiologic examination. The characteristic vascular pathology in diabetic neuropathy was first described by Fagerberg in 1959 [25]. He described "vascular changes in the form of hyalinization, caliber reduction and thickening of the wall to be more common in diabetic neuropathy than in other groups". He proposed that progressive degenerative vascular change leads to structural damage to myelin and hence results in neuropathy.

Evidence from early autopsy studies from the peripheral nervous system supported an ischemic pathology [26]. Multifocal fascicular lesions were demonstrable in the lumbosacral trunk, posterior tibial nerve, and sural nerve obtained at autopsy from diabetic patients in a proximal-distal gradient similar to pattern seen in vasculitic neuropathy. The perineurium and surrounding epineurium were damaged, reinforcing an important role for ischemia in the pathogenesis of diabetic neuropathy [27–29]. Ultrastructural studies by Dyck and colleagues supported the "ischemic hypothesis" by demonstrating reduplication of basement membrane and perineurial damage akin to that described in diabetic retinopathy. There were no significant changes in number and the distribution of microvessels. The authors concluded that these mural changes of endoneurial microvessels were specific to diabetes and contributed to the functional alterations of the blood-nerve barrier and endoneurial microenvironment [30]. Assessment of local blood flow in sural nerves by Doppler flow metry demonstrated reduction in blood flow only in the stage of severe polyneuropathy and plexopathies but not in the early stages [31].

Chronic hyperglycemia was argued to produce endoneurial hypoxic changes by Dyck and co workers [32]. Experimental studies in diabetic rats supported a role for hypoxia, as oxygen supplementation partially reversed the reduction in nerve conduction velocities and resistance to ischemic conduction blocks. Reduction in levels of endoneurial glucose, sorbitol and fructose was also demonstrable [33].

It is now known that endothelial cells, by their strategic location between the circulating blood and the vessel wall, are important regulators of vascular function and structure. They synthesize and release biologically active substances to maintain vascular homeostasis, ensuring adequate blood flow and nutrient delivery while preventing thrombosis and leukocyte diapedesis. A healthy endothelium has vasodilatory, antiatherogenic, and anti-inflammatory properties by regulating vascular smooth muscle tone, platelet activation, leukocyte adhesion, thrombogenesis, and inflammation [34].

In focal or asymmetrical diabetic neuropathy syndromes, vascular pathology or autoimmunity have a major role in pathogenesis [35]. The unique anatomical peculiarities of the peripheral nervous system vasculature in all its three compartments – the epineurium, perineurium and endoneurium render the peripheral nerve susceptible to ischemia. Transperineurial arterioles that traverse the perineurium are few in number and lack autoregulation. Impairment of autonomic innervation that occurs early in diabetes, affects epineurial microvessels impairing blood supply to the nerve [36, 37].

Mononeuropathies of upper and lower limbs result from carpal tunnel syndrome or cubital tunnel syndrome and pressure effects. Repetitive shear forces, and excessive stiffness of the enveloping connective tissues are also implicated in mononeuropathies.

Involvement of dorsal root ganglia by diabetes can also contribute to the neuropathy. Two features in the dorsal root ganglia render it vulnerable to diabetic complications. The ganglia possess fenestrated vessels, and the distal efferents being free of perineurium are directly exposed to toxins in the environment. Ganglionopathy can lead to truncal sensory neuropathies.

10.2.2.1 Alteration in Vasomotor Regulation

The microcirculation in peripheral nerve is regulated by central and local mechanisms. The central regulation is through the autonomic sympathetic and parasympathetic innervation of the vascular smooth muscle. The endothelial cells regulate locally through continuous production of vasodilators (nitric oxide) and vasoconstrictors like endothelin-1. These regulatory mechanisms adjust microvascular flow instantaneously to meet the metabolic needs of the tissue [38]. Diabetes, by affecting the autonomic nervous system, leads to a chronic hypercontracted state of the peripheral vasculature due to dysregulation of vascular tone. There is also decreased bioavailability of NO, and enhanced production of the vasoconstrictor endothelin-1. It has been shown experimentally that hyperglycemia acutely inhibits the production of NO from arterial endothelial cells [39]. The bioavailability of NO within the endothelial cells is reduce by presence of insulin deficiency or defective insulin signaling due to insulin resistance [40]. The consequent chronic vasoconstriction

also leads to hypertension and reduced blood flow to other tissues. A similar chronic state of vasoconstriction has also been also found in patients with the metabolic syndrome [41].

10.2.2.2 Vascular Basement Membrane Thickening (Ultrastructural Findings)

Capillary basement membrane thickening associated with prolonged hyperglycemia is a structural hallmark of diabetic microvascular disease. Thickening of the basement membrane impairs the selective transport of metabolites and nutrients from the circulation into the tissue [42].Transport of substances from the circulation into the interstitium, across the microvessel wall, is regulated by the pressure, flow, the size and charge specificity. Paradoxically, though microvessels display basement membrane thickening in diabetes, there is increase in microvascular permeability caused by alterations in the meshwork and changes in the electrical charge surrounding the pores between endothelial cells. These allow extrusion of large molecules into the endoneurial compartment that are normally excluded from transport across the microvasculature. Similarly transcapillary leak of albumin in the kidney causes albuminuria which therefore is an important indicator of widespread microvascular disease.

10.2.2.3 Endothelial Dysfunction and Hypercoagulable State

Endothelial dysfunction due to diabetes can lead to a hypercoagulable state due to activation of platelets and clotting factors in the blood. High plasma levels of beta-thromboglobulin, platelet factor 4, and thromboxane B2, reflect platelet activation. Coagulation activation markers, such as prothrombin, thrombin–anti-thrombin complexes, and clotting factors such as fibrinogen, factors VII, VIII, XI, XII, and von Willebrand factor are elevated in diabetes [43]while anticoagulant mechanisms are reduced tilting the balance to a hypercoagulable state [44].

Early detailed electron microscopic studies from sural nerve biopsies from patients with diabetic neuropathy revealed prominent alterations in the endothelium. [45]. The finding of prominent hyperplasia of endothelium almost occluding lumina of small vessels, would lead to reduction in blood flow through the microcirculation and ischemic damage to the endoneurial contents. Secondly, a careful study revealed pericyte damage, endothelial desquamation and small luminal thrombi. The desquamation of endothelium, on the one hand, expose the luminal blood to the underlying basement membrane activating coagulation cascade and on the other, reduced fibrinolytic activity which compromised the intraneural blood flow within the microcirculation.

Seepage of plasma and its components across the damaged vessel wall into the interstitium promotes fibrosis and results in the characteristic collagen deposition around the basement membrane. This is similar to the deposition of fibrin, albumin and globulins in the glomerular basement membrane in the kidney and the retinal

leaky vessels that are demonstrable on fluorescein angiographic studies. The pericyte pathology noted on electron microscopic studies are important. Pericytes in capillaries function like contractible smooth muscle cells to regulate vascular tone. Hence pericyte destruction would lead to aneurysmal dilation and rupture of microvessels as noted in diabetic retinopathy [46].

10.2.2.4 Role of Inflammation

Diabetes is now considered to be a state of chronic, low-level inflammation. Long standing hyperglycemia activates protein kinase C (PKC) pathway, which activates the transcription factor NF-κB and the enzyme NADPH oxidase. NF-κBrecruit circulating pro-inflammatory leukocytes, and promote apoptotic death of endothelial cells and pericytes. NADPH oxidase promotes production of reactive oxygen species (ROS) that further promote NF-κB transcription activity, setting up a vicious cycle. Further, elevated AGE levels cause activation of the AGE receptor (RAGE), which in turn promotes NF-κB transcription of pro-inflammatory genes.

Obesity associated with diabetes, induces the production of several cytokines (termed adipokines), in addition to tumor necrosis factor-α, interleukin 1β, interleukin 6, and plasminogen activator inhibitor 1 (PAI-1), that promote an inflammatory response [47]. With increase in fat mass, the levels of pro-inflammatory cytokines proportionately rise. Adiponectin, with anti -inflammatory properties is decreased in obese subjects [48]. This altered balance exacerbates the chronic inflammatory nature in obesity. These cytokines also have endocrine, autocrine and paracrine properties with effect on adjacent tissues.

10.2.2.5 Oxidative Stress and Free Radical Injury

Oxidative stress is currently the unifying factor in the development of diabetic complications. It appears to be the final common pathway of several processes. Hyperglycemia-induced mitochondrial ROS production activates each of the four major pathways implicated in hyperglycemia induced damage namely polyol pathway; formation of AGEs; activation of protein kinase C; and the hexosmine pathway. [49]. At the subcellular level, complications of diabetes, is linked to mitochondrial dysfunction. Hyperglycemia leads to increase ROS production [50, 51]. Pro-inflammatory cytokines and activated leukocytes enhance the production of reactive oxygen species (ROS) and free radicals that are toxic to the cell components, such as plasma membrane and organelles.

Results of a number of clinical trials have shown that strict glycemic control attenuated the onset and progression of diabetic neuropathy in T1DM, but not in T2DM patients, suggesting pathogenetic differences between the two [52]. Genetic factors have been explored to determine why some individuals develop a more severe polyneuropathy than others with similar diabetic status. Polymorphisms of the AKR1B1 gene have been found, which codes for aldose reductase, and are strongly associated with type 1 diabetes [53].

10.3 Cliniocopathogenetic Correlation

Understanding the pathophysiology in the context of the clinical subtypes of diabetic PN is enumerated below with case illustrations:

10.3.1 Hyperglycaemic Neuropathy

Patients with severe uncontrolled hyperglycaemia develop positive sensory symptoms in the lower limbs that are rapidly corrected by normalizing blood sugar levels suggesting direct association with hyperglycaemia. Electrophysiologic testing reveal reduction in nerve conduction velocity and increased resistance to ischaemic conduction failure attributed to switch to anaerobic glycolysis within the diabetic nerve. The positive sensory symptoms are attributed to hyperglycemic hypoxic changes that cause acidification of axoplasmic contents, alters fast K+ conductance and generates ectopic impulses to produce positive sensory symptoms [13]. Experimental models of acute, severe hyperglycemia can produce reduction in nerve conduction velocity and axonal shrinkage. Impaired glucose tolerance can also cause neuropathy which is milder, with small-fiber involvement [5].

10.3.2 Distal Sensory/Autonomic Polyneuropathy (DSPN)

10.3.2.1 Clinical Illustration

A 65 year old male presented with progressive burning sensation in soles of both feet till the ankle and hands. No weakness or cranial nerve symptoms were noted. He was a diabetic on oral hypoglycemic agents from the last 2 years. Examination revealed loss of pain and temperature in the extremities. Deep tendon reflexes were absent. No motor deficits were seen. Nerve conduction studies revealed symmetric sensory axonal neuropathy. Sural nerve biopsy revealed significant depletion of myelinated fibers (small fiber predominant) without any evidence of axonal regeneration. The loss was in sectorial pockets surrounding hyalinized vessels suggesting perfusion defect. Endoneurial and epineurial microvasculature showed prominent basement membrane thickening. There was no evidence of vasculitis (Fig. 10.1a, b).

10.3.2.2 Pathogenesis

Distal symmetric sensory polyneuropathy (DSP) is the commonest type of diabetic neuropathy. Itis characterized by length-dependent loss of sensation in a “stocking-glove” pattern. The longest axons to the feet are affected first, followed by involvement

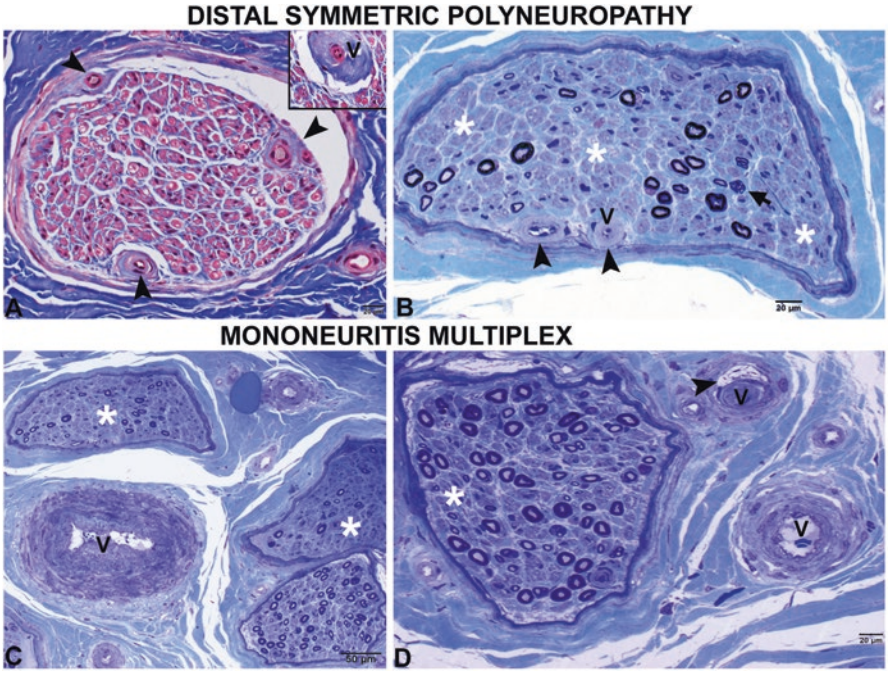

Fig. 10.1 (**a**, **b**) Sural nerve biopsy findings in length-dependent distal symmetric diabetic polyneuropathy. There is prominent endoneurial microvessel basement membrane thickening (*arrowheads*, **a**, **b**); narrowing of endoneurial microvessel reflects diabetic microangiopathy (**a**, *inset*). The myelinated fiber loss is prominent, in multifocal pockets (**b**, *) and small fiber predominant with characteristically mild axonal regeneration (**b**, *arrow*). [**a**: Masson trichrome, scale bar = 20 µm, **b**: Toluidine blue, scale bar = 20 µm] [v = vessel]. (**c, d**) Sural nerve biopsy in a diabetic with mononeurtis multiplex. Transverse sections from resin embedded nerve biopsy shows striking sectorial pockets of myelinated fiber loss (*asterix*, **c**, **d**), affecting fascicles in non uniform manner reflecting ischemia. The epineurial vessels (V) show concentric sclerosis. Also note the foam cells accumulating within smooth muscle cells of arterioles reflecting heralding atherosclerosis (**d**, *arrowhead*]. [**c**, **d**: Toluidine blue, **c**, scale bar = 50 µm. **d**, scale bar = 20 µm]

of the hands. Loss of sensation progresses in a distal-to-proximal fashion. Clinical manifestations depend on the nerve fibers involved. Neuropathy involving the small fibers appears first characterized by hyperalgesia and allodynia [54]. With progression, there is degeneration of larger myelinated nerve fibers that correlates with loss of deep tendon reflexes, sensory ataxia, and reduced proprioception [16]. Patients ultimately develop complete loss of sensation.

Histopathology revealsa distal axonal degeneration of dying back type [55]. There is central-peripheral distal axonopathy with involvement of the dorsal columns of the spinal cord and peripheral nerve but relative preservation of dorsal root ganglion cells [56].

Several histological abnormalities are described. These include axonal degeneration, progressing from axonal distension in early stages to axonal loss due to deple-

tion of microfilaments (cytoskeletal actin and myosin filaments) [27]. Importantly, distal sprouting of the proximal axonal stump following degeneration of the distal axon occurs only in the early stages when patients complain of paresthesias and pain. In later stages, there is conspicuous absence of regeneration [57, 58] heralding irreversible nerve damage.

Myelin alterations also occur with primary demyelination due to Schwann cell dysfunction, or secondary segmental demyelination due to impaired axonal control of myelination. There is Schwann cell proliferation, and atrophy of denervated bands of Schwann cells. Remyelination, onion-bulb formation, and hypertrophy of the basal lamina surrounding individual fibers are also described on ultrastructural studies.

It is debated if DSP is due to a direct metabolic effect or secondary to hypoxia following microangiopathy. The Diabetes Control and Complications Trial (DCCT) demonstrated that strict glycemic control with daily insulin injections can prevent or reduce the risk of developing neuropathy [59].The inability to maintain the axon has been attributed to metabolic abnormalities such as accumulation of sorbitol from increased flux in the polyol pathway, demonstrated in animal models. However the quantities of sorbitol detected in human diabetic nerve are insufficient to produce osmotic damage and, trials with aldose reductase inhibitors that reduce the production of sorbitol have so far failed to show any substantial improvement of the polyneuropathy [60]. Reduced nerve myoinositol concentrations by lowering Na+K+−ATPase activity, lead to “axoglial dysjunction” with paranodal swelling, axonal atrophy, and eventually nerve fiber degeneration [61]. However, the reduction of nerve myoinositol concentrations, paranodal swelling and axoglial dysfunction that was found was in experimental diabetes in rats has not been confirmed in human diabetic nerve.

Reduction in nerve perfusion occurs due to basement membrane thickening, pericyte loss and reduction in endoneurial capillary blood flow to C fibers. Hypoxia has been held responsible for the demyelination associated with diabetic PN [32, 62, 63]. Although histologically microvascular pathology with basement membrane thickening is a consistent finding, ischemic injury is not favored as the cause of DSP as ischemic injury usually causes predominant motor involvement and not sensory/autonomic neuropathy. Secondly, it would be difficult to explain the occurrence of a central-peripheral distal axonopathy on an ischemic basis and summation of multiple proximal nerve trunk lesions has been proposed [64].

The most important aspect of diabetic distal sensory polyneuropathy is the failure of axonal regeneration that contributes to the lack of reversibility of the neuropathy once established, even with good glycaemic control. It is not yet resolved whether this is due to alterations in the microenvironment or reduction in capacity of the neurons to mount a regenerative response. King et al. have elegantly summarized the various pathomechanisms to explain this phenomenon which include metabolic failure of neurons, ischemic effects due to microangiopathy, and glycation of Schwann cells and extracellular matrix [65]. The glycated collagen of extracellular matrix, is less susceptible to protease digestion and therefore acts as a barrier to penetration and elongation of the newly sprouting axons into the matrix. The next step for successful axonal regeneration involves adhesion to the Schwann

cell basal lamina. An intact basal lamina acts as a guide for regenerating axon to connect with the end organ. Glycation of the Schwann cell basal lamina by AGE, interferes with this step. Additionally, connection by regenerating axon is also prevented by glycation of cytoskeleton of the growth cones.

Dorsal root ganglia lack blood brain barrier and are hence vulnerable to damage from high glucose concentrations. In contrast, motor neurons in spinal cord are protected from high circulating glucose due to intact blood brain barrier [65]. The fenestrated blood vessels allow leakage of proteins into endoneurium. Experimental evidence however shows only mild loss of dorsal root ganglion cells [66]. Alteration in dorsal root ganglion cation channel signaling have been documented in rat models and reduction in blood flow into dorsal root ganglia [67, 68].

10.3.3 Focal and Multifocal Neuropathies

10.3.3.1 Clinical Illustration: Diabetes with Mononeuritis Multiplex

A 56 year old man with well controlled diabetes presented with paresthesias along palmar aspect of the medial two fingers of left hand and paresthesias along the sole of the right foot for 6 months. He worsened in the last 3 months and developed bilateral wrist drop and right foot drop. Examination revealed sensory loss along ulnar, radial and median nerve distribution in upper limb with wasting of thenar and hypothenar muscles. There was right common peroneal neuropathy with foot drop. His glycosylated Hb (HbA1c) was 6.5. Work up for systemic vasculitis, paraprotein and malignancy was negative. Nerve conduction studies revealed mononeuritis multiplex. He received steroids with glycemic control with no appreciable improvement in symptoms.

Sural nerve biopsy revealed strikingly non uniform degree of myelinated fiber loss, in large sectorial pockets reflecting ischemic damage. The fiber loss involved predominantly small diameter fibers. The endoneurial micro vessels revealed diabetic micorangiopathic changes with basement membrane hyalinization. No vasculitis was detected in the biopsy but the epineurial vessels revealed concentric thickening of the media, sclerosis and neovascularization. In addition, the medium sized muscular arterioles showed accumulation of foamy macrophages in the muscle coat suggesting atherosclerotic changes, due to macroangiopathy (Fig. 10.1c, d).

10.3.3.2 Clinical Illustration: Diabetic Lumbosacral Plexopathy

A 65 year old man presented with lancinating pain in the hip, buttock and thigh, involving right side more than the left since the last 10 days. He had lost almost 8 kg of weight in the last 2 months. He was a diabetic for the last 10 years, requiring insulin. His glycemic control was poor (glycosylated Hb was 10.0). On examination there was asymmetric weakness and wasting of the thigh muscles. Clinical

diagnosis was ‘diabetic plexopathy’. He received immunomodulaion with dramatic response.

Superficial peroneal nerve biopsy revealed necrotizing vasculitis with fibrinoid necrosis and transmural infiltration of nutrient vessel by lymphocytes, histiocytes and few plasma cells. There was florid neovascularization around inflamed vessels. Diabetic microangiopathic changes were not prominent. The fascicles showed sectorial pockets of myelinated fiber loss (small fiber predominant) with acute axonal and myelin degeneration (Fig. 10.2a, b).

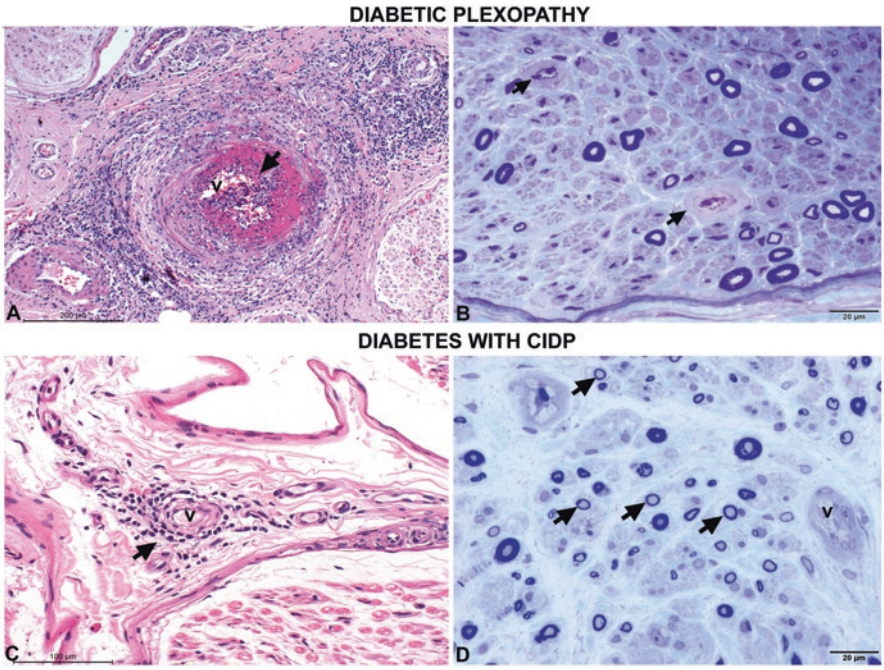

Fig. 10.2 (**a, b**) Diabetes with lumbosacral plexopathy. Cross section of a paraffin-embedded superficial peroneal nerve biopsy of a patient with diabetic plexopathy, shows prominent necrotizing vasculitis of the nutrient vessel (**a**, *arrow*) with transmural lymphoplasmacytic inflammatory infiltrate and fibrin deposition. Note inflammation enclosing adjacent small caliber vessels. There is severe degree of small fiber predominant loss of myeliinated fibers with conspicuous absence of axonal regeneration (**b**). Note hyalinised endoneurial microvessels (**b**, *arrows*). [**a**: Hematoxylin and eosin, scale bar = 200 μm, **b**: Toluidine blue, scale bar = 20 μm]. (**c, d**) Sural nerve biopsy in a diabetic patient with chronic inflammatory demyelinating polyneuropathy (CIDP). There are perivascular lymphomononuclear inflammatory infiltrates around small epineurial arteriole (**c**, *arrow*). There is prominent loss of myelinated fibers (demyelination) with several thinly myelinated fibers (signifying remyelination) (*arrows*, **d**). Endoneurial vessels (V) show prominent endothelium (**d**) reflecting to ongoing response to immune mediated damage. [**c**: Hematoxylin and eosin, scale bar = 100 μm, **b**: Toluidine blue, scale bar = 20 μm]

10.3.3.3 Pathogenesis

Focal peripheral nerve lesions are more common in diabetic patients and can present with a wide spectrum of manifestations that include cranial neuropathies of the third and seventh cranial nerves, thoraco-abdominal neuropathies, focal limb neuropathies, and proximal lower limb motor neuropathy (diabetic amyotrophy/lumbosacral plexopathy). The focal limb neuropathies are often at common sites of entrapment or external compression.

In diabetic cranial neuropathies, the abrupt onset suggests an ischaemic basis. However, histological studies have shown focal demyelination, which explains the recovery that can occurs, presumably by remyelination. As nerve ischemia generally gives rise to axonal loss rather than selective demyelination, it has been hypothesized that the demyelination in diabetic cranial neuropathies results from reperfusion injury which is known to produce demyelination [69].

In patients with proximal neuropathy of the lower limbs (plexopathies), biopsy specimens of the intermediate cutaneous nerve of the thigh and sural nerve revealed nerve ischemia, with inflammatory infiltrates around epineurial and perineurial blood vessels with admixture of B and T lymphocytes along with macrophages suggesting an autoimmune process. The reason for the predilection of these lesions in lower spinal roots, lumbar plexus and nerves of the lower limbs in proximal diabetic neuropathies is unclear [63, 70, 71].

Other focal peripheral nerve lesions have been attributed to result from an abnormal susceptibility of diabetic nerve to compression. The thickened basal lamina in diabetic nerves is abnormally rigid, due to cross linking of collagen by abnormal glycation end products. The reduced compliance of the basal lamina tubes around the myelinated fibers, renders them more vulnerable to mechanical shearing and damage.

10.3.4 Superimposed Chronic Inflammatory Demyelinating Polyneuropathy

10.3.4.1 Clinical Illustration

A 62 year old male, known diabetic presented with history of difficulty in walking of 6 months duration which was progressively worsening. On examination he had proximal weakness without wasting, with areflexia. CSF examination revealed elevated protein (62 mg%) with low cells (2 cells/cu mm). Nerve conduction studies showed slowing of conduction velocities with conduction block and prolonged latencies. F-waves were absent. Work up for systemic vasculitis, paraprotein and malignancy were negative. Provisional clinical diagnosis considered was chronic inflammatory demyelinating neuropathy (CIDP). Nerve biopsy revealed marked loss of myelinated fibers (small fiber predominant) with several thinly myelinated fibers (reflecting de/remyelination). Axonal regeneration was inconspicuous. Mild inflammation was noted around epineurial venules and arterioles. Diabetic microangiopathic changes

with basement membrane thickening were prominent. Biopsy features were suggestive of CIDP with diabetic vascular changes (Fig. 10.2c, d).

10.3.4.2 Pathogenesis

Chronic inflammatory demyelinating polyneuropathy (CIDP) is suspected in diabetic patients with a predominantly motor distal polyneuropathy, with markedly reduced nerve conduction velocities and conduction block [72]. An autoimmune process is suspected though only non specific antibodies are described till date. Patients improve with immunomodulatory therapy similar to CIDP patients without diabetes. Hence it is debated whether this is related to diabetes or an independent complication. Some authors believe that diabetic patients are more susceptible to develop CIDP, though reasons are unknown.

In summary, the metabolic and ischemic mechanisms lead to diabetic neuropathies. Metabolic factors operate in DSP, whereas an ischemic with superimposed inflammatory process is responsible for focal neuropathies, but there is a degree of overlap. The hyalinization of the walls of small blood vessels, due to reduplication of the basal lamina, is a diffuse process and leads to irreversible structural damage to the nerve whereas the early metabolic phase of diabetic neuropathy is reversible.

There has been some debate regarding whether the primary lesion in diabetic neuropathy includes the axon or Schwann cell/myelin. It is more likely that both components are affected depending on which mechanism is operative in individual cases, with overlap causing heterogeneity. Hyperglycemia induced metabolic derangements can directly affect Schwann cells causing myelin pathology, while alterations at nodes of Ranvier, and axoplasmic flow can lead to axonal degeneration. Structural changes in endoneurial microvessels leading to hypoxia and ischemia produce focal fascicular lesions. Alteration in the blood-nerve barrier introduces an immunological and inflammatory process producing a spectrum of focal neuropathies. In proximal diabetic neuropathy, the core pathology is an inflammatory vasculitis and ischemic nerve fiber degeneration. Truncal radiculopathy is secondary to an inflammatory polyganglionopathy. In III cranial nerve neuropathy, there is ischemia, though reversible. Mononeuropathies of upper and lower limbs result from carpal tunnel syndrome or cubital tunnel syndrome and pressure effects. Repetitive shear forces and excessive stiffness of enveloping connective tissues are also implicated in mononeuropathies.

Differences in pathogenesis have been noted between type 1 and type 2 diabetes. In type 2 diabetes, neuropathic complications occur late in the disease process. The initial deficits are milder due to evolving metabolic complications. As ischemic changes set it, deficits become irreversible. In type 1 diabetes, structural changes occur early and are of greater severity leading to early axonal atrophy and paranodal changes that are not seen in type 2 diabetes. These differences have been attributed to differences in insulin action, signal transduction and posttranslational modifications of nociceptive peptides in type 1 diabetes [73].

10.4 Treatment Prospects

10.4.1 Distal Symmetric Sensory Polyneuropathy

Results of a number of clinical trials have shown that maintaining strict glycemic control attenuated the onset and progression of diabetic neuropathy [59]. However, once neuropathy is established, it fails to improve significantly even with satisfactory glycaemic control. After 25 years of diabetes, about 50% of patients will have established neuropathy [74].Therapeutic trials based on proposed pathogenetic mechanisms have been carried out.

Accumulation of polyols, observed in animal model of diabetes, led to clinical trials with aldose-reductase inhibitors to treat diabetic polyneuropathy but failed to produce any clinical improvement [75]. In 2006, AKR1B1 gene polymorphism was detected in a study of Type 1 diabetic adolescents that could influence the treatment response to aldose reductase inhibitors [53].

Glucose and myo-inositol have structural similarity and in acute streptozotocin induced rats, increase in glucose within the nerve competitively reduces myo-inositol uptake in the diabetic nerve which impairs sodium/potassium ATPase in the axolemma, and slowing of conduction velocity and was corrected with dietary myoinositol supplementation [76]. Myoinositol accumulation has not been demonstrated in human nerve biopsies nor have clinical trials with myoinositol shown any benefit [77].

γ-linolenicacid is an essential fatty acids necessary for the maintenance of cell membrane. In diabetes, defective conversion of linoleic to γ-linolenic acid has been demonstrated and in animal models, administration of γ-linolenic acid was shown to improve nerve conduction velocity, by enhancing vascular perfusion in peripheral nerve [78].But this improvement was not demonstrable in human diabetic subjects.

Abnormal glycation of axonal neurofilament collagen of blood vessels and matrix resulting from hyperglycemia act on specific receptors, inducing monocytes and endothelial cells to produce cytokines and adhesion molecules and activate matrix metalloproteinases, which damage nerve fibers [65].Trials with Aminoguanidine, that inhibits AGE production improved blood flow and conduction velocity in experimental diabetic rats but not in humans [79].

Administration of growth factors such insulin-like growth factor I (IGF-I) in animal models of diabetic neuropathy has been shown to improve axonal regeneration suggesting a role in axonal maintenance [80, 81].

Modulation of mitochondria in dorsal root ganglia has been attempted with inconsistent results [82].Clinical trials of antioxidants such as α-lipoic acid, a powerful antioxidant that scavenges free radicals to regenerate glutathione improved nerve conduction velocity and had some positive effects on neuropathic symptoms, but not consistently [83–85].

10.4.2 Neuropathies Related to Dysimmune Mechanisms

The detection of inflammation/vasculitis in the peripheral nerves of patients with proximal plexopathy or superimposed CIDP and in autonomic ganglia in severe autonomic neuropathy has led to the use of immunomodulatory treatment. Treatment of patients with plasma exchange, corticosteroids, I ntravenous human immunoglobulin, or cytotoxic drugs (cyclophosphamide, azathioprine) either alone or in combination has been relatively successful [86]. However, the natural history of this disorder is often one of spontaneous improvement and a controlled clinical trial is now clearly needed.

10.4.3 Use of Growth Factors

Studies on animal models of diabetes have shown that use of IGF I enhanced regeneration with a beneficial effect. Preliminary evidence from phase II clinical trials using human recombinant NGF indicated amelioration in symptoms related to small fiber dysfunction [87]. Future treatment regimens could consider combinations of growth factors—NGF for improving small fibers function and brain derived neurotrophic factor (BDNF)—for targeting large fiber neuropathy.

Till date, no effective treatment strategies have been shown to reverse or arrest progression of diabetic neuropathy, once it sets in and prevention remains the only effective modality. It would be helpful to discover biomarkers to help to identify those patients who are more susceptible to develop this complication—for instance, genetic predisposition, and individualize treatment [88].

10.5 Animal Models of Diabetic Neuropathy

Mouse models of diabetes are critical for our understanding of peripheral neuropathy due to this metabolic disorder, and develop novel treatment strategies. In response to the explosion in research in diabetes and the burgeoning numbers of affected persons, the National Institutes of Health formed the Diabetic Complications Consortium (DiaComp; www.diacomp.org last accessed 10/08/2013) whose primary goal was to identify and characterize novel animal models of diabetic complications, provide a central resource for phenotyping protocols and establish criteria to define diabetic complications to minimize inter-investigator variability and will serve to screen the efficacy of the potential therapeutic interventions [89].

The most useful model of DN should exhibit the key feature present in human pathology and mimic the all major pathogeneses of human diabetic neuropathy. There are several established mouse models for diabetic neuropathies, and new mouse models that more faithfully reflect human disease process are being devel-

oped. Animal models are of two classes: induced models and genetic models. Induced models are drug-induced or diet-induced models of DN. Some commonly used animal models include streptozotocin-induced rat models, nutrition-induced models, or a combination of chemically- and nutrition-induced model. Recent models include the Zucker diabetic fatty rat model, type 1 insulinopenic BB/Wor and type 2 hyperinsulinemic diabetic rat models, and transgenic/knockout models [90].

There are advantages and disadvantages to each model for the investigation of rodent DN. The neuropathy phenotype in rodents is influenced by the strain, diet composition, insulin/C-peptide deficiency, hyperglycemia, coexisting hypertension and duration of diabetes. Several insights into the disease have emerged from the animal models.

An important pathogenetic role of impairment of insulin action rather than hyperglycemia per se, is suggested by the more severe abnormalities in type 1 diabetes rat models that are insulin deficient compared to type 2 DM models that are hyperinsulinemic [91].

Noninvasive monitoring of cutaneous nerve fiber loss was achieved using *thy1*-YFP mice that express yellowish-green fluorescent protein (YFP) in the sensory and motor neurons. Significant small fiber loss in the leg was observed at 3 months following the onset of diabetes, but loss of temperature (particularly heat) and pain perception occurred within 1 month of onset of diabetes, indicating that functional impairment precedes cutaneous nerve fiber loss.

10.6 Conclusion

A wide variety of syndromes involving the peripheral nerves are encountered in patients with diabetes mellitus, paralleling the diverse range of underlying causative mechanisms. The pathogenesis of diabetic neuropathies appears complex. Available clinical trials have not shown any promising efficacious treatment as there is interplay of more than one factor in pathogenesis. Hence a combination of several inhibitors maybe required. It is hoped that intensive research in diabetic PN will yield robust biomarkers that will determine the pathomechanism operating and thus individualize treatment. There are also pathogenetic differences between types 1 and 2 diabetes and these differences should be considered in the design of interventional paradigms.

Multiple types of models specific for the various forms of diabetic PN will eventually provide insights into the disease process and impact the translation of bench discoveries to the bedside. Advances in the technology will aid discovery of ‘neuropathic disease–modifying agent’ strategies, such as regeneration. Reversal and regeneration of damage to peripheral nervous system is the ultimate goal.

10.7 Perspectives

After perusal of enormous literature on biology of diabetic neuropathy, one realizes that we are still in muddy waters, with no clear discernible vision about the pathology.

The pathology of peripheral nerves in diabetes, especially – Type 2 DM, is varied with overlapping clinical features. The disease is heralded by hyperglycemic state, but the pattern of evolution leads to different syndrome complexes, the common denominator being axonopathy of motor, sensory, autonomic or a mixture of these with or without myelinopathy and reparative process.

The pathogenesis is multifactorial, the major events being metabolic toxicity and vascular ischemia, at the microvasculature level. There is considerable variation in the nature of pathologic alterations among types of diabetic neuropathies suggesting that they are heterogeneous. The participation of cytokine and autoimmunity mediated inflammation, altered vasoactive spasm of microvessels mediated by nitrous oxide, the metabolic oxidative damage, all participate in various proportions to result in final clinical manifestation. We need to invoke genetic susceptibility to explain varied clinical manifestations. Though animal models provide some insight in the distance evolution, the applicability to human biology is doubtful. Till date we have no accepted treatment strategy applicable especially because of various syndrome manifestations.

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Part III
Molecular Mechanisms

Chapter 11
The Renin Angiotensin System and Diabetes

Ana Cristina Simões e Silva, Rodrigo Novaes Ferreira, and Aline Silva Miranda

Abstract The Renin Angiotensin System (RAS) is clearly implicated in the physiopathology of diabetes mellitus (DM). The frequent association of diabetes mellitus (DM) with hypertension, retinopathy, nephropathy, and cardiovascular disease has implicated the RAS in the initiation and progression of these complications of DM. This has been supported by clinical trials in which RAS inhibitors significantly reduced the incidence of vascular complications in DM patients. The main RAS mediator, Angiotensin II (Ang II), exerts several deleterious actions in patients with DM, including increase in insulin resistance, endothelial damage and deterioration of renal function. On the other hand, only few studies have reported the potential protective role of the stimulation of the conter-regulatory RAS axis formed by the enzyme homologue to ACE, ACE2, the heptapeptide Angiotensin-(1-7) [Ang-(1-7)] and its receptor, the proto-oncogene Mas. In this review, we report recent experimental and clinical evidence in relation to ACE2 stimulation and Mas receptor agonists as potential therapeutic targets for DM.

Keywords Diabetes mellitus • Renin angiotensin system • Angiotensin II • Angiotensin-(1-7) • ACE2 • Mas receptor • Diabetic nephropathy

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11.1 Introduction

The renin-angiotensin system (RAS) has been implicated in complications linked with diabetes mellitus, including insulin resistance, endothelial damage and diabetic nephropathy [1–3]. Measurements of the RAS components in diabetic patients have shown conflicting results: some have found elevated levels, others, reduced, and others yet, found no change [4–6]. The picture can be further confusing given the activity of local and independently regulated RASs [7, 8]. However, the significant reno and cardioprotection that have been achieved by blockade of the RAS with angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor antagonists (ARAs) are strong compelling evidence for the role of the RAS in this disease [2, 9, 10].

Many studies have shown that Angiotensin (Ang) II exerts physiological and biochemical actions that may contribute to cardiovascular and renal damage [11]. The Angiotensin type 1 (AT_1) receptor mediates the main actions of Ang II [12]. Over the recent past, our view of Ang II has changed from being a simple vasoconstrictor to that of a complex growth factor mediating effects through diverse signaling pathways [8]. It has also become clear that Ang II is a key player in vascular inflammation. Through increased generation of reactive oxygen species (ROS) and activation of redox-sensitive transcription factors, Ang II promotes expression of cell adhesion molecules and induces synthesis of proinflammatory mediators and growth factors [8, 12]. These processes increased vascular permeability, leukocyte recruitment and fibrosis leading to tissue injury and structural remodeling. Targeting some of these signaling events with novel therapeutic strategies may provide important tissue protection in many forms of cardiovascular, renal and metabolic diseases.

On the other hand, it was originally thought that Ang II mediates all actions of the RAS. Over the past few years, other angiotensin peptides, like Ang III, Ang IV, and especially Ang-(1-7), were shown to selectively mediate different RAS effects [11, 13]. In regard to Ang-(1-7), this heptapeptide can be formed from Ang I by neutral-endopepdidase 24.11 or prolyl-endopeptidase or from Ang II via prolyl-endopeptidase, prolylcarboxypeptidase [14] or mainly by ACE2, an enzyme homologue to ACE [15, 16]. Ang-(1-7) binds to a G-protein coupled receptor, named Mas receptor [17], and, in general, plays a counter-regulatory role in the RAS by opposing the vascular and proliferative effects of Ang II [11, 13]. Currently, RAS is conceived as a system formed by two opposite axes: the first and classical one composed by ACE, Ang II and AT_1 receptor and the second and counter-regulatory axis comprising ACE2, Ang-(1-7) and Mas receptor [11, 13]. Experimental studies clearly support a role for the counter-regulatory RAS axis in diabetes. However, a limited number of studies have evaluated the components of ACE2-Ang-(1-7)-Mas receptor axis in diabetic patients [18–25], and the majority of them investigated only ACE2 [18–20, 23–25]. In this chapter, we report evidence for the role of ACE2-Ang-(1-7)-Mas receptor axis in diabetes mellitus and its complications, including poor glycemic control, diabetic nephropathy and cardiovascular alterations.

11.2 Role of ACE2-Angiotensin-(1-7)-Mas Axis in Glycemic Control

Identification of a local pancreatic RAS has led to a better understanding of the role of the RAS in the physiopathology of diabetes. RAS blockers seem to be able to reverse Ang-II-induced impairment in insulin sensitivity, insulin secretion, and pancreatic β-cell function [26–29]. However, the role of RAS blockers in this context is an ongoing matter of debate due to studies showing the inefficiency of RAS blockers in controlling hyperglycemic symptoms [30, 31].

On the other hand, ACE2 has received significant attention over the past years, being considered a promising target due to its beneficial role in glycemic control [32]. ACE2 was discovered in 2000 and shares 42% sequence homology with ACE, but cannot be inhibited by ACE inhibitors [15, 16].ACE2 gene is located on the Xchromosome and cleaves various substrates, including Ang II, angiotensin I (Ang I), apelin, neurotensin, and des-Arg bradykinin with the highest catalytic efficiency towards Ang II [15, 16]. Indeed, ACE2 is the main enzyme responsible for the conversion of Ang II into Ang-(1-7) in many organs and tissues [33].

ACE2 overexpression has been shown to reverse the detrimental phenotypes in cardiovascular disease [34, 35], diabetes [36, 37],and its related complications, an effect known to occur by suppressing the overactive Ang II levels [38]. The beneficial effects of ACE2 have been attributed to its capacity to increase Ang-(1-7) levels [15, 16, 33]. It has reported that Ang-(1-7) improves insulin sensitivity and glucose tolerance in experimental animal models, possibly by stimulating the insulin signaling via Mas receptor [39, 40]. Supporting this hypothesis, mice with genetic deletion of Mas receptor exhibit disturbances in glucose and lipid metabolism [41]. Furthermore, the increase in circulating levels of Ang-(1-7) improves glucose tolerance and dyslipidemia [42]. Even under physiological conditions, mice with genetic deletion of ACE2 progressively reduce insulin secretion and glucose tolerance [43]. However, when these knockout animals were under a high-fat high-sucrose diet,the degree of glucose intolerance is higher than in wild type mice [44]. This effect was attributed to the reduced skeletal muscle levels of GLUT4 and myocyte enhancer factor 2A expression [44]. It should be mentioned that the administration of Ang-(1-7) restored glucose tolerance [44]. These results support the importance of Ang-(1-7) signaling in maintaining glucose tolerance and insulin sensitivity.

The importance of the counter-regulatory ACE2-Ang-(1-7)-Mas axis in maintaining pancreatic β-cell function has been investigated in vitro [45]. The results showed that an upregulation in the expression of ACE2 and of Mas is associated with an increase in insulin secretion at high glucose concentrations [45]. In experimental models of diabetes, ACE2 levels decrease as the disease progresses, leading to an uninhibited rise in the activity of the classical ACE-Ang II-AT_1 axis [36, 46]. Thus, it can be speculated that, as the Ang II levels increase in hyperglycemic state, ACE2 is also upregulated as a compensatory mechanism and aids in the degradation and reduction of Ang II. Moreover, ACE2 gene therapy and the administration of

ACE2 activators, including xanthenone and diminazene aceturate [47], exerted beneficial effects in the face of diabetes [36, 48] and its complications [49, 50].

Various mechanisms have been proposed by which ACE2 elicits opposing effects on Ang II signaling. Oxidative stress has been reported to be one of the predisposing factors of pancreatic β-cell dysfunction during hyperglycemic states [51, 52]. Ang II activates reactive oxygen species (ROS) [53]. ACE2 over expression reduced oxidative stress and corrected Ang II-induced imbalance in the relationship between the expressions of AT_1 receptor and of ACE2 [37]. Both mechanisms improved glycemic control [37]. Pharmacological inhibition of ACE2 and of Mas receptor increased ROS formation induced by Ang II [54], further supporting the hypothesis that ACE2 reduces thecapacity of Ang II to form ROSby converting Ang II into Ang-(1-7).

Other mechanisms of impaired glucose homeostasis include endoplasmic reticulum stress, tissue fibrosis and inflammation [55–57]. The lack of ACE2 has been reported to exacerbate fibrosis and inflammation in the kidney [58, 59]and in the heart [60], whereas the overexpression of ACE2 decreased fibrosis in the heart [61], lungs [62], and pancreas [63].Moreover, Ang-(1-7) improved insulin sensibility, at least in part, via its anti-inflammatory properties in the liver [64].This effect was also associated with an up regulation in ACE2 expression in the liver. On the other hand, there are scarce information on the role of ACE2-Ang-(1-7)-Mas axis in preventing fibrosis in pancreatic islets [63]. The role of ACE2 in modulating endoplasmic reticulum stress, fibrosis, and inflammation in the islets warrants further investigation.

Figure 11.1 summarizes the main actions of the classical and the counter-regulatory RAS axes in the control of the glycemia and of the insulin secretion.

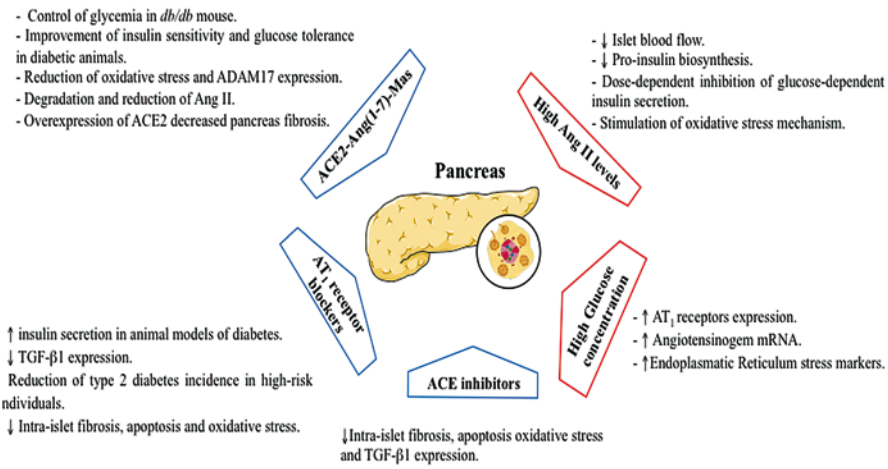

Fig. 11.1 The role of components of the classical and the counter regulatory renin-angiotensisn system axes in the control of glycemia Nd of the insulin secretion

11.3 Role of ACE2-Ang-(1-7)-Mas Receptor Axis in Diabetic Nephropathy

Diabetic nephropathy is one of the most common causes of end-stage renal disease and one of the main complications of diabetes, but the factors responsible for the development of diabetic nephropathy have not been fully elucidated [65].

There is growing interest in a possible role of ACE2 in diabetic kidney disease [19, 46, 65]. Activation of the classical RAS axis, ACE-Ang II- AT_1 receptor, is widely believed to contribute to kidney injury in diabetes [66]. ACE2 may act as a negative regulator of the classical RAS axis, exerting a renoprotective action [65]. Obese *db/db* mice (C57BLKS/JLepr) have been used as a model of type 2 diabetes, and their lean littermates (*db/m*) have served as nondiabetic controls [18, 19, 24, 65]. In renal cortical tubules of *db/db* mice, the pattern of ACE and ACE2 expression was characterized by low ACE, but increased ACE2 protein [65]. These alterations in ACE2 protein in renal tubules from diabetic mice are accompanied by corresponding changes in enzymatic activity [19]. Ye and co-workers [24]examined the localization of ACE and ACE2 in the glomerulus of control and diabetic mice. The glomerulus is the site of the nephron where the lesions of diabetic nephropathy appear earlier, and an increase in glomerular permeability is an early manifestation of diabetic kidney disease as reflected by the presence of albuminuria. The authors found that in glomeruli from *db/db* mice, ACE staining was higher than in control mice, while strong ACE2 staining in glomeruli from diabetic mice was less frequently seen than in controls [24]. In addition, the same research group reported that chronic blockade of ACE2 with the enzyme inhibitor, MLN-4760, in control or diabetic mice produced albuminuria and matrix proteins deposition [18]. In this regard, Wong and co-workers [67] examined the effect of deletion of the *ACE2* gene on diabetic kidney injury. In this study, ACE2 knockout mice [$ACE2^{-/-}$]were crossed with Akita mice ($Ins2^{WT/C96Y}$), a model of type 1 diabetes mellitus, and four groups of mice were studied at 3 months of age: $ACE2^{+/y}Ins2^{WT/WT}$, $ACE2^{-/y}Ins2^{WT/WT}$, $ACE2^{+/y}Ins2^{WT/C96Y}$, and $ACE2^{-/y}Ins2^{WT/C96Y}$. $ACE2^{-/y}Ins2^{WT/C96Y}$ mice exhibited increased mesangial matrix scores, glomerular basement membrane thicknesses, glomerular deposit of fibronectin and a twofold augmentation in the urinary albumin excretion rate compared with $ACE2^{+/y}Ins2^{WT/C96Y}$ [67]. The treatment with an AT_1 receptor blocker, irbesartan, reversed the alterations in renal histology and reduced proteinuria in $ACE2^{-/y}Ins2^{WT/C96Y}$ mice [67]. More recently, ACE2 knockout mice with streptozotocin-induced diabetes presented an increase in serum creatinine, urea levels and albuminuria in comparison with wild type diabetic animals [66]. In addition, glomerular and tubulointerstitial injuries and macrophage infiltration were significantly more severe inACE2 knockout mice than in wild type controls. AT_1receptor blocked with olmersartan attenuated the effects of ACE2 deficiency, but only partially [66]. Taken together, these studies suggested that ACE2 plays a protective role in the diabetic kidney, and ACE2 is an important determinant of diabetic nephropathy.

Some studies have also suggested a close correlation between albuminuria and ACE2. The treatment of HK-2 cells with bovine serum albumin has led to significant changes in ACE/ACE2 expression favoring Ang II formation [52]. More recently, Marquez and co-workers [68] showed that insulin increases ACE2 gene, protein expression, and enzymatic activity in cultured podocytes and these increases were maintained over time. In the presence of albumin, the beneficial effect of insulin on ACE2 expression and activity disappeared [68]. Therefore, ACE2 reduction might increase urinary albumin excretion, while albuminuria, in turn, could disrupt the balance of ACE/ACE2 expression [68]. In this regard, Riera and co-workers [69] studied the non-obese diabetic mice model, since these animals develop autoimmune diabetes that resembles human type 1 diabetes. At an early stage of diabetes, diabetic mice exhibited tenfold increase in urinary albumin excretion, glomerular enlargement, increased glomerular filtration rate and higher blood pressure in comparison to controls [69]. At a later stage, diabetic mice had a 20-fold increase in albuminuria, mesangial expansion and reduced podocyte number. Circulating and urine ACE2 activity were markedly increased at early and late stage of diabetes. Insulin administration prevented albuminuria, markedly reduced GFR, blood pressure, and glomerular enlargement at the early stage; and prevented mesangial expansion and the reduced podocyte number at the late stage of diabetes. The increase in serum and urine ACE2 activity was normalized by insulin administration at the early and late stages of diabetes. The authors conclude that diabetic mice develop features of early kidney disease associated with increased activity of ACE2 in both serum and urine and these alterations can be completely prevented by the administration of insulin.

Ang-(1-7) has also a role in experimental models of diabetes. The administration of Ang-(1-7) was able to normalize creatinine clearance and significantly attenuate proteinuria in Zucker diabetic fattyrats, a model of type 2 diabetes and diabetic nephropathy [70]. Diabetic rats treated with Ang-(1-7) displayed markedly reduction in renal fibrosis, presenting levels of extracellular matrix proteins similar to control animals [70]. Levels of TNF-α, IL-6, endothelin-1, and hypoxia inducible factor (HIF)-1α in the kidneys were also decreased to levels similar of those of control animals. The same effect was observed in renal and urinary levels of neutrophil gelatinase-associated lipocalin (NGAL), a marker of kidney damage [70]. Accordingly, chronic infusion of Ang-(1-7) also had significant protective effects in leptin deficient *db/db* mice, another model of type2 diabetes and diabetic nephropathy [25]. Animals treated with Ang-(1-7) for 28 days normalized urinary albumin excretion and significantly decreased kidney weight and mesangial expansion. Phosphorylation of STAT3 and renal fibrosis were also significantly reduced, as well macrophage infiltration in perirenal adipose tissue [71]. These findings suggest that both elevated levels of Ang II and decreased levels of Ang-(1-7) may contribute to renal damage [68].

In contrast to experimental studies, limited data were obtained in regard to ACE2-Ang-(1-7)-Mas axis in patients with diabetic nephropathy [22]. Most studies measured urinary levels of ACE2 in patients with type 2 diabetes [18, 20, 23, 24] and few others investigated mRNA and/or protein expression for ACE2 in human renal tissue [21, 25].

Concerning the studies that measured ACE2 in urine, Park and co-wotkers investigated whether urinary ACE2 levels are associated with abnormal glucosehomeostasis and urinary albumin excretion [23]. The authors found that urinary ACE2 levels were an independent predictor of microalbuminuria afteradjusting for other clinical risk factors in patients with type 2 diabetes [23]. In patients with type 2 diabetes and chronic kidney disease, Abe and co-workers showed that the treatment with the AT_1 receptor antagonist olmesartansignificantly increases urinary ACE2 levels independently of blood pressure andplasma aldosterone levels and reduces albuminuria, urinary liver-type fatty acidbinding protein, and plasma aldosterone levels [18]. The authors raised the possibility that increased ACE2 contributes to renoprotection elicited by olmesartan [18]. More recently, Liang and co-workers reported that urinary levels of ACE2 are increased in type 2 diabetic patients with various degrees of albuminuria [20]. Furthermore, the treatment with RAS inhibitors reduced urinary ACE2 excretion [20]. The authors concluded that urinary ACE2 measurement mightpotentially function as a marker for monitoring the metabolic status and therapeutic response to RAS inhibitors in diabetes [20]. Only one study investigated ACE2 in patients with type 1 diabetes and found that urinary ACE2 activity and protein expression are increased prior to the onset of clinical complications [19]. None of these studies have investigated the mechanisms that promote the elevation of ACE2 in the urine of diabetic patients. A possible explanation is that the augmentation of urinary ACE2 levels might be a compensatory mechanism in response to kidney injury in diabetic patients.

In regard to the evaluation of Ang-(1-7) and Mas receptor in diabetic patients, Mizuiri and co-workers reported that the proximal tubules from type 2 diabetic patients with nephropathy exhibited higherexpression of ACE and lower expression of ACE2, Ang-(1-7) and Mas receptor in comparison to healthy controls and to patients with minimal change nephrotic syndrome [21].

Figure 11.2 displays the effects of the classical and the counter-regulatory RAS axes in diabetic nephropathy.

11.4 Role of ACE2-Ang-(1-7)-Mas Receptor Axis in Diabetic Cardiovascular Disease

Diabetes mellitus is associated with substantial risk of heart failure and has been described as the leading cause of morbidity and mortality related with cardiovascular diseases (CVD) worldwide. Diabetic CVD includes myocardial infarction, mainly associated with premature atherosclerosis, and diabetic cardiomyopathy, characterized by left ventricular (LV) remodeling and dysfunction, both leading to heart failure [72, 73]. Indeed, diabetes has been considered not only a risk factor for CVD, but also a cardiovascular event equivalent, since diabetic subjects had a risk of cardiovascular complications similar to patients with previous myocardial infarction [74]. Accordingly, diabetic patients with myocardial infarction have worse prognosis than non-diabetic patients with myocardial infarction [75].

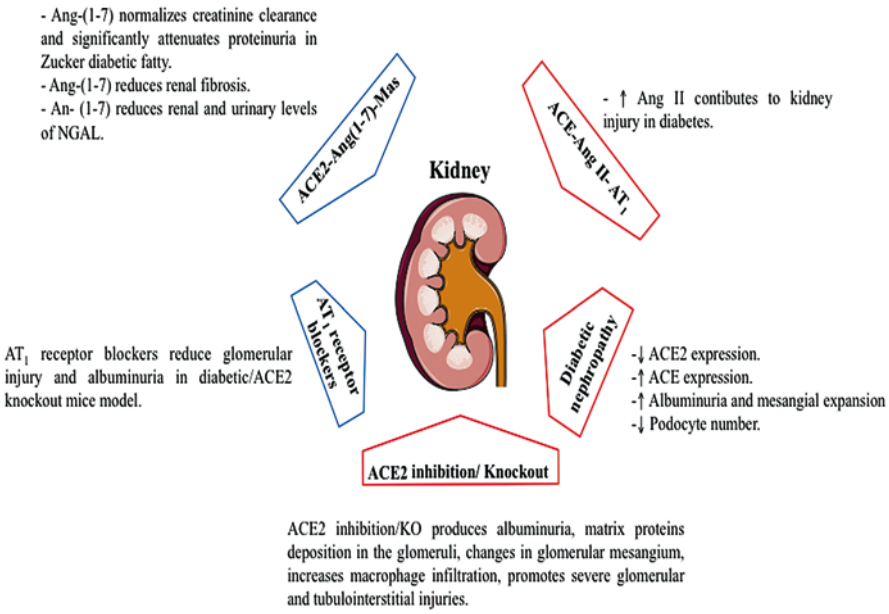

Fig. 11.2 The role of components of the classical and the counter regulatory renin-angiotensisn system axes in diabetic nephropathy

The pathophysiological mechanisms underlying diabetic CVD remain poorly elucidated. The discover that RAS key components are also locally expressed in different organs, including the heart, opens the road for the hypothesis that RAS exerted both hemodynamic and non-hemodynamic effects [76]. In fact, RAS components, including renin, angiotensinogen, ACE and Ang II receptors, were upregulated in the heart after cardiac injury, volume overload, myocardial infarction, and heart failure [77–80]. In the context of diabetes, over the past decades, clinical and experimental studies have been linked the classical RAS axis to diabetic CVD pathophysiology. For instance, ACE inhibitors, like perindopril, and AT_1 receptor blockers improved cardiovascular morbidity and mortality in patients with diabetes [81, 82] and prevented atherosclerosis and myocardial infarction in diabetic apolipoprotein E-deficient mice and in a streptozotocin-induced diabetes model [83–85]. There is evidence that Ang II by binding to its AT_1 receptors might mediate cardiovascular damage by inducing reactive oxygen species generation, tissue inflammation, fibrosis, and apoptosis [83, 84, 86–89].

A more modern concept has been supported that diabetic CVD depends on a balance between both RAS axes, the classical (ACE-Ang II-AT_1 receptor) and the counter-regulatory (ACE2-Ang-(1-7)-Mas receptor) [50, 90, 91].In line with this view, an elegant study demonstrated a significant reduction in cardiac ACE2 expression and activity along with elevated circulating levels of AngII and reduced Ang-(1-7) concentration in the heart in streptozotocin-induced diabetic mice. The changes in RAS components in response to diabetes induction were associated with

a significant cardiovascular damage, which included thinning of the LV wall, mild ventricular dilatation, increased cardiomyocyte apoptosis and compensatory heart hypertrophy [90]. Interestingly, the induction of diabetes by streptozotocin in mice genetically deficient for ACE2 did not change Ang II and Ang-(1-7) concentrations; neither led to cardiovascular dysfunction. Moreover, the absence of ACE2 also prevented the accelerated atherosclerosis found in diabetic apolipoprotein E-deficient mice. Altogether, these findings suggest that ACE2 might be a key factor in RAS activation in diabetic CVD, mainly by regulating cardiac levels of Ang II and of Ang-(1-7) [91]. Accordingly, in a model of human diabetes by employing the Akita mice with the loss of ACE2 expression increased plasma and heart tissue levels of Ang II, leading to systolic dysfunction on a background of impaired diastolic function [91]. The cardiovascular systolic alterations were associated with increased oxidative stress, degradation of the extracellular matrix activation of protein kinase C and loss of Akt and endothelial nitric oxide synthase phosphorylation, all of which prevented by the administration of the AT_1 receptor blocker, irbesartan [90]. Similarly, diabetes induction by streptozotocin in male Wistar rats resulted in diastolic dysfunction, cardiac hypertrophy and fibrosis along withACE2/ACE ratios imbalance, ERK1/2 phosphorylation and changes in the AMP-activated protein kinases, AMPK-α and AMPK-β1 expression. All these changes were prevented by the oral administration of the ACE2 activator XNT, suggesting that increase in ACE2 activity might be a promise therapy for diabetic CVD [50]. This hypothesis was supported by further studies showing that ACE2 over expression induced by a gene therapy with adenovirus was superior to losartan in attenuating diabetic cardiomyopathyas indicated by a decrease in myocyte hypertrophy, myocardial fibrosis, and LV remodeling and an improvement in LV systolic and diastolic function [92]. A protective effect was also found following an oral administration of the ACE2 activator, diminazene aceturate (DIZE), reflected by the improvement in cardiac electrical function in streptozotocin-induced diabetic rats [93].

Emerging evidence have been supported the idea that the beneficial effects of ACE2 is related with its capacity to convert AngII into Ang-(1-7). For instance, increased plasma levels of Ang-(1-7) were independently associated with a protection of left ventricular function in patients with type 2 diabetes mellitus [94]. Moreover, a growing body of experimental studies showed that the administration of Ang-(1-7) or of the Mas receptor oral agonist, AVE0991, significantly protects against diabetes-induced cardiovascular dysfunction [95–99]. Importantly, the opposite effect was observed with the administration of the Mas receptor antagonist, A779 [95, 98]. In this scenario, the elevation of Ang-(1-7) levels might also represent a promise therapeutic strategy for diabetic CVD.

The mechanisms underlying Ang-(1-7) cardiac protection might rely on the inhibition of inflammation and of oxidative stress by decreasing the transcript factor NF-kB activity and the NADPH oxidase activation, by restoring lipid profile alterations, and by reducing collagen and fibronectin-1 production, and TGF-β1 expression [95, 98, 99]. More recent studies, by employing the *db/db* mice, a well-established model of type 2 diabetic cardiomyopathy, showed that Ang-(1-7) improves myocardial hypertrophy and fibrosis by decreasing the lipotoxicity and

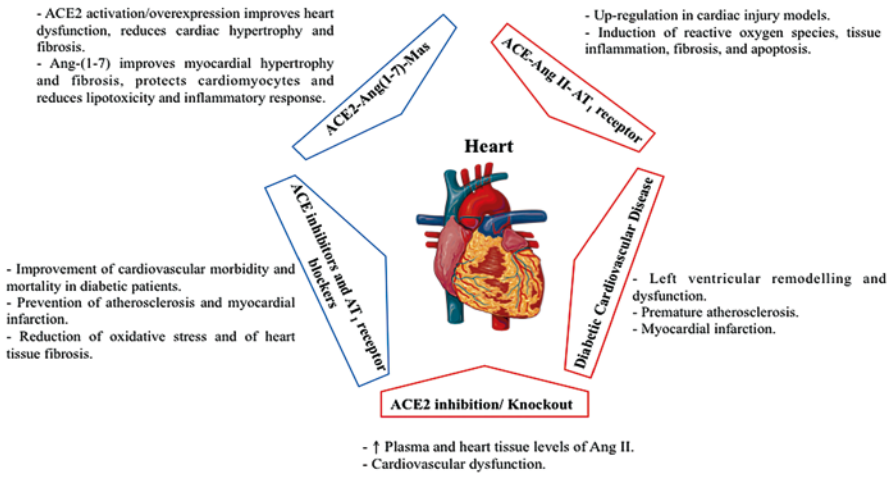

Fig. 11.3 The role of components of the classical and the counter regulatory renin-angiotensisn system axes in heart alterations in diabetes

the inflammatory response [100, 101]. Similar findings were reported by an in vitro study showing that Ang-(1-7) protects cardiomyocytes against high glucose-induced injuries by inhibiting the activation of the reactive oxygen species-activated leptin-p38 MAPK/ERK1/2 pathways [102].

It has been also reported that the cardioprotective effects of Ang-(1-7) may result from a complex interaction between AT2 and Mas receptors with a subsequent down-regulation of ACE expression and activity and of AT_1receptor expression, as well as up-regulation of ACE2 expression and activity [98, 103]. In addition, an increase in AT2 expression was associated with higher apoptosis rate of cardiomyocytes in diabetic rats [104]. In fact, the exogenous Ang-(1-7) significantly increased myocardial ACE2 activity and Ang-(1–9) levels, possibly via its effect on AT2 receptor. The increased activity of ACE2 leads to higher conversion rate of Ang II into Ang-(1-7), thus forming a positive feedback that elevates Ang-(1-7) levels, which, in turn, produce protective effects in diabetes-induced CVD [98].

Figure 11.3 shows the role of the classical and the counter-regulatory RAS axes in heart alterations of diabetes.

11.5 Conclusion

Despite available treatments for diabetes, a substantial population is still suffering from renal injury, cardiovascular alterations and other associated comorbidities. The inhibition of the classical RAS axis with angiotensin receptor blockers and/or ACE inhibitors is not effective for all cases and, in such conditions, we may speculate the usefulness of therapies to activate the counter-regulatory RAS axis.

Therefore, different ways to activate ACE2-Ang-(1-7)-Mas receptor axis emerge as a promise therapeutic strategy for diabetes and its co-morbidities.

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Conflict of Interest None declared

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Chapter 12
Adipokines and Vascular Disease in Diabetes

Camille M. Balarini

Abstract Adipose tissue is currently considered an endocrine organ. It releases adipokines with autocrine, paracrine and endocrine functions that regulate, among other homeostatic processes, inflammation, fat distribution, satiety and vascular function. It is important to highlight that, during obesity, the pattern of secreted molecules can change towards an overproduction of proinflammatory, diabetogenic and pro atherogenic ones. In diabetic patients, vascular dysfunction is an important risk factor for cardiovascular diseases. Considering the importance of adipokines in vascular function, here we present a brief review of the relevant aspects involved in the influence of adipokines in the establishment of vascular diseases in diabetes.

Keywords Adipokines • Diabetes • Vascular function • Inflammatory cytokines • Adiponectin • Leptin • TNF α • IL-6

12.1 Introduction

The view of adipose tissue has changed a lot in the past few years and we now know that this tissue is very complex, metabolically active and presents potent secretory features [1]. It can be considered not only an energy storage organ but also and endocrine organ [2]. Adipokines are a group of molecules released by adipose tissue with autocrine, paracrine and/or endocrine functions. Their target organs include adipose tissue itself, vessels, brain, liver and muscles. These molecules, which include adiponectin, tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), leptin and others, are involved in the regulation of important homeostatic processes such as inflammation, satiety, fat distribution and vascular function [3–5]. Although adipose tissue can constitutively secrete adipokines, during obesity the patter of secreted molecules can change towards an over production of proinflammatory, diabetogenic and pro atherogenic ones [3, 6]. Chronic silent inflammation is now

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considered a key feature in diabetes mellitus, cardiovascular diseases, obesity and metabolic syndrome [7].

Obesity is characterized by increase in fat mass deposition and it is closed related to insulin resistance and diabetes [8]. In this context, the expansion of adipose depots involves hyperplasia and hypertrophy of adipocytes and increasing number of other cell types such as macrophages, fibroblasts and lymphocytes [9]. In addition to visceral and subcutaneous fat, perivascular adipose tissue (PVAT) occurs naturally and increases proportionally to the increase in fat mass [10].These depots of fat around large, medium and small vessels may be considered important sources of vasoactive substances and adipokines as well. Therefore, PVAT plays an important role on vascular function, regulation of flow distribution and regional vascular (or *vasocrine*) signaling both by endothelium-dependent and independent mechanisms [10, 11]. Experimental studies revealed that PVAT is capable to release diverse anti contractile and/or vasodilator substances such as adiponectin, hydrogen sulfide, angiotensin 1–7 or prostacyclin. On the other hand, PVAT can also be considered as a source of vasoconstrictor agents. Although the identity of these substances are not as good documented yet as the vasodilators, it was described that COX inhibitor or α-adrenergic receptor antagonist blocked PVAT-induced vasoconstriction, suggesting adrenaline and COX-derived vasoconstrictor prostaglandins may be involved [12]. Moreover, perivascular fat may contribute both to insulin resistance and to macrovascular disease [11]. It is important to highlight that the profile of adipokines secreted by PVAT is different from other fat depots, suggesting that PVAT is a unique type of adipose tissue, with an independent impact on adjacent vasculature [10]. In obesity PVAT showed increased expression of TNFα and IL-6 along with reduced production of adiponectin [13].

In diabetic patients, vascular disease is clinically manifested by atherosclerosis, myocardial infarction, stroke, retinopathy, erectile dysfunction and other complications. Moreover, endothelial dysfunction can contribute to insulin resistance by reducing blood flow to target tissues, resulting in impaired delivery of glucose and insulin [14]. Considering the importance of adipokines released both by visceral, subcutaneous and PVAT in vascular function, here we present a brief review of the relevant aspects involved in the influence of adipokines in the establishment of vascular diseases in diabetes.

12.2 Adipokines and Vascular Function

12.2.1 Adiponectin

Adiponectin can be considered the most adipocyte-specific of all adipokines since it is expressed almost exclusively in adipose tissue. Nevertheless, Mori and colleagues demonstrated that adiponectin is located in endothelial cells in the normal vasculature [2]. Adiponectin concentration in plasma presents an inversely proportional relation with fat mass, in contrast with other adipocyte-derived substances [1, 15].

There is a suggestion that adiponectin is more closely related to insulin resistance than the fat mass *per se*. However, hypoadiponectinemia is a significant predictor of peripheral and coronary endothelial dysfunction independent of insulin resistance, body mass or dyslipidemia [6]. In men with type 2 diabetes, a moderate decrease in risk of coronary heart disease is associated with increased adiponectin levels [15].

There are two different receptors for adiponectin, known as AdipoR1 and AdipoR2. AdipoR1 is ubiquitously expressed in different tissues and at a high level in skeletal muscle [16, 17]. The intracellular pathway of this receptor involves the activation of AMPc production and adenosine monophosphate-activated protein kinase (AMPK) [18]. AdipoR2 is expressed mostly in liver [16, 17]. It was reported that it activates peroxisome proliferator-activated receptor alpha (PPARα) [18].

The vasoprotective effects of adiponectin are attributed to its actions on the vascular system including endothelial cells, smooth muscle cells, macrophages, leukocytes and platelets [6]. It was reported that administration of adiponectin to obese rats increase endothelium-dependent relaxation, a marker of vascular function [19]. On the other hand, dysfunctional endothelium can alter the profile of adipokines secreted by adipose tissue due to the release of proinflammatory cytokines. This contributes to a self-perpetuating cycle that exacerbates inflammation and contributes to the progression of vascular disease [4]. It was reported that AdipoR2 expression was reduced in diabetic patients compared to controls [20].TNFα and IL-6 are considered potent inhibitors of adiponectin expression and secretion [15].

The activation of AMP kinase intracellular pathway by adiponectin leads to an increase in endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production. It favors the phosporilation of eNOS at Ser1177 and Ser633, contributing to eNOS activation [6]. It is also involved in restoration of eNOS uncoupling, a phenomena usually related to increase in oxidative stress. Thus, adiponectin can contribute to increase in NO due to a direct and an indirect pathway related to reduced oxidave stress [6, 21]. It also suppresses the oxidation of low density lipoproteins (LDL), an important step that triggers inflammation during atherogenesis [5, 13].

All tissues are affected by adiponectin, directly or indirectly and improvements of tissue function are not only related to increase in adiponectin but also a direct consequence of it [1]. Adiponectin has been pointed to present anti-inflammatory, anti diabetic, antioxidant, vasodilatory and vasoprotective activities [6]. In absence of obesity adiponectin is particularly involved in vasodilatory, anti-inflammatory and anti-proliferative effects of PVAT [10]. It increases the number and function of endothelial progenitor cells, contributing to endothelium repair and angiogenesis [6, 22–24]. Adiponectin is capable to reduce the proliferation of vascular smooth muscle cells (VSMC), a feature usually observed in atherosclerosis progression [6].

Adiponectin is considered an inhibitor of leukocyte migration though endothelial wall once it reduces the expression of E-selectin and vascular cell adhesion molecule-1 (VCAM-1). This effect can be, at least in part, attributed to the fact that adiponectin can inhibit TNFα, resistin and IL-8-induced expression of endothelial adhesion molecules [6, 15, 25, 26]. On the other hand, it was described that acute treatment of endothelial cells with adiponectin can enhance the expression of adhe-

sion molecules. These contradictory results may be attributed to different forms of adiponectin found in plasma [6].

Considering that endocanabinoid system regulates food intake and energy homeostasis, and based on previous works that describe the beneficial effects of cannabidiol (CBD) and delta-9-tetrahydrocannabivarin (THCV) in obesity, insulin resistance and diabetes, Joodan and colleagues evaluated the effects of CBD and THCV in patients with type 2 diabetes. Although no differences in markers of vascular function were found, THCV alone was efficient in reducing fasting plasma glucose while CBD alone or in combination with THCV had no effect on glycaemic control. Also, THCV increased adiponectin despite no changes in leptin and resistin. CBD alone caused a reduction in resistin but had no effect on adiponectin or leptin. Both cannabinoids, alone or in combination, had no effect on TNFα or IL-6 [27]. This study emphasizes the importance of clinical trials, once basic and preclinical tests do not always present reproducible responses in humans. Also, it is important to clarify the correlations between adipokines and vascular function since changes in adipokines do not always lead to the expected amelioration in vascular function *in vivo*.

12.2.2 TNFα

TNFα is related to diverse aspects of vascular dysfunction in many tissues. During obesity and insulin-resistance, PVAT is involved in the release of proinflammatory adipokines such as IL-1, IL-6 and TNFα. Particularly, TNFα is produced as a 26-kD cell surface transmembrane protein which is released to plasma upon cleavage by TNFα converting enzyme (TACE, also known as A Disintegrin and Metalloprotease 17 – ADAM17) to produce a 17-kD biologically active form [8, 15, 28, 29]. It was suggested that adipocytes were the main source of TNFα in obesity. However, it has been recently recognized that infiltrated macrophages are the primary source of adipose tissue-derived TNFα [8]. Apart from its role in insulin resistance, TNFα is an important adipokine involved in vascular dysfunction during diabetes.

Interestingly, ADAM17 presents only one endogenous inhibitor: tissue inhibitor of metalloproteinase 1 (TIMP1) [29]. It was described that TIMP1 activity is reduced in obesity, diabetes and insulin resistance [30–32]. Adiponectin, on the other hand, increases TIMP1 expression in macrophages due to upregulation of IL-10, an anti-inflammatory cytokine [33]. Thus, adiponectin indirectly suppresses TNFα-induced endothelial dysfunction.

In obesity, TNFα from PVAT inhibits insulin-stimulated NO synthesis via a vasocrine signaling, which links insulin resistance to vascular disease [11]. Thus, in this situation, PVAT is thought to modulate insulin vasodilator effects through TNFα, instead of causing vasoconstriction itself. This diminished insulin-mediated vasodilation (or even vasoconstriction) could be a mechanism to protect the muscle from excess substrate delivery, although it contributes to exacerbation of insulin resistance [11]. TNFα can also impair endothelial function through activation of TNFR, leading to reduced eNOS expression due to suppression of promoter activity

and destabilization of mRNA. It reduces endothelium-dependent relaxation in vivo. Also, eNOS activity is reduced by TNFR activation by increasing of its endogenous inhibitor ADMA (asymmetric dimethyl arginine) [34].

Activation of NF-κB cascade by TNFα enhances expression of intercellular adhesion molecules such asICAM-1, VCAM-1 and E-selectin. Moreover, endothelial permeability to LDL is also altered by TNFα, contributing to transmigration of these particles to sub endothelial space and to the initiation of atherogenesis [5, 35]. NF-Kb activation can increase NADPH oxidase activity, contributing to oxidative stress, which uncouples eNOS and decreases NO bioavailability [34]. In obese women, visceral body fat correlates with endothelial dysfunction and the proposed mechanism underlie in the inappropriate cytokine secretion [15]. These patients showed increase in TNFα, IL-6, ICAM-1, VCAM-1 and impaired vascular response to L-arginine, the substrate of eNOS [15, 36].

In diabetes, structural alterations in renal microvasculature may be present even before the detection of urinary albumin. In this context, Tatsch and colleagues suggest that markers of oxidative DNA damage could be a better indicative of microvascular complications. The increase inoxidative DNA damage was also associated to a greater degree of inflammation (including higher TNFα levels) and high insulin resistance in the studied patients [37]. Interestingly, apart from its effects on plasma cholesterol, simvastatin was described to reduce TNFα and TNFα-induced apoptosis of endothelial progenitor cells, which are involved in endothelium recovery after injuries [38, 39]. These finding suggest that modulation of TNFα secretion and activity could contribute to the prevention of vascular dysfunction in diabetes.

12.2.3 IL-6

IL-6 is secreted by adipocytes in a "size-dependent" manner in which larger cells secrete higher amounts of IL-6 [40, 41]. Approximately 30 % of plasma IL-6 is attributed to be produced by adipose tissue [8], although it can also be produced by smooth muscle cells, particularly those found in atherosclerotic plaques [42]. PlasmaIL-6 correlates positively with insulin resistance and can be considered as a predictive of type 2 diabetes [8, 15].

Although many cells do not express the specific IL-6 receptor (IL-6R) they can be activated by a complex formed by IL-6 bonded to soluble IL-6R, which is released into serum by ADAM17-mediated shedding. This phenomenon is known as trans-signaling [41, 43, 44] and plays an important role in vascular dysfunction, since IL-6 can act through this pathway in virtually any cell type, including endothelial cells [45, 46]. Trans-signaling pathway is implicated in proinflammatory responses [45]. Activation of nuclear factor-kappaB (NF-kappaB) signaling pathway is associated with the production of IL-6 in vessels [47] and IL-6 is elevated in patients with type 2 diabetes [48].

In endothelial cells, IL-6 elicits an increase in the expression of adhesion molecules and lymphocyte migration through endothelial wall [44, 49, 50]. Moreover, it

can reduce NO production due to increase in TNFα [45, 48], which is considered a hallmark of endothelial dysfunction [51]. Administration of an antibody against IL-6R demonstrated beneficial effects on endothelial function and attenuated aortic stiffness [45]. On the other hand, Cotter and colleagues demonstrated that IL-6 given to diabetic rats was efficient in improving endothelium-derived hyperpolarizing factor (EDHF)-dependent vasodilation in renal artery, although no differences in NO-induced vasodilation nor adrenergic vasoconstriction were found [52]. In VSMC from atherosclerotic plaques, IL-6 acts an autocrine substance to further accelerate inflammation [45].

Although IL-6 anti-inflammatory and regenerative properties have been recognized, it is mostly considered a proinflammatory substance and an increase in plasma IL-6 has been characterized as a marker of metabolic disorder and cardiovascular disease [45]. According to Zhang and colleagues, IL-6 was the main factor that influenced genotype-dependent endothelial dysfunction among type 2 diabetic patients [48]. There is evidence that, in patients with diabetes, IL-6 levels show significant association to macrovascular complications. It is suggested that this could be a potential target to pharmacological approach and that IL-6 antagonists might reduce the risk of myocardial infarction, stroke or death [53]. IL-6 anti-inflammatory pathway is usually associated to classic signaling pathway (in cells that do express IL-6R), while trans-signaling is associated to proinflammatory actions. In this context, ADAM17 over-activity shifts IL-6 signaling balance towards trans-signaling, contributing to vascular inflammation [45]. It is important to highlight that the activity of ADAM17 only endogenous inhibitor, TIMP1, is reduced in diabetes [30–32].

In 1997 Bhagat and Vallance described that instillation of IL-6 in healthy volunteers during 1 h had no effect of its own on endothelial function but, when combined with TNFα and IL-1β, produced long-lasting impairment of endothelium-dependent relaxation [54]. It was demonstrated that, although IL-6 cannot act as a vasoconstrictor per se, it promotes an increase in phenylephrine-induced vasoconstriction in diabetic animals, suggesting this adipokine might be involved in the increased risk of hypertension development observed in diabetes [55]. Considering that ADAM17 expression and activity can be enhanced by angiotensin II [29], there is clearly and correlation between IL-6, hypertension and diabetes. IL-6 is considered an independent predictor for peripheral arterial disease and its genetic polymorphism is implicated in increasing susceptibility of this clinical manifestation among type 2 diabetic patients [50].

This adipokine also increases the production of C-reactive protein and TNFα by liver. Moreover, it also presents a positive feedback loop with intimal cells and macrophages to increase its own production [50]. IL-6 is related to EPC impaired function, which also favors vascular dysfunction [56] once it compromises endothelial repair. IL-6 is a stimulator of matrix metalloproteinase, contributing to vascular remodeling [41, 57].

12.2.4 Leptin

Leptin was firstly described in the classic paper by Friedmand and colleagues [58]. It is considered as an adipose-tissue specific adipokine which production is positively correlated to body fat mass [59, 60]. Leptin can cross blood-brain barrier and, in hypothalamus, decrease appetite and increase energy expenditure [61]. It is important to highlight that during obesity, although leptin levels are increased, its central anorectic action is imbalance while other activities are maintained, a phenomena known as selective resistance [61, 62].

Blood vessels express leptin receptors (named Ob-R) and its activation is considered to be proatherogenic, prothrombotic and angiogenic [59, 61, 63]. In endothelial cells, leptin impairs NO-dependent vasodilation and increases endothelin [63, 64]. Leptin can also augment oxidative stress due to increase in NADPH oxidase expression and activity [65, 66] which, in turn, contribute do decrease in NO [51].This adipokine also promote VSMC proliferation and migration, contributing to atherosclerosis and vascular remodeling. In diabetic patients, leptin was considered a determinant factor of intima-media thickness [67].

It was described that leptin increases the expression of angiotensin II type-1 receptor (AT1R) in smooth muscle cells [68]. This contributes to direct deleterious effects on vascular function and hypertension. Also, considering that angiotensin II can increase ADAM17 expression and activity [29], leptin can potentiate proinflammatory cascades mediated by ADAM17, such as those dependent of TNFα and IL-6. Additionally, angiotensin II increases leptin synthesis, creating a self-perpetuating cycle of oxidative stress and vascular dysfunction [61].

Although diabetes per se does not affect plasma leptin levels [69], they were are increase in obese diabetic individuals when compared to non-obese diabetic [69, 70]. Also, it was increased in hypertensive ones when compared to normotensive [69]. Interestingly, Morioka and colleagues (2014) suggested that leptin exerts vasodilator and positive endothelial effects in overweight diabetic patients. This effect is attributed to the release of NO, EDHF and/or prostacyclin by endothelium. These results are contradictory and can be attributed to the degree of obesity and age in the study. In fact, the patients were not very hyper lectinemic. This leads to the hypothesis that selective leptin resistance may not be limited to central nervous systems actions in controlling apatite and energy expenditure but may also involve hemodynamic effects of leptin [70, 71]. It was observed that in obese mice deficient in leptin (ob/ob mice), endothelial dysfunction was reversed by leptin repletion [72].

Leptin favors the migration on monocytes through endothelial layer not only by increasing the expression of adhesion molecules such as VCAM-1, ICAM-1 and E-selectin [63] but also indirectly, by increasing the secretion of proinflammatory substances including TNFα, IL-6 and MCP1 9 [61, 73]. Importantly, leptin can potentiate thrombus formation due to enhancement of platelets activation and aggregation and PAI-1 expression [64, 74], which is an important feature in atherosclerosis and contribute to the evolution of subclinical cases to stroke or acute myocardial infarction.

12.3 Conclusion

In conclusion, it is a consensus nowadays that adipose tissue present important secretory functions, acting like an endocrine gland. In the majority of cases, obesity and excess adiposity are involved with a proinflammatory profile of secreted adipokines that may compromise vascular function and contribute to increase cardiovascular risk. It is especially important in diabetic patients, a group which is already at an increased risk. These patients should maintain weight control to avoid further macro and microvascular complications.

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Chapter 13
G-Proteins in Vascular Complications of Diabetes

Madhu B. Anand-Srivastava

Abstract Vascular complications including impaired contractility and increased cell proliferation are the most common complications associated with diabetes, and chronic hyperglycemia appears to be an important contributing factor in this process. However, the precise mechanism(s) responsible for hyperglycemia-induced vascular dysfunction remains poorly characterized. Guanine nucleotide regulatory proteins (G-proteins) play a key role in the regulation of various signal transduction systems including adenylyl cyclase/cAMP and phospholipase C (PLC)/phosphatidyl inositol turnover (PI) which are implicated in the regulation of a variety of vascular functions including cell proliferation, hypertrophy, vascular tone and reactivity and the aberration of these pathways contribute to vascular complications in diabetes. The levels of inhibitory G-proteins (Giα-2 and Giα-3) are decreased in several tissues from streptozotocin diabetic rats and diabetic subjects. A relationship between the development of diabetes and Giα protein expression is also shown and suggests a role of decreased levels of Giα proteins in the pathogenesis of diabetes. In addition, exposure of aorta as well as VSMC with high glucose that simulate diabetic state also decreased the levels of Giα-2 and Giα-3 proteins. A correlation between the levels of glucose (in vivo and in vitro) and decreased expression of Giα proteins exists and suggests that hyperglycemia may be a contributing factor in diabetes-induced decreased expression of Giα proteins. The decreased levels of Giα proteins and associated adenylyl cyclase signaling in diabetes/hyperglycemia are attributed to the enhanced levels of vasoactive peptides. In addition, hyperglycemia-induced enhanced nitroxidative stress also contributes to the decreased expression of Giα proteins induced by high glucose. Furthermore, the basal adenylyl cyclase activity and cAMP levels are decreased in VSMC exposed to high glucose. On the other hand, Gqα proteins and the downstream molecules PKC and DAG are upregulated in different tissues from STZ-induced diabetic rats. In addition, VSMC exposed to high glucose also have enhanced expression of Gq/11α, PLCβ-1 and PLCβ-2 proteins. The enhanced levels of vasoactive peptides induced by

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hyperglycemia through oxidative stress, c-Src, growth factor receptor activation and MAP kinase signaling contribute to the enhanced expression of Gqα, PLCβ proteins in VSMC. The enhanced expression of Gqα and PLCβ has been shown to contribute to VSMC hypertrophy. It is thus suggested that the decreased levels of cAMP, Giα proteins and as well as overexpression of Gqα and PLCβ may be the contributing factors responsible for the vascular complications of diabetes/hyperglycemia. This review briefly summarizes some of the key studies on the modulation of G protein expression involving oxidative stress, growth factor receptor activation and associated signaling pathways in diabetes/hyperglycemia in VSMC and their potential role in the development of vascular complications observed in diabetes.

Keywords G-proteins • Hyperglycemia • Diabetes • Signalling pathway • Vascular disease • Vascular smooth muscle • Oxidative stress

13.1 Introduction

Chronic hyperglycemia is an important contributing factor in impaired contractility and cell proliferation leading to various cardiovascular complications associated with diabetes [1] and [2]. However, the precise mechanism(s) responsible for hyperglycemia-induced vascular dysfunction remains poorly characterized. Guanine nucleotide regulatory proteins (G proteins) play an important role in the regulation of vascular tone, and aberrations in these mechanisms may contribute to vascular complications in hyperglycemia/diabetes.

Guanine nucleotide regulatory proteins (G-proteins) are a family of guanosine triphosphate (GTP) binding proteins that play a key regulatory role as transducers in a variety of signal transduction system. These include adenylyl cyclase/cAMP system [3] the receptor-mediated activation of phospholipase C and A2 [4, 5] and a number of hormone and neurotransmitter-regulated ionic cannels [6, 7] G proteins are heterotrimeric proteins composed of three distinct subunits; α (alpha), β (beta) and γ (gamma) subunits [8]. The α-subunits bind and hydrolyse GTP and confers specificity in receptor and effector interactions [8]. The GDP bound form of α binds tightly to βγ and is inactive, whereas the GTP bound form of α dissociates from βγ and serves as a regulator of effector proteins. All α-subunits possess intrinsic GTPase activity and hydrolyse the terminal phosphate of bound GTP to yield bound GDP and free inorganic phosphate (Pi). Upon hormone binding and receptor activation, the receptor interacts with the heterotrimeric protein to promote a conformational change and dissociation of bound GDP from the guanine nucleotide binding site. GDP is released and replaced by GTP. Binding of GTP to α-subunit induces a conformational change and promotes the dissociation of hormone receptor complex (HR) and the holo G protein into α and βγ. Both α-GDP and βγ subunits can interact with effectors. This activation cycle is terminated by intrinsic GTPase activity of α-subunit. The GDP-bound form of α -subunit has high affinity for βγ and then

reassociates with the βγ dimmer to form the heterotrimer in the basal resting state. The family of G-protein α-subunits can be subclassified according to functional or structural relationship. More than 20 mammalian Gα gene products and several alternatively spliced isoforms have been identified. These can be divided into four major subfamilies according to amino acid homology and are represented by Gsα, Giα,Gqα/α11 and α 12/α13. The G proteins Gs and Giα are implicated in the regulation of adenylyl cyclase/cAMP signal transduction system whereas Gqα, G11α are implicated in the regulation of phosphatidylinositide signaling.

The hormone-sensitive adenylyl cyclase system is composed of three components: the receptor, the catalytic subunit, and G-proteins – stimulatory (Gs) and inhibitory (Gi). Molecular cloning has revealed four different forms of Gsα having molecular weights of 45, 45, 52 kD resulting from the different splicing of one gene [9–11]. Gsα is positively coupled to adenylyl cyclase and mediates the stimulatory responses of hormones on adenylyl cyclase [12, 13]. The Gs-mediated activation of adenylyl cyclase results in the increase formation of cAMP. cAMP activates cAMP-dependent protein kinase A that induces the phosphorylation of contractile filaments, sarcolemmal and sarcoplasmic proteins, and regulates intracellular calcium homeostasis [14]. In addition, Gsα was also shown to open the Ca^{2+} channels directly by cAMP-independent mechanism [15]. In contrast, Giα protein is associated with adenylyl cyclase inhibition [12, 13]. Three distinct forms of Giα, namely, Giα-1, Giα-2, and Giα-3 have been cloned and encoded by three distinct genes [16–18]. All three forms of Giα; Giα 1–3 have been shown to be implicated in adenylyl cyclase inhibition [19] and activation of atrial Ach-K^+ channels [20]. Both the Gα and Gβγ dimer mediate G-protein signaling. Five different β subunits of 35–36 kDa and 12 γ subunits of 8–10 kDa have been identified by molecular cloning. The βγdimer is tightly associated with GDP bound chain and facilitate interaction of G-protein with a receptor molecule. The effectors regulated by Gβγ include K^+ channels, phospholipase C-β, and adenylyl cyclase [21–23]. Like α-subunit the γ-subunit is subject to a cascade of posttranscriptional modification including isoprenylation and myristoylation that contributes to βγ membrane association and the interaction of the subunits [24].

G-protein α-subunits also possess specific residues that can be covalently modified by bacterial toxins. Cholera toxin catalyzes the transfer of ADP-ribose moiety of NAD to a specific arginine residue in certain α-subunits, whereas pertussis toxin ADP-ribosylates those α-subunits that contain a specific cysteine residue near to carboxy terminus. Modification of α-subunit by cholera toxin persistently activates these protein by inhibiting their GTPase activity, whereas pertussis toxin inactivesGiα protein and thereby results in the uncoupling of receptor from the effector. G protein α-subunits are regulated by covalent modifications by fatty acids myristate and palmitate. These lipid modifications serve to anchor the subunits to the membrane and increase the interaction with other protein and also increase the affinity of α subunit for βγ. In this regard, the myristoylation of Giα is required for adenylyl cyclase inhibition in cell free assay [25].

13.2 G Proteins and Adenylyl Cyclase Signaling in Diabetes

Several abnormalities in G-proteins expression and adenylyl cyclase activity has been shown in various pathophysiological conditions including diabetes [26, 27]. The decreased expression of Giα proteins has been reported in hepatocytes from human diabetics and STZ-diabetic rats [26, 27], whereas an increase in the levels and functions of Giα was shown in diabetic adipocytes from a genetic model of diabetes [28] Livingstone et al. [29] have shown a decreased expression of Giα proteins in platelets from diabetic subjects as compared to non-diabetic subjects. In addition, diabetic retina has been shown to exhibit decreased levels and functions of Giα [30]. Hattori et al. [31] have also reported a similar decrease in Gi protein in aorta from long-term diabetic rats, however, these investigators did not examine adenylyl cyclase Gi-protein signaling in their studies. However, Weber and McLeod [32]were unable to observe any changes in the levels of Giα proteins in aorta or caudal artery from 12 to 14 week-STZ-diabetic rats as compared to control rats. Further support and involvement of Giα-2 protein in pathogenesis of diabetes has been provided by the studies showing that the overexpression of constitutively activated Giα-2 ameliorates STZ-induced diabetes in rats [33]. In addition, a complete knockout of the Giα-2 gene that has been reported to produce a metabolic state resembling type II diabetes suggests the relationship between the decreased levels of Giα protein and diabetes [34]. However, Hashim et al. [35] showed that the aorta from STZ-diabetic rats exhibits decreased expression of Giα-2 and Giα-3 but not of Gsα proteins. An unaltered expression of Gsα in hearts from STZ-induced diabetic rat have also been reported [35, 36]. A relationship between the development of diabetes and Giα protein expression was also demonstrated [35]. The rats treated with STZ showed enhanced blood glucose levels within 2 days after injection with a concurrent decrease in the levels of Giα proteins, suggesting that the decrease in the levels of Giα proteins is associated with the development of diabetes. Subsequent increase in the levels of blood glucose through day 5 resulted in further decrease in the levels of Giα proteins suggesting a close relationship between decreased levels of Giα proteins and severity of diabetes [35]. The decreased levels of Giα proteins were reflected in decreased Gi functions whereasGsα–mediated stimulatory effects of hormones on adenylyl cyclase were augmented in STZ-aorta as compared to control aorta resulting in an enhanced levels of cAMP, whereas the basal cAMP levels were reduced in diabetic aorta [35].

13.3 G Proteins and Adenylyl Cyclase Signaling in Hyperglycemia

Furthermore, aorta as well as A10 VSMC exposed to high glucose (26 mM) that simulate diabetic state also exhibited decreased levels of Giα-2 and Giα-3 proteins, whereas the levels of Gsα were not altered [37]. A correlation between the levels of

glucose (in vivo and in vitro) and decreased expression of Giα proteins was also reported and suggests that hyperglycemia may be a contributing factor in diabetes-induced decreased expression of Giα proteins [37].In addition, aortic VSMC from STZ-diabetic rats alsoshowed decreased expression of Giα proteins [38], suggesting that aortic VSMC cultured from STZ-diabetic rats retained the diabetic phenotype.

Hyperglycemia was also shown to stimulate adenylyl cyclase activity in bovine aortic endothelial cells which causes an inhibition of glucose-6-phosphate dehydrogenase and thereby results in decreased levels of NADPH that may be responsible for hyperglycemia-induced apoptosis [39]. In addition, an increased stimulation of cAMP levels by OP-1206, alpha CD, an analog of prostaglandin E1 (PGE1), was reported in sciatic nerve from STZ-diabetic rats that were shown to increase Na^+/K^+ ATPase activity [40]. In support of these studies, Hashim et al. [37]reported an increased stimulation of adenylyl cyclase activity by isoproterenol and glucagon in A10 vascular smooth muscle cells exposed to high glucose (26 mM) as compared to the cells exposed to normal glucose (5.5 mM). These enhanced stimulations were attributed to the decreased expression of Giα-2 and Giα-3 levels and not to increased levels of Gsα proteins, because the levels of Gsα proteins were not altered in hyperglycemic cells. Taken together, it may be suggested that hyperglycemia may be a contributing factor in diabetes-induced decreased expression of Giα proteins. However, Mancusi et al. [41]were unable to show any changes in Gi protein expression in human umbilical vein endothelial cells (HUVEC) exposed to high glucose for 15 days.

High glucose-induced decreased expression of Giα proteins was also reflected in decreased Gi functions [42]because angiotensin II (Ang II), oxotremorine (Oxo) and C-$ANP_{4–23}$ (a ring deleted peptide of ANP)-mediated inhibitionswere almost completely abolished in cells exposed to high glucose [43–45]. In addition, hyperglycemia-induced down-regulation of, vascular NPR-C, AT1 and arginine-vasopressin (AVP) receptors may also be responsible for a complete attenuation of inhibitory responses on adenylyl cyclase. In this context, acute hyperglycemia induced by STZ or alloxan has been shown to decrease the levels of vascular NPR-C, AT1 and arginine-vasopressin (AVP) receptors [46, 47]. Hyperglycemia has also been shown to impair voltage gated K^+ channel current in rat small coronary VSMC [48]. Since Giα proteins are implicated in the activation of K^+ channels, it may be possible that the impairment of K^+ channel activity may be attributed to the decreased levels of Giα protein induced by high glucose. On the other hand, basal adenylyl cyclase activity was significantly decreased in A10 VSMC or aorta exposed to high glucose [37]. Since decreased cAMP levels have been shown to augment cell proliferation [49], it may be possible that the decreased basal adenylyl cyclase activity and thereby decreased cAMP levels induced by high glucose may be a contributing factor in increased cell proliferation observed under hyperglycaemic conditions and diabetes [50].

13.4 Oxidative/Nitrosative Stress and G- Protein-Adenylyl Cyclase Signaling in Diabetes and Hyperglycemia

Increased oxidative stress has been shown to be an important contributing factor in the development of micro- and macrovascular complications of diabetes, which include nephropathy, retinopathy, and neuropathy as well as endothelial and vascular smooth muscle dysfunction [51–53]. Enhanced oxidative stress induced by hyperglycemia has also been reported in cultured VSMC and different tissues from STZ-diabetic rats [38, 51–53]. In addition, the contribution of enhanced production of superoxide anion (O_2^-) in the decreased expression of Giα proteins has also been reported in aortic VSMC from STZ-diabetic rats and A10 cells exposed to high glucose [38]. Antioxidants such as α-tocopherol, NAC; scavengers of O_2^- and DPI, an inhibitor of NADPH oxidase that were shown to restore the enhanced levels of O_2^- induced by hyperglycemia also restored the hyperglycemia-induced decreased expression of Giα-2 and Giα-3 to control levels [38].These studies suggest the implication of NADPH oxidase/O_2^- in hyperglycemia-evoked decreased expression of Giα proteins. In addition, hyperglycemia-induced decreased expression of Giα proteins was also shown to be attributed to the increased levels of peroxynitrite because scavengers of peroxynitrite; uric acid and MnTBAP restored the hyperglycemia-induced decreased expression of Giα proteins to control levels [38], suggesting a role of nitrosative stress in decreased expression of Giα proteins in hyperglycemia. Further, there is an accumulating evidence that supports the hypothesis that diabetes is associated with increased nitrosative stress and increased peroxynitrite formation in several tissues both in experimental animals and humans [54]. The increased levels of nitrotyrosine, a relatively specific marker of peroxynitrite formation has also been shown in different tissues from STZ-diabetic rats and diabetic subjects [55]. For example, an increased nitrotyrosine plasma levels were shown in type 2 diabetic patients [56] and increased production of iNOs-dependent peroxynitrite was shown in platelets from diabetic individuals [57]. In addition, hyperglycemia has also been reported to induce increased nitrotyrosine formation in the artery well of monkeys [58]. Taken together, it may be possible that the levels of peroxynitrite, formed by the interaction of NO and O_2^- may contribute to hyperglycemia-induced decreased expression of Giα proteins in VSMC.

Thus, we have shown that diabetes/hyperglycemia decreased the expression of Giα proteins and associated adenylyl cyclase signaling which may be attributed to the augmented levels of Ang II that enhance the nitroxidative stress by increasing the levels of O_2^- and $ONOO^-$ (Fig. 13.1). The treatment with antioxidants reversed the hyperglycemia-induced decreased expression of Giα proteins and adenylyl cyclase signaling to control levels. In this regard, the overexpression of constitutively activated Giα-2 has also been shown to improve STZ-induced diabetes in rats [33, 51].Taken together, it may be suggested that antioxidants by augmenting the decreased levels of Giα proteins-induced by high glucose may have beneficial effects in improving the cardiovascular complications of diabetes.

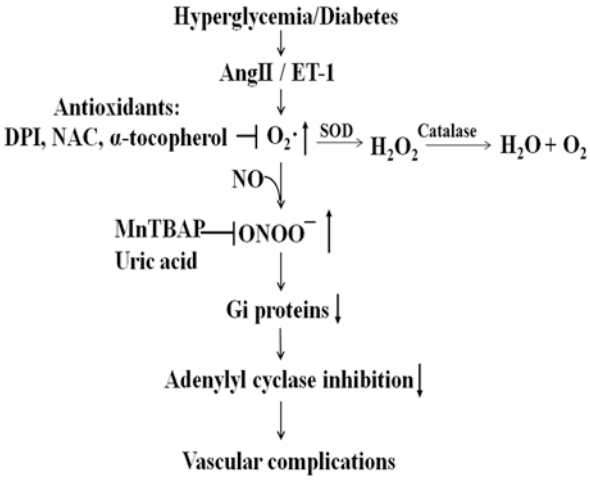

Fig. 13.1 Schematic diagram summarizing the possible mechanisms by which hyperglycemia/diabetes decreases the expression of Giα proteins and adenylyl cyclase signaling. *NO* Nitric Oxide, *ONOO-* Peroxynitrite, *SOD* Superoxide Dismutase

13.5 Gqα Proteins and Phosphatidyl Inositide Signaling in Diabetes/Hyperglycemia

The activation of Gqα by a G-protein coupled receptor (GPCR) stimulates PLCβ which hydrolyzes inositol biphosphates (PIP_2) to produce the second intracellular messengers, inositol triphosphates (IP_3) and diacylglycerol (DAG) [59, 60]. IP_3 stimulates the release of the calcium (Ca^{2+}) from the intracellular stores and DAG activates the protein kinase C (PKC). The release of intracellular calcium activates the calcium channels localized at the cell surface thus allowing the uptake of extracellular calcium inside the cell [61].

Gqα and associated signaling has been shown to play an important role in the regulation of cardiovascular functions. Alterations in the levels of $G_q\alpha$ protein and associated signaling pathways appear to contribute to the impaired cellular functions in several pathological states including diabetes, hyperglycemia, and cardiac hypertrophy [40, 62–65].A genetic ablation of $G_q\alpha$ in mice has been shown to result in a cardiac malformation and craniofacial defects [66],whereas an overstimulation of the G_q pathway in mice was shown in the development of hypertrophic cardiomyopathy [63].The $G_q\alpha$ protein and associated signaling pathway activated by several hormones such as Ang II, endothelin-1 (ET-1), and phenylephrine has also been implicated in the development and progression of cardiac hypertrophy and heart failure [67–71]. Cardiac over expression of $G_q\alpha$ in transgenic mice has also been reported to result in hypertrophy and induction of classic hypertrophy gene expression profile [72]. In addition, the transgenic over expression of a $G_q\alpha$ dominant negative mini gene that resulted in the lack of hypertrophy response to transverse aortic constriction (TAC) [62] further supports the implication of $G_q\alpha$ in hypertrophy. In addition, the $G_q\alpha$ signaling components including IP_3-Ca^{2+} and DAG-PKC have also been shown to play an important role in the development of cardiac hypertrophy in the stroke-prone spontaneously hypertensive rat (SHRSP) [73].

Furthermore, vascular G_q-coupled signaling has also been shown to contribute to the development of cardiac hypertrophy by using transgenic mice with vascular-specific $G_q$nhibitor expression [74]. We also showed the implication of G_q and MAPK/phosphatidylinositol 3-kinase signaling in VSMC hypertrophy induced by vasoactive peptides in A10 VSMC [75] In addition, Ang II-induced VSMC hypertrophy has also been shown to involve Gq signalling [76].

Diabetes-induced alterations in enhanced expression and activity of Gqα, PKC and DAG have been reported in different tissues from STZ-induced diabetic rats as well as in Bio-Breeding (BB) rats [77–81].In addition, high glucose treatment has also been reported to enhance the activity of PKC and DAG in cultured aortic endothelial and vascular smooth muscle cells (VSMC) [78, 82, 83]. The aortic VSMC from STZ diabetic rats were shown to exhibit enhanced expression of Gqα and PLCβ1 proteins [64]. Furthermore, Descorbeth and Anand-Srivastava [64] have reported that VSMC exposed to high glucose exhibit enhanced expression of Gq/11α, PLCβ-1 and PLCβ-2 proteins, the upstream signaling molecules of PI turnover and enhanced formation of IP_3 by ET-1 [64]. In addition,an increased expression of $G_q\alpha$ in sciatic nerves [77] and hearts [80, 81] from STZ-diabetic rats was also shown. However, Ceccarelli et al. [84] showed a decreased expression of $G_q\alpha$ in bovine retinal pericites exposed to 25 mmol/l of glucose [84]. In addition, an unaltered or a decreased expression of G_q/11α protein was also shown in gastric VSMCs from 10-week STZ-diabetic rats and a genetic model of non-insulin-dependent diabetes (11–12 months) WBN/Kob diabetic rats [85], respectively. The apparent discrepancies may be attributed to the difference in the cell type used and its origin.

Vasoactive peptides such as Ang II and endothelin-1 (ET-1), which can be synthesized locally in the vasculature, have been implicated in diabetes-associated vascular dysfunctions, including vascular remodeling, hypertrophy, and proliferation of VSMCs [86–91], leading to an impaired relaxation to vasodilators or an exacerbated response to vasoconstrictors. The levels of ET-1 and Ang II that are elevated in plasma from both type 1 and type 2 diabetes and also in experimental models [92–94], as well as in aortic endothelial and smooth muscle cells in the presence of high glucose [95–97], were shown to contribute to the vascular complications of diabetes. In addition, the enhanced expression of G_q/11α and PLC-β proteins in VSMC exposed to high glucose was also shown to be attributed to the enhanced levels of endogenous Ang II and ET-1 [95–97], because losartan, a selective AT_1 receptor antagonist, BQ123 as well as BQ788, ET_A and ET_B receptor antagonists, respectively, completely prevented high glucose-induced enhanced levels of G_q/11α and PLC-β protein in VSMCs and suggests that these effects may be mediated by an autocrine production of Ang II and ET-1.In this regard, the levels of various vasoactive peptides including Ang II and ET-1 which are augmented in diabetes and under hyperglycemic conditions [93–97].The underlying mechanism(s) by which AT_1 and ET_A/AT_B receptor activation by high glucose induces increased expression of Gqα and PLCβ1 proteins and vascular dysfunction involve oxidative stress because Ang II and ET-1 have increase oxidative stress by activating NADPH oxidase, an enzyme responsible for the production of superoxide anion and other reactive oxygen species [98].

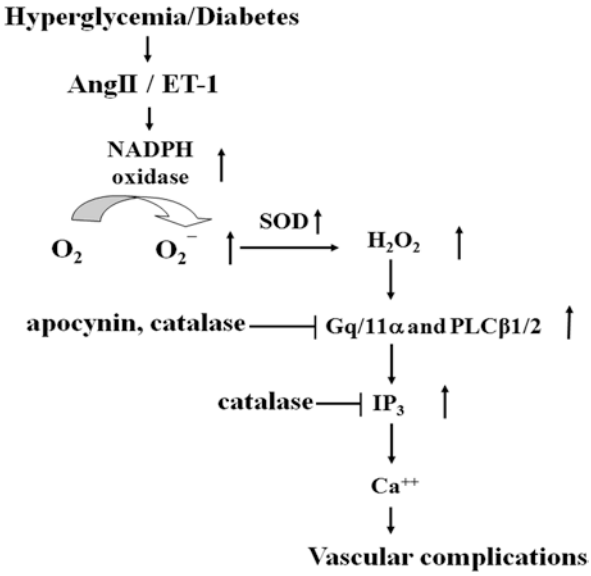

Fig. 13.2 Schematic diagram summarizing the possible mechanisms by which hyperglycemia/diabetes increases the expression of Gq/11α and associated cell signaling

The implication of enhanced oxidative stress in high glucose-induced enhanced activity/protein levels of PKC and DAG in VSMC was demonstrated by the study [99] showing that intraperitoneal injection of the antioxidant α-tocopherol into diabetic animals or the incubation of VSMCs with α-tocopherol prevented the increase in the levels of DAG and PKC due to diabetes and hyperglycemia, respectively [99]. In addition, antioxidants: apocynin, an NADPH oxidase inhibitor, and catalase, a scavenger of hydrogen peroxide but not^{111}Mn-tetrakis(benzoic acid porphyrin) (MnTBAP) and uric acid, scavengers of peroxynitrite restored the hyperglycemia/diabetes-induced enhanced levels of Gq/11α and PLCβ1/2 proteins to control levels [100] further suggest that O_{2-} and H_2O_2 but not peroxynitrite contribute to the enhanced expression of Gq/11α and PLCβ proteins in diabetes/hyperglycemia (Fig. 13.2).

13.6 Growth Factor Receptor Transactivation and Associated Cell Signaling in Gqαand PLC Protein Expression and Vascular Complications in Hyperglycemia

The role of growth factor receptor activation in VSMC hypertrophy and proliferationhas been shown [101, 102]. High glucose has also been reported to induce growth factor receptor activation/phosphorylation [103–106]. Belmadani et al. [107] showed that endothelial and VSMC from mesenteric resistance artery as well as coronary artery from db/db mice exhibited an enhanced phosphorylation of EGF-R. In addition, enhanced levels of PDGFβ-R were also shown in aortic VSMC from

STZ-diabetic rats compared with normal cells [106]. Furthermore, the implication of PDGF-R and EGF-R in high glucose-induced enhanced levels of Gq/11α and PLCβ protein as well as enhanced PLC signaling activated by ET-1 has been shown [64] and suggest that growth factor receptor activation through Gqα and PLCβ1 proteins may also contribute to VSMC hypertrophy in hyperglycemia. In addition, VSMC under hyperglycemic conditions as well as VSMC from diabetic ratsalso exhibit exaggerated cell proliferation compared to control cells [108–110]. The implication of EGF-R and PDGF-R in vascular dysfunction including remodeling, migration and proliferation of VSMC has been reported in diabetes [111–113]. This was further supported by the study showing that the inhibition of growth factor receptors, EGF-R and PDGF-R attenuated the enhanced proliferation of aortic VSMC from STZ-diabetic rats [100].

A role of MAPK and PI3K in protein synthesis and cell proliferation has been well established [42, 114–116]. Hyperglycemia has also been shown to increase the phosphorylation of ERK1/2 and AKT in human VSMC [117] and in VSMC from mesenteric resistance arteries and in A10 VSMC [100, 118] that contributes to vascular dysfunctions [118] and [117] . Campbell et al. [117]have also shown that high glucose-induced increased AKT and ERK1/2 activity was associated with VSMC chemotaxis. In addition, high glucose-induced phosphorylation of ERK1/2 was also shown to result in increased deposition of collagen in mesenteric resistance arteries [118]. In addition, MAPK, and PI3K/AKT are implicated in the hyperproliferation of VSMC from STZ-diabetic rats. The activation of MAPK and PI3K pathway by high glucose has also been shown to enhance the expression of Gqα/PLCβ proteins [119]suggesting that high glucose through Gqα/PLCβ proteins and MAPK/PI3K signaling result in VSMC hypertrophy.

The precise mechanism by which high glucose increased AKT and MAPK activity is yet not known. However, the implication of growth factor receptors in the activation of MAPK and PI3-K/AKT pathways has been shown by several studies [120–122]. Zhang et al. [123] have shown that PDGF-induced cardiac fibroblast and aortic VSMC proliferation was mediated through the activation of ERK1/2 and AKT pathways. In addition,the study showing that high glucose-induced enhanced phosphorylation of AKT and ERK1/2 was restored to control levels by growth factor receptor inhibitors suggests the implication of EGF-R and PDGF-R transactivation in high glucose-induced increased phosphorylation of AKT and ERK1/2. Taken together, it can be suggested that high glucose-induced transactivation of EGF-R and PDGF-R through the activation of AKT and ERK1/2 pathways may be responsible for high glucose-induced increased expression of Gq/11α and PLCβ proteins in VSMC. The enhanced expression of Gqα,PLCβ1 and PKCδ involving oxidative stress, growth factor receptor activation and MAP kinase signaling has been shown to contribute to VSMC hypertrophy in spontaneously hypertenive rats [101, 124, 125]. In addition, Ang II has also been reported to induce VSMC hypertrophy through Gq signaling pathway [76]. Taken together, it may be suggested that the enhanced expression of Gqα. PLCβ as well as PKC in VSMC exposed to high glucose or from diabetic rats may also contribute toVSMC hypertrophy resulting in vascular remodeling.

A role of c-Src has also been shown in high glucose-induced activation of EGF-R and PDGF-R as well as in the enhanced expression of Gq/11α and PLCβ proteins [119], and suggest that c-Src may be an upstream signaling molecule in high glucose-induced EGF-R and PDGF-R transactivation that contributes to the enhanced expression of Gq/11α and PLCβ proteins. In addition, a role of c-Src in ET-1-induced enhanced PLC signaling under hyperglycaemic conditions has also been reported [119].Furthermore, a role of Src kinases in the transactivation of EGF-R and PDGF-R has been shown [126]. In addition,Suzuki et al. [127] have shown that rat mesangial cells exposed to high glucose, exhibited an increased activity of c-Src that is associated with the increased activity of ERK5. In addition, the implication of c-Src in the proliferation of aortic VSMC from STZ-diabetic ratshas also been shown [119]. The fact that the enhanced phosphorylation of EGF-R and PDGF-R in VSMC exposed to high glucose was also attenuated by the inhibitors of c-Src suggests that c-Src through the transactivation of growth factor receptors may contribute to the enhanced cell growth in STZ-diabetic rats. In addition, the mechanism by which high glucose transactivates c-Src and thereby EGF-R and PDGF-R appears to involve oxidative stress because DPI, an antioxidant restored the high glucose-induced enhanced phosphorylation of c-Src to control levels.

Thus, we showed that high glucose transactivates EGF-R and PDGF-R through c-Src, which by activating MAPK and PI3K signaling may be responsible for the high glucose-induced enhanced expression of Gq/11α and PLCβ. The increased expression of Gq/11α and PLCβ results in enhanced production of IP_3, which by increasing the intracellular levels of Ca^{2+} may contribute to the vascular complications observed in diabetes (Fig. 13.3). In addition, the increased expression of Gqα and PLCβ may also contribute to VSMC hypertrophy and hyperproliferation, the key players of vascular remodeling resulting in vascular complications.

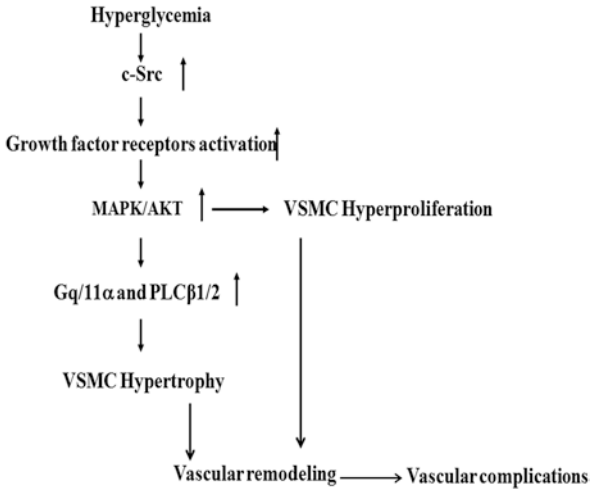

Fig. 13.3 Schematic diagram summarizing the role of C-Src and growth factor receptor activation in hyperglycemia/diabetes-induced increased expression of Gq/11α and associated – cell signaling

13.7 Conclusions

In conclusion, we have discussed the alterations in heterotrimeric G-proteins and associated functions in diabetes/hyperglycemia. We have mainly focused on Giα, Gsα and Gqα proteins which are implicated in the regulation of the adenylyl cyclase/cAMP and PLC/PI turnover, signal transduction systems respectively that play an important role in the regulation of vascular functions, including vascular tone, reactivity, VSMC proliferation and hypertrophy, the key players of vascular remodelling resulting in vascular complications. The levels of Giα-2 and Giα-3 proteins are decreased in several tissues including VSMC and aorta from STZ- induced diabetic rats as well as in VSMC exposed to high glucose., whereas the basal activity of adenylyl cyclase is attenuated resulting in the decreased levels of intracellular cAMP which may contribute to the hyperproliferation and hypertrophy of VSMC as well as to the increased vascular reactivity in diabetes and hyperglycemia (Fig. 13.4). On the other hand, the levels of Gqα/11 and PLCβ are augmented in diabetes and hyperglycemiaresulting in enhanced production of $IP_{3,}$ which by increasing the intracellular levels of Ca^{2+} may contribute to the vascular complications observed in diabetes (Fig. 13.4). In addition, the over expression of Gqα and PLCβ that has been shown to contribute to cardiac hypertrophy may also result in VSMC hypertrophy in diabetes. Taken together, it can be concluded that the alterations in the expression and functions of Giα and Gqα/PLCβ proteins in diabetes/hyperglycemia contribute to the vascular complications which may lead to cardiac failure.

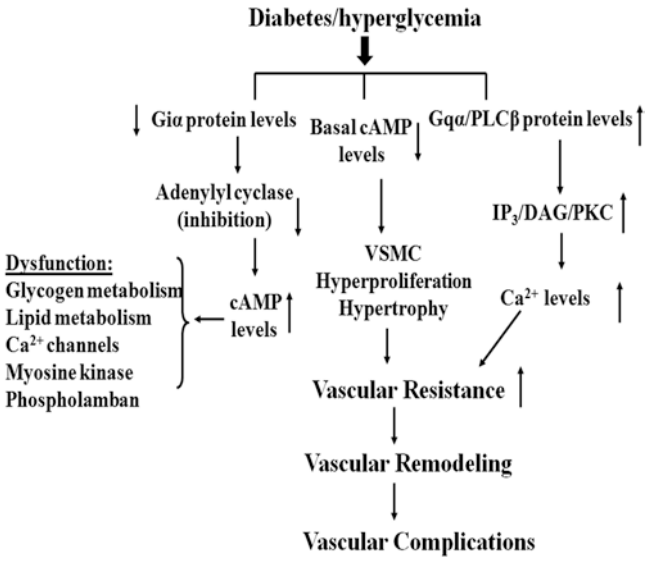

Fig. 13.4 Schematic diagram summarizing the possible mechanisms and role of altered expression of G-proteins in vascular remodeling and resultant vascular complications in diabetes/Hyper glycemia

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Chapter 14
Histone Deacetylases in Vascular Pathophysiology: Regulation by Vasoactive Peptides and Growth Factors

Paulina Pietruczuk and Ashok K. Srivastava

Abstract Vascular disease represents one of the major complications of diabetes. Chronic or spiking postprandial hyperglycemia- induced increase in protein glycation, oxidative stress, inflammation and alterations in the levels/action of vasoactive peptides and growth factors have been suggested as potential mediators of vascular dysfunction. Phenotypic modulation of vascular smooth muscle cells (VSMC) from a quiescent, contractile to a synthetic proliferative state has been implicated role in vessel remodeling that is a hall mark of vascular disorders. Vasoactive peptides and growth factors activate signaling pathways that induce expression of genes linked with growth and proliferation of VSMC. Recent studies have suggested that deacetylation/acetylation of histones plays an important role in the regulation of gene expression and contributes to vascular dysfunction. This review highlights some key observations on the role of histone deacetylases in the cardiovascular biology and discusses their potential role in the pathogenesis of vascular disease.

Keywords Vascular dysfunction • Histone deacetylases • Vasoactive peptides • Growth factors • Vascular smooth muscle

14.1 Introduction

Cardiovascular disease (CVD) is one of the major complications of diabetes and persistent hyperglycemia, which is often associated with diabetes, has been suggested to play a role in this process [1–4]. The precise mechanism by which

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hyperglycemia contributes to CVD remains elusive; however, several mechanisms, including production of advanced glycation end products (AGE), excessive production of reactive oxygen species (ROS) and increased inflammation, have been suggested to play an important role in the pathobiology of CVD [4–6]. All these events also induce alterations in the levels and activities of vasoactive peptides and growth factors [7–11]. Aberrant growth, proliferation and migration of vascular smooth muscle cells (VSMCs) are crucial events that have been implicated in the pathogenesis of CVD [12, 13]). Vasoactive peptides, such as angiotensin II (AngII), and growth factors, such as epidermal growth factor (EGF), platelet derived growth factor (PDGF) and insulin-like growth factor-1 (IGF-1), activate signaling pathways that induce the expression of genes linked to cell growth and proliferation [10, 14–16]. In recent years, there has been growing interest in understanding the role of epigenetic mechanisms in modulating the expression of genes that regulate cell growth and proliferation [17]. These epigenetic processes include the reversible posttranslational modification of histone structures within nucleosomes in chromatin [18]. Such modifications include acetylation and methylation, which are respectively catalyzed by histone acetyltransferases (HATs) and histone methyltransferases (HMTs). These enzymes transfer acetyl- or methyl- groups mostly to lysine or arginine residues within the N-terminal tail of histone [18]. Both acetylation and methylation reactions are reversible because histone deacetylases (HDACs) and histone demethyltransferases (HDMTs) also exist in the cells and catalyze the opposing reactions resulting in deacetylation and demethylation of amino acids in histone or, in some cases, non–histone proteins [18]. Recently, there has been a surge of interest in investigating the role of HDACs in cardiovascular biology. Therefore, the goal of this article is to provide an overview on HDACs and to highlight some key observations on the involvement of HDACs in the cardiovascular physiology and pathophysiology.

14.2 General Overview of Histone Modifications

In eukaryotic cells, to accommodate the large mass of genetic material within the nucleus, DNA is packaged into chromatin. Chromatin is composed of a combination of DNA, histone proteins and some RNA. Histones can be covalently modified by a variety of processes including acetylation, methylation, SUMOylation, ubiquitination and citrullination [18], thereby providing a regulatory mechanism for controlling gene expression. These modifications alter the structure of the DNA-histone complex, which in turn affects DNA transcription. Each of these histone modifications may be involved in epigenetics; however acetylation has been shown to be particularly important in CVDs [19]. As mentioned above, acetylation of lysine residues neutralizes the charge on histones, which increases chromatin accessibility [19]. This is achieved by HATs, which attach acetyl groups to conserved lysine residues on histone tails. Conversely, HDACs remove these groups and therefore reduce DNA transcription. Several studies have indicated the involvement of HDACs in

vasoactive peptide/growth factor-mediated aberrant vascular smooth muscle cell (VSMC) proliferation and hypertrophy, linking HDACs to vascular pathophysiology [20–22].

14.3 HDAC System and Classes

As mentioned above, HDACs are a group of enzymes that lead to deacetylation of lysine residues on histones. They have also been shown to deacetylate non-histone proteins as well [23]. At least 18 different mammalian HDAC genes have been identified, which can be categorized into four classes based on structure, function and the associated yeast orthologue. The four classes are class I HDACs (HDAC1, 2, 3 and 8), class II (HDAC4, 5, 6, 7, 9, 10), class III HDACs (sirtuins), and class IV HDAC (HDAC11). Classes I, II and IV HDACs have been termed classical HDACs as they require zinc as a cofactor, while class III HDACs require NAD+ as a cofactor [24].Class II HDACs are further subdivided into class IIa (HDAC 4, 5, 7 and 9) and class IIb (HDAC 6 and 10) based on their primary structure. In contrast to class IIb HDACs, class IIa HDACs contain a large N-terminal regulatory domain involved in protein-protein interactions, in addition to a C-terminal catalytic domain [25]. Class IIa HDACs seem to have critical roles in many disease processes, including CVDs. For example, HDAC4 and HDAC5 have been shown to be critical in promoting VSMC proliferation and migration in response to growth factors and vasoactive peptides [20, 21, 26] and HDAC7 has been linked to PDGF-BB-induced endothelial cell migration [27].

14.3.1 HDAC Localization

HDAC localization, which can either be nuclear or cytoplasmic, greatly affects HDAC activity. HDACs that are able to shuttle in and out of the nucleus have been shown to regulate various cytoplasmic processes and function as signal transducers [28]. Localization varies depending on the class. Class I HDACs are primarily located in the nucleus with the exception of HDAC3, which can also translocate into the cytoplasm. In contrast, class II HDACs is able to shuttle in and out of the nucleus. Some Class III and IV HDACs can also shuttle between the nucleus and cytoplasm, while others are confined to either the nucleus or the cytoplasm [24].

14.3.2 HDAC Structure

Despite the fact that it has been over 50 years since Allfrey and coworkers first proposed the idea that the acetylation status of the histone proteins that make up chromatin is correlated with the transcriptional status of a given gene [29], information regarding HDAC structure has only become available more recently. Due to their comparable enzymatic activities, it would be expected that the proteins within the HDAC super family share some structural features. A study of the available structural information reveals that this is indeed true. In humans, HDACs are divided into separate classes based on sequence similarities. The Class I proteins (HDAC1, HDAC2, HDAC3, HDAC8) have a sequence similarity that extends over 300 residues with the yeast Rpd3 protein. Within this 300 residue sequence, there is an especially marked homology within an internal 70 residue stretch [30]. To be specific, 80% similarity was found between yeast Rpd3 and human HDAC1 within the 300 residue region of conservation and 99% homology in the 70 residue stretch [30]. The Class II HDAC proteins (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10) have a comparable sequence with the yeast Hda1 protein. Additionally, mammalian Class I and II HDACs were also found to be related to *Saccharomyces cerevisiae* Hos1, Hos2 and Hos3 proteins, which have 35–49% identity to Rpd3 and 21–28% identity to Hda1 [31]. The Class III proteins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7) and the yeast Sir2 protein have a comparable sequence [23]. The Class IV protein (HDAC11) has a comparable sequence to both Class I and II proteins [23].

Class I and II HDACs have some structural similarities to Class III HDACs in that they all contain a central parallel β sheet network that is flanked on opposite sides by helix-rich segments [30]. Moreover, sequence conservation studies suggest that, in all three HDAC classes, substrate binding and catalysis is mediated by the domain proximal to the C-terminal tip of the β sheet. This domain is rich in loop segments that appear to be critical for function as they form protein cavities that are implicated in acetyl-lysine binding [32]. Despite these marked similarities, there are also striking structural differences between the Class I/II and Class III HDACs. Most notably is the small globular region in Class III HDACs, which is absent in Class I/II HDACs. This region is important for class III HDAC deacetylase activity and, due to its sequence and structural divergences, it plays a modulatory role in substrate specificity within the class III HDAC family [30]. The absence of a corresponding domain in Class I/II HDACs suggests that other proteins may substitute for this domain in vivo. This is consistent with the observation that Class I/II HDACs associate with other proteins in vivo for catalytic activity [30].

14.3.3 HDAC Expression and Regulation

14.3.3.1 Class I HDACs

In terms of HDAC expression and regulation, Class I HDACs are ubiquitously expressed nuclear enzymes; however, it has been shown that HDAC8 is generally poorly expressed [28]. Not a great deal is known about HDAC3 and HDAC8 regulation, but the other Class I HDACs (HDAC1 and HDAC2) form homo- and heterodimers between each other [33, 34], which is a requirement for HDAC activity. Dissociation of the dimer will impede HDAC activity, which is something viruses take advantage of to hinder HDAC activity. For example, it has been shown that, by binding to the N-terminal region of HDAC1 and likely dissociating the dimer, the adenoviral protein GAM1 impedes HDAC1 activity [35]. The level of HDAC1 and HDAC2 heterodimers is likely cell type-specific, because it was demonstrated that 80–90% of HDAC1 and HDAC2 proteins formed dimers in the nucleus of human breast cancer MCF-7 cells [36], whereas, in mouse embryonic fibroblasts, 40–60% of HDAC1 and HDAC2 proteins were shown to be free from each other [37]. Additionally, Class I HDACs depend on the presence of a catalytic Zn2+ ion for their activity and have been shown to be sensitive to inhibition by a family of small molecule compounds that have homology to trichostatin (TSA) such as suberoylanilide hydroxamic acid (SAHA) and trapoxin (TPX) [38].

14.3.3.2 Class II HDACs

Like Class I HDACs, Class II HDACs also require the presence of a catalytic Zn2+ ion for their activity. Class II HDACs, as previously mentioned, shuttle between the nucleus and cytoplasm and have tissue-specific expression and functions [38]. Class IIa HDACs (HDAC4, HDAC5, HDAC7 and HDAC9) are important signal transducers. In the regulatory N-terminal domain of these HDACs, there are several conserved serine residues that are subject to reversible phosphorylation. An array of kinases and phosphatases acting downstream of diverse biological pathways have been demonstrated to act on these HDACs, regulating their nucleocytoplasmic trafficking through the modification of their phosphorylation status [39, 40]. In the case of HDAC4, this phosphorylation occurs on serine 246 (S246), S467 and S632 by several isoforms of the calcium/calmodulin-dependent kinase (CaMK) family [41, 42], by Aurora B on S265 [43], by Mirk/dtrk1B on S266 [44], and by glycogen synthase kinase 3β (GSK3β) on S298 and S302 [45]. The critical residues on HDAC5 are S259 and S498 and have been shown to be phosphorylated by CaMK-1, -II and -IV, protein kinase D (PKD) and AMPK [46–48]. Protein kinase C (PKC), an upstream regulator of PKD, has also been shown to phosphorylate S259 directly [49]. For HDAC7, the critical residues are S181, S155, S358 and S486 [50]. The latter three sites can be modified by CaMK1, whereas PKD can phosphorylate all four residues. Less is known about HDAC9, but it has been suggested that HDAC9

residues S239, S240 and S253 are phosphorylated by Aurora B, Mirk/dtrk1B and PRK1, respectively [50]. Class IIa HDAC phosphorylation leads to the binding of 14-3-3 proteins, the nuclear export of these HDACs and the de-repression of their target genes [51].

14.3.3.3 Class IIb HDACs

Class IIb HDACs (HDAC6 and HDAC10) have duplicated catalytic domains, however the duplication is only partial in the case of HDAC10 [52]. HDAC6 and HDAC10 shuttle between nucleus and cytoplasm, but their location is primarily cytoplasmic [53], Not much is known regarding the regulation of class IIb HDAC [53].

14.3.3.4 Class III HDACs

Class III HDACs, also known as sirtuins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, SIRT7), are NAD+ dependent and the availability of NAD^+ in cells is a limiting step in the activation of sirtuin catalytic activity [54]. The basal intracellular NAD+ levels are maintained relatively constant [55] through NAD+ biosynthetic and salvaging pathways. By utilizing NAD^+, sirtuins regulate the fluctuation of the NAD^+/NADH ratio [56]. Additionally, sirtuin gene expression has been shown to be under the control of numerous transcription factors involved in cell cycle regulation and apoptosis. Among them, the transcription factor E2F1, which induces cell cycle progression from G1 to S phase, directly binds to the Sirt1 promoter upregulating its gene expression in cells treated with the topoisomerase II inhibitor etoposide [57]. Furthermore, the tumour suppressor p53, which is one of the most extensively mutated proteins in cancers, has also been shown to affect Sirt1 gene expression. Two functional p53-binding sites have been identified in the regulatory region of the Sirt1 promoter and studies have indicated that in nutrient-deprived mammalian cells p53 stimulates Sirt1 gene expression [58]. On the contrary, in normal nutrient conditions, p53 mediates repression of Sirt1 gene expression [59]. Sirt1 levels are regulated by E2F1 and p53 at the transcriptional level as well as the translational level.

Sirtuin activity is also regulated by posttranslational modifications. In vitro evidence has demonstrated that cyclin B/Cdk1-mediated dephosphorylation at specific sites decreases Sirt1 and Sirt2 deacetylase activity [60]. Additionally, Sirt1 has also been shown to be phosphorylated by the c-Jun N-terminal kinase 2 (JNK2) [61] and casein kinase 2 (CK2) [62]. JNK2-mediated phosphorylation of Sirt1 is associated with the regulation of its protein stability [61]. Furthermore, several conserved phosphorylation sites have been found within Sirt1 that are possible targets for a variety of kinases such as ATM, casein kinase 1 (CK1), DNA-dependent protein kinase (DNA-PK), extracellular-signal-regulated kinase (ERK1), GSK-3, IκB kinase (IKK), and MAPK [60]. It is not known, however, if these kinases phosphorylate only Sirt1 or other members of the sirtuin class as well. In addition to phosphorylation, other posttranslational modifications may regulate the catalytic activity of sirtuins.

14.3.3.5 Class IV HDACs

The Class IV HDAC (HDAC11) has sequence similarity to Class I and II HDACs. Aside from this, little is known of its function and regulation [23].

14.4 HDAC Activation and Signaling in Vascular Pathophysiology

14.4.1 Vasoactive Peptide-Induced HDAC Activation and Signaling

AngII, a potent vasoconstrictor, has been shown to activate several signaling pathways linked to cellular hypertrophy, growth, migration and proliferation in various cell types including VSMCs. One of its targets is CaMKII, which transduces downstream signaling responses of AngII upon activation [63]. A potential transcriptional target for CaMKII is myocyte enhancing factor 2 (MEF2). MEF2, a DNA binding transcription factor that likely promotes the synthetic phenotype of VSMCs, can either act as an activator or a repressor of transcription depending on its interaction with co-activators or co-repressors, respectively [64]. HDAC4 and HDAC5 have been shown to directly interact with MEF2 in the nucleus to promote its repressive activity. Consequently, HDAC4 and HDAC5 phosphorylation and nuclear export increases MEF2 transcriptional activity and leads to VSMC hypertrophy [64]. As recently reported, CaMKII is capable of mediating AngII-dependent increases in HDAC4 (S467) [65] and HDAC5 (S498) [47] phosphorylation and subsequent nuclear export. This derepresses MEF2, increasing MEF2 DNA binding activity and transcription. Additionally, it was also demonstrated that HDAC5 phosphorylation is mediated by HDAC4 in VSMCs, suggesting a potential regulatory mechanism involving protein-protein interaction [47].

Based on evidence from different cell types, class IIa HDACs have been shown to have many transcription factor targets in addition to MEF2, including serum response factor (SRF), which has been linked to CaMKII and HDAC4 in cardiomyocytes [66]. Davis et al. showed that HDAC4 interacts with SRF in cardiomyocytes and this interaction is enhanced by AngII stimulation [66]. Thus, MEF2 is unlikely to be the only transcription factor targeted by CaMKII and class IIa HDACs in VSMCs.

As suggested above, there are many pathways and factors involved in propagating hypertrophic and proliferative signals in VSMCs. As evidenced by Pang et al., G-protein coupled receptor (GPCR)-kinase 2 interacting protein-1 (GIT1) has been shown to be involved in mediating HDAC5 phosphorylation by AngII [21]. AngII stimulation leads to GIT1 phosphorylation, causing PLCγ activation which is required for elevation of intracellular Ca2+ and activation of CaMKII. Once CaMKII is activated, it phosphorylates HDAC4 and HDAC5 leading to nuclear export [64].

In contrast to CaMKII-dependent HDAC4 and HDAC5 phosphorylation, other studies have shown that AngII is able to induce HDAC5 phosphorylation and nuclear export in VSMCs in a calcium- independent manner [67] via the protein kinase C (PKC)-protein kinase D1 (PKD1) pathway. PKD1 has been shown to phosphorylate HDAC5 at Serine 259/498 [67]. PKC has also been shown to phosphorylate Serine 259 directly in failing hearts [68]. Once phosphorylated, these residues serve as docking sites for 14-3-3 chaperone proteins. This results in an increase in MEF2 transcriptional activity, which derepresses VSMC growth genes and consequently leads to VSMC hypertrophy [26].

14.4.2 Growth Factor-Induced HDAC Phosphorylation and Activation

In addition to vasoactive peptides, like AngII, growth factors, such as platelet-derived growth factor-BB (PDGF-BB), have also been implicated in regulating HDAC4 and HDAC5 function by inducing their phosphorylation [69]. PDGF-BB is a key mediator of VSMC phenotype switching from the contractile state to the synthetic state [70]. This switch is associated with suppressed expression of VSMC marker genes, such as smooth muscle α-actin and smooth muscle myosin heavy chain, as well as increased proliferation and migration rates of cultured VSMCs. Yoshida et al. have reported that PDGF-BB represses the expression of smooth muscle cell differential marker genes through the recruitment of HDAC4 and HDAC5 to the promoters of these genes [71]. HDAC-induced hypoacetylation inhibits the accessibility of transcription factors, notably SRF, to interact with the promoters of these genes, reducing transcription [72]. Additionally, HDAC5 has been shown to directly interact and inhibit myocardin resulting in decreased transcription of differentiation marker genes [73]. In terms of proliferation and migration, PDGF-BB- induced HDAC4 phosphorylation was reported to mediate proliferation and migration responses in VSMC [74]. HDAC4 knockdown was shown to inhibit PDGF-induced expression of cyclin D1, a cell cycle regulatory protein required for the progression of the G1 phase, which has an inhibitory effect on proliferative signals [74, 75]. Furthermore, Usui et al. also reported the involvement of HDAC4 in PDGF-BB-induced VSMC migration and cytoskeletal reorganization [74]. Both migration and cytoskeletal reorganization were shown to be significantly inhibited by HDAC4 siRNA as well as MC1568, a Class IIa HDAC inhibitor [74].

In addition to VSMC phenotype switching, it is likely that PDGF-BB also increases reactive oxygen species (ROS) production by upregulating NADPH oxidase (NOX) activity in an HDAC4-dependent manner, as evidenced by HDAC4 siRNA studies [76]. Once HDAC4 is phosphorylated by PDGF-BB in a CaMKII-dependent manner, it is believed that HDAC4 might upregulate NOX activity via p47 phox or Rac-1 activation (79). Upregulation of NOX activity will cause

increased ROS, which can stimulate VSMC proliferation and migration via the activation of p38MAPK/HSP27 signals [77].

In terms of the specific signaling cascades activated during PDGF-BB-mediated vascular pathophysiology, CaMKs and PKC/PKD are known to be activated by PDGF-BB leading to VSMC migration and proliferation [78, 79]. HDAC4 has been shown to be a substrate for activated CaMKII [80]. It has been suggested that PDGF-BB stimulation causes CaMKIIδ to sequester HDAC4 to the cytoplasm, thereby stimulating MEF2 activity in VSMCs [64].

14.4.3 Prostanoids and B-Adrenergic Agonists-Induced HDAC Activation and Signaling

In contrast to vasoactive peptides and growth factors, which contribute to vascular disease, prostanoids, such as prostacyclin, and β-adrenergic agonists decrease cardiovascular risk [81]. Prostacyclin and β-adrenergic agonists activate protein kinase A (PKA), which has been shown to minimize the incidence of cardiovascular disease by opposing VSMC proliferation [81]. Although the mechanisms underlying this process have not yet been fully elucidated, PKA has been shown to promote HDAC4-induced repression of MEF2-dependent gene expression and thus may contribute to the anti-proliferative effects exerted by PKA [82].

14.5 Involvement of HDACs in Cardiovascular Pathophysiology

The majority of CVDs, such as hypertension and atherosclerosis, are the result of vascular remodeling mediated in part by VSMC proliferation and migration. HDACs, notably Class IIa HDACs, have been associated with this process [38].

14.5.1 Hypertension

In particular, HDAC4 has been implicated in promoting vascular hypertensive disease [83]. It was reported that subcutaneous administration of trichostatin (TSA), a non-specific inhibitor of HDACs, reduced the elevated blood pressure in spontaneously hypertensive rats (SHR) compared to normotensive controls and this reduction was associated with an attenuation of ROS generation and suppression of augmented mesenteric artery contraction [83]. In addition, a recent report has demonstrated that MC1568, a class IIa- selective HDAC inhibitor, reduced systolic blood pressure in an AngII-induced rat model of hypertension [84]. In these studies,

MC1568 was shown to reduce HDAC4 phosphorylation, as well as the levels of VSMC proliferation and expression of cell cycle regulating genes such as cyclin E, E2F3 [84].Vascular inflammation is also known to be important in the pathogenesis of hypertension. In particular, inflammation induced by ROS has been suggested to have a critical role in the development of hypertension via promoting proliferation and migration of VSMCs [85]. Class IIa HDACs have been linked to this process and HDAC4 is of particular importance because it has been demonstrated that HDAC4 promotes ROS-dependent vascular inflammation and mediates VSMC proliferation and migration through the activation of p38 MAPK/ HSP27 [86]. These studies suggest a likely role of HDAC4 and HDAC5 in the development of hypertension by regulating inflammatory, proliferative and growth promoting responses in vascular system.

14.5.2 Atherosclerosis

VSMCs undergo a switch from the contractile to the synthetic phenotype in response to various stimuli, such as shear stress, lipids, ROS and cytokines, giving rise to a state favoring migration and proliferation [87]. VSMCs recruited from the media to the intima, as well asthose proliferating within the intima, contribute to progression of atherosclerosis. They secrete large quantities of extracellular-matrix components, such as collagen, resulting in atherogenic lipoprotein retention and aggregation [88]. Additionally, migrating and proliferating smooth muscle cells beneath the endothelium form a fibrous cap, cutting the blood supply from the plaque [89]. Thus, it is evident that smooth muscle cell proliferation and migration are key events in the development of atherosclerosis. HDACs are implicated in this process as studies have shown that they play a critical role in the VSMC phenotype switch needed for the progression of atherosclerosis. Notably, Class IIa HDACs have been shown to promote the phenotype switch of smooth muscle cells though Ca2+ signals [90]. As previously mentioned, in addition to the phenotypic switch, VSMC migration and proliferation are also integral to the development of atherosclerotic vascular disease. Interestingly, MC 1568 has been shown to prevent intimal lesion formation in ligated carotid areteries of mice [74]. Moreover, studies demonstrating that phosphorylation of HDAC4 and HDAC5, promotes PDGF-BB-induced VSMC proliferation and migration ultimately resulting in neointimal hyperplasia further supports the notion that this class of HDACs may have a role in vascular remodeling and the atherosclerotic plaque formation [53, 91].

14.6 Conclusion

CVD ranks among the most common complications of diabetes and persistent hyperglycemia, which is often associated with diabetes, has been suggested to play a role in this process.The precise mechanism by which hyperglycemia contributes

to CVD remains elusive; however, several mechanisms, including production of advanced glycation end products (AGE), excessive generation of reactive oxygen species (ROS) and elevated levels and action of vasoactive peptides and growth factors have been suggested to play a role in this process.Aberrant growth, proliferation and migration of VSMC are crucial events that have been implicated in the pathogenesis of CVD. Recent studies have demonstrated that HDACs play an important role in regulating the proliferation and migration of VSMC proliferation and by using pan- inhibitors or in some cases, class- specific inhibitors of HDACs, a potential role of HDAC in the development of hypertension has also been reported. In addition, HDAC inhibition, either by pharmacological blockade or by siRNA has revealed that HDACs participate in the transcription of genes that regulate cell cycle and inflammatory responses in VSMC. However, despite these encouraging reports, additional studies are needed to establish a role of HDACs in the pathogenesis of CVD.

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Chapter 15
Role of Non-coding RNAs in Vascular Complications of Diabetes Mellitus

Sumi Surendran and C.C. Kartha

Abstract Non-coding RNAs (ncRNAs) have emerged as an important component of gene regulatory networks. These regulatory RNAs orchestrate different functions of the vascular system by regulating target gene expression. Significant dysregulation of ncRNAs is associated with hyperglycemia, angiogenesis and vascular repair and contribute to vascular disease in patients with diabetes. The functional roles of only a very few ncRNAs such as miRNAs are well studied in vascular biology; studies on other ncRNAs are limited. In this article, we outline the known roles of ncRNAs in diabetes associated vascular complications, as well as their potential use as biomarkers and therapeutic targets. We also discuss the strategies and challenges in the possible use of these microregulators for clinical application in patients with diabetes associated vascular diseases.

Keywords Micro RNA • Non coding RNAs • Vascular disease • Diabetes • Hyperglycemia • Insulin resistance • Atherosclerosis

15.1 Introduction

Diabetes associated vascular complications are broadly classified as macrovascular diseases which include coronary artery disease, cerebrovascular disease and peripheral vascular disease and microvascular diseases comprising of diabetic neuropathy, nephropathy, and retinopathy. Vascular diseases accounts for 80% of mortality from diabetes related complications [1]. Chronic hyperglycemia, insulin resistance and dyslipidemia induce systemic inflammation and elevated oxidative stress [2]. These pathophysiological alterations lead to vascular dysfunction in patients with diabetes mellitus. Moreover, hyperglycemia impairs the endogenous repair mechanisms [3]

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such as angiogenesis and endothelial progenitor cell (EPC) responses [4–6] which normally counteract vascular damages in ischemic conditions and arterial endothelial denudation.

Molecular mechanisms of diabetic vascular diseases has been a research hotspot in recent years, for discovering biomarkers enabling early diagnosis, accurate prognosis and for developing better therapeutic targets. Mounting evidences suggest that epigenetic mechanisms such as DNA methylation, post-translational modifications of histones and non-coding RNAs (ncRNAs) are crucial in the initiation and progress of macro- and micro-vascular complications of diabetes mellitus [7].

The recent advancements in high throughput sequencing techniques have revealed that only 2% of the transcribed genomic sequences code for proteins [8–10]. The vast majority of non-protein coding sequences are transcribed as non-coding RNAs; these include long non-coding RNAs (lncRNAs), microRNAs, snoRNAs, piRNAs, snRNAs etc. Rather than being the 'junk nucleotides' as they were regarded earlier, the ncRNAs are now regarded as tiny yet powerful riboregulators of gene expression [11]. The discovery of the epigenetic control of gene regulation by ncRNAs has added a new layer of complexity to our understanding of diabetes associated pathological complications. This article focuses on ncRNAs in a hyperglycaemic scenario and their 'micro-signature' on the pathogenesis of vascular complications in high risk patients with diabetes. Their plausible roles as biomarkers and novel therapeutic targets to manage diabetes vascular disease are also discussed.

15.2 Non-coding RNA Family

Broadly ncRNAs are classified based on their housekeeping and regulatory functions. NcRNAs such as tRNAs, rRNAs, snRNAs, snoRNAs, tmRNA are considered 'housekeeping' as they function in translation and RNA recycling processes. Housekeeping RNAs are constitutively expressed while regulatory ncRNAs are induced only at requirement. Regulatory ncRNAs are also arbitrarily defined by their transcript length (Fig. 15.1). They can be classified as small ncRNAs comprising of less than 200 nucleotide sequences and long ncRNAs whose sequences are longer than 200 nucleotides [12].

Small ncRNAs such as micro RNAs (miRNAs) and Piwi-interacting RNAs (piRNAs) are called 'micromanagers' of gene expression. They are involved in transcriptional and posttranscriptional gene silencing through specific base pairing with target genes.

Long ncRNAs (lncRNAs) such as long intervening RNAs (lincRNAs), natural antisense transcripts (NATs or asRNA) are the class of ncRNA which constitute the largest fraction of the non-coding transcriptome in mammals. Unlike small ncRNAs, lncRNAs are not generally conserved among species [13–15]. Several mechanisms

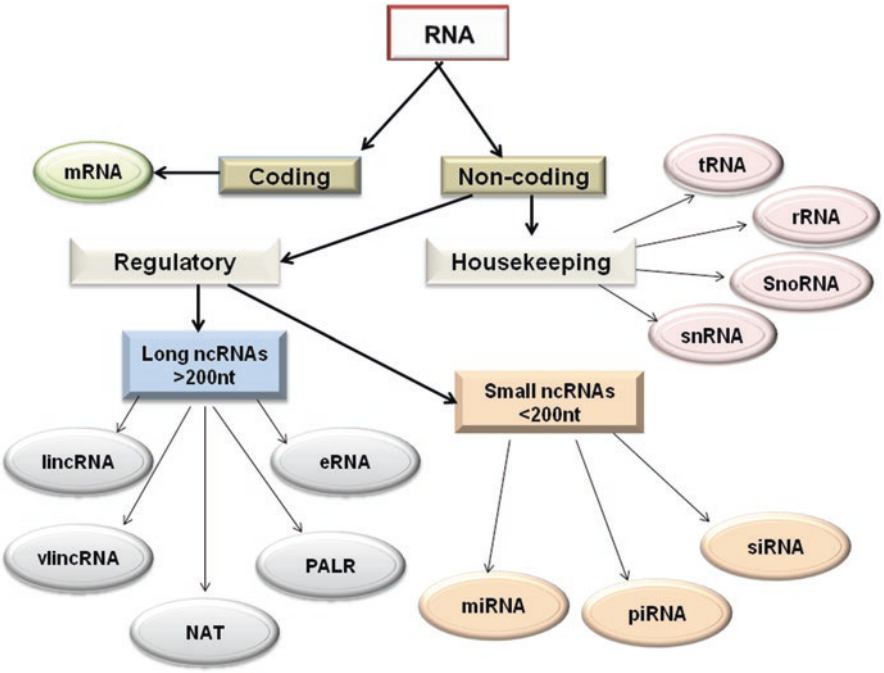

Fig. 15.1 Different types of RNA molecules
Noncoding RNAs are arbitrarily classified into regulatory and housekeeping subtypes. Housekeeping ncRNAs include *snRNA* Small nuclear *RNA*, *snoRNA* Small nucleolar *RNAs*, *rRNA* Ribosomal *RNA*, *tRNA* Transfer *RNA*. Regulatory *ncRNAs* are further classified based on their size. Long ncRNAs include *lincRNA* Long intergenic non-coding RNAs, *vlincRNA* Very long intergenic noncoding RNA, *NAT* Natural antisense *ncRNA*, *PALR* Promoter-associated long *RNA*, *eRNA* Enhancer-associated RNA. Small ncRNAs include *piRNA* Piwi-interacting *RNA*, *siRNA* Small interfering *RNA*, *miRNA* MicroRNA

of transcriptional regulation by lncRNAs have been proposed. In general, lncRNAs are proposed to function as molecular scaffolds, assembling different groups of regulatory proteins into thermodynamically stable functional units.

A great deal of data is now available on the basic biology of ncRNAs. Yet the knowledge to identify the impact of these ncRNAs in disease states is preliminary and the capability to effectively target them *in vivo* is inadequate at present. However, what we do know about the intrinsic biology of these ncRNAs makes them potentially attractive targets for pharmacologic manipulation. The control of many cellular functions is being linked to ncRNAs and, not surprisingly, they are emerging as key molecules in human pathology . The most widely studied classes of ncRNAs are miRNAs, lncRNAs and recently snoRNAs. Their regulatory roles in chronic hyperglycemia and their impact on vascular function will be discussed.

15.3 Micro RNAs

15.3.1 Micro RNAs: Biogenesis, Function and Cellular Location

Micro RNAs are RNA molecules comprising of approximately 20–22 nucleotides. Victor Ambros [16] and Gary Ruvkun [17] independently in 1998 *discovered Lin-7* as the miRNA in *Caenorhabditis elegans*. After 7 years of the first discovery, Ruvkun and Amy Pasquinelli detected another miRNA (*let-7*) in *C. elegans* and multiple other animal species as well as in humans [18]. Shortly after these breakthrough reports, hundreds of miRNAs were identified in various organisms. More than 2000 miRNAs are so far documented in humans.

15.3.1.1 Biogenesis

The biogenesis of miRNA is well studied. Briefly, miRNAs are transcribed by RNA polymerase II enzyme as primary miRNAs (pri-miRNAs). Primary miRNAs are further cleaved by Drosha RNase III endonuclease enzyme and its binding partner DGCR8 (DiGeorge syndrome critical region 8) to precursor miRNAs (pre miRNAs) and later processed into a single stranded mature miRNA by Dicer RNase III endonuclease enzyme and Transactivator RNA binding protein (TRBP) [19].

15.3.1.2 Function

MicroRNAs function by post-transcriptional gene silencing, by controlling the translation of mRNA into proteins and it also render RNA stability [19]. They control translation of more than 60% of protein-coding genes [20]. They are involved in regulating major biological processes such as cell proliferation, differentiation and apoptosis. Some miRNAs regulate specific individual mRNA targets while other miRNAs regulate the expression levels of hundreds of mRNAs simultaneously [21], thereby functioning as ‘master regulators’ of a process.

15.3.1.3 Localization

MicroRNAs are found both intracellular and extracellular. Normal RNAs are extremely unstable in extracellular fluids as they are prone to cleavage by RNases, whereas miRNAs are stable in circulation. Circulatory miRNAs are actively secreted by cells as exosomes or as microparticles sometimes referred to as ‘apoptotic bodies’. miRNAs associated with exosomal or apoptotic bodies are transported to neighbouring cells, where they exert their influence on gene expression [22]. Due to its stability in circulation, blood miRNA profile is expected to reflect underlying pathological conditions. Their sequences are highly conserved between species.

15.3.2 miRNAs in Diabetes Associated Vascular Diseases

First miRNA to be implicated with diabetes was miR-375 which was discovered by Poy and colleagues in 2004 [23, 24]. They demonstrated that miR-375 regulate pancreatic α and β cells functioning, insulin synthesis and insulin secretion. During the past decade, several research groups have reported that miRNAs orchestrate various stages in the pathogenesis of both type 1 and type 2 diabetes mellitus and associated complications. A majority of these miRNAs are secreted by blood cells [25, 26], pancreatic cells [23, 24] and vascular cells [27]. Another fraction of miRNAs are produced by liver [28], skeletal muscle [29], and adipose tissue [30], all of which are tissues whose function are known to be affected in type 2 diabetes.

Vascular complications associated with diabetes often begin with endothelial dysfunction. Hyperglycemia, oxidative stress and insulin resistance are the major risk factors for endothelial dysfunction and vascular complications in patients with diabetes mellitus [31].

The effect on vascular integrity by miRNAs produced during hyperglycemia alone or in conjunction with other risk factors is discussed below.

15.3.2.1 Pro/Antiangiogenic miRNAs Regulated by Hyperglycemia

Anti Angiogenic mRNAs

(i) *miR-320:* Wang et al. showed for the first time that miRNAs are differently expressed in endothelial cells primed with high glucose. Microarray analysis has revealed that patients with hyperglycemia have a deregulated miRNA profile which is antagonistic to vascular reparative processes and endothelial homeostasis. One of the major differentially expressed miRNA is miR-320 which is highly expressed in endothelial cells exposed to high glucose. miR-320 inhibits angiogenic factors such as vascular endothelial growth factor and insulin like growth factor-1 and affect the proliferation and migration in vascular endothelial layer [32].

(ii) *miR-221/222 cluster:* These high glucose induced miRNAs inhibit proangiogenic activation, proliferation, and migration of endothelial cells and precursors. Their mode of action is mainly by targeting c-kit receptors. Several other antiangiogenic mechanisms of miR-221/222 are postulated which include upregulation of transcription factor GAX which inhibits endothelial cell activation. Studies show that miR-221/222 downregulate ZEB2 which is the transcription repressor of GAX thereby increasing GAX expression [33]. miR-221/222 is associated with reduced recruitment of endothelial cells during vascular repair and is also implicated in increased apoptosis of endothelial cells. This miRNA cluster also facilitate in switching vascular smooth muscle cells (VSMC) to proliferative ‘synthetic’ phenotype from the quiescent ‘contractile’ phenotype and activate the formation of neointima in atherosclerotic vessels [34].

(iii) *miR-492:* In hyperglycemic conditions, there is a notable reduction in miR-492 expression and thereby a significant overexpression of resistin which is a key factor in atherosclerosis. It has also been demonstrated that miR-492 upregulation reduced endothelial lipid accumulation and cell migration during plaque formation [35].

Proangiogenic mRNAs

In contrast to the general concept that only upregulated miRNAs exert pathological effects, recent studies have identified a few miRNAs associated with reduced risk for disease.

(i) *miR-126:* This miRNA is seen highly expressed in endothelial cells and progenitor cells [36]. MiR-126 is interesting by its contrasting role in embryonic and adult angiogenesis. In embryonic blood vessel formation, miR-126 directs the differentiation of stem cells to EPCs and then to endothelial cells and also supports maturation of endothelial cells. Interestingly, in adult angiogenesis, miR-126 helps in maintaining quiescent endothelial phenotype and thereby in vascular homeostasis [37]. miR-126 is also known to reduce apoptosis in EPCs and it also promotes EPCs related vascular repair activities [38]. miR-126, when delivered by apoptotic bodies give protection against diet induced atherosclerosis in $Apoe^{-/-}$ mice models [39].

Recent reports suggest that expression of miR-126 in EPCs is inhibited by high glucose and glycation end products [40]. In the light of all the above facts, we can speculate that miR-126 could be an excellent biomarker for diabetic vascular disease.

(ii) *miR-17-92 cluster:* This cluster comprises of seven miRNAs: miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-9b and miR-92-1 [41]. A very recent study in knock-out mice models revealed that miR-17-92 is a key regulator of pancreatic β cell homeostasis and glucose-stimulated insulin secretion [42]. Another study on miR-17–92 iEC-KO mice suggests that miR-17–92 cluster has a proangiogenic effect during both developmental and adult angiogenesis [43]. These findings imply the significance of this miRNA cluster in diabetic vascular diseases.

Aforementioned studies on pro- and anti-angiogenic miRNAs cumulatively indicate that modulation of hyperglycemia-specific miRNAs can improve angiogenesis and vascular remodelling by EPC repair.

15.3.2.2 Proinflammatory and Proatherogenic Effect of miRNAs Modulated by Hyperglycemia

A balanced redox system contributes to a protective function in organisms; its imbalance obviously leads to several diseases especially severe vascular impairment. Various components of diabetes mellitus such as hyperglycemia, dyslipidemia and dysregulated nitric oxide signalling increase oxidative stress in endothelial cells and VSMCs. miRNA profiling studies based on hyperglycemia induced oxidative stress demonstrate the central role played by miRNA in cell response stimulated by a redox imbalance.

(i) *miR-200 family:* Studies performed in VSMCs of db/db mice indicate an upregulation of miR-200 family members such as miR-200b, miR-200c and miR-429 [44]. Upregulated miR-200 inhibits ZEB1 which is a transcriptional repressor protein. This results in the increased transcription of inflammatory genes such as monocyte chemo attractant protein-1 (MCP-1) and cyclooxygenase-2 (COX-2) which induce monocyte binding to vascular wall, stimulating inflammatory and atherogenic signalling cascades. This finding is of significance, as a similar pattern in miR-200 increase and ZEB1 decrease is observed in renal arteries of patients with diabetes.

(ii) *miR-125b:* In a study by Wang et al., it was found that miR-125b is significantly upregulated in serum samples from both patients with type 2 diabetes mellitus as well as those with diabetes associated microvascular diseases [45]. miR-125b is stimulated by oxidative stress in high glucose conditions. The protein target of miR-125b is histone H3 lysine-9 methyltransferase Suv39h1. Villeneuve et al. reported that in a diabetic setting, Suv39h1 has a reduced promoter affinity in genes coding for inflammatory signalling resulting in the overexpression of inflammatory proteins in endothelial cells and VSMCs [46].

15.3.2.3 Role of miRNAs Induced Due to Insulin Resistance Resulting from Hyperglycemia

(i) *miR-143/145:* Another important miRNA family involved in insulin resistance is miR-143/145. This miRNA family was demonstrated to target angiotensin converting enzyme (ACE) and inhibit angiotensin II formation [47]. miR-143/145 has also been recently implicated in defective diabetic wound healing [48].

(ii) *miR-503:* miR-503 is upregulated in 3 T3-L1 insulin-resistant adipocytes and in the muscles of insulin-resistant diabetic patients [29, 49, 50]. A study conducted by Caporali et al. implicated miR-503 in hyperglycemia induced endothelial dysregulation and impairing endothelial cell cycle [51]. miR-503 expression was found increased in the limb muscles and plasma samples of patients with diabetes who had limb ischaemia when compared with calf biopsies of non-diabetic/non-ischaemic controls. Experiments in diabetic mice models have shown that inhibiting miR-503 could help in post-ischaemic neovascularisation [52].

Table 15.1 Key micrornas associated with diabetes associated vascular diseases as identified in animal models, *in vitro* culture studies and human plasma/tissue samples

	MicroRNAs	
Diseases	Upregulated (pathogenic)	Downregulated (protective)
Atherosclerosis	miR-155 [53], miR-503 [54]	miR-16 [55], miR-126 [56]
Cerebrovascular diseases	miR-21 [57, 58]	miR-221 [58]
Peripheral vascular diseases	miR-503 [51], miR-130 [59]	miR-126 [36], miR-15a/16 [60]
Diabetic retinopathy	miR-34,miR-200 family [61]	miR-93 [62]
Diabetic nephropathy	miR-29, miR-21, miR-377 [63]	miR-93, miR-192 miR-14, miR-200a [63, 64]

Very less is known about the regulatory role of miRNAs in diabetes and its complications when compared to its role in other diseases such as cancer. Eventhough several miRNAs have been related with diabetic complications, the question whether they are simply the effect of disease process or they are actually involved in the pathogenesis of the disease is yet unclear and is currently investigated. MicroRNAs hitherto implicated in diabetes associated vascular complications and their known targets are given in Table 15.1.

15.3.3 Micro RNAs as Biomarkers in Diabetic Vascular Diseases

Currently, there is a paucity of reliable plasma biomarkers for detecting early stages of endothelial dysfunction and vascular diseases in patients with diabetes. Levels of miRNAs in the serum of humans have been shown to be stable and consistent in healthy individuals, qualifying them as predictive markers of disease progression. Identifying miRNAs specific to disease pathways can predict the development and progression of diabetes related complications at a very early stage.

A pioneering study by Zampetaki and colleagues in 2010 has demonstrated a specific miRNA signature of five miRNAs (miR-15a, miR-29b, miR-126, miR-223, and miR-28–3p) in plasma samples which distinguish subjects with a high probability of developing diabetes mellitus from others. They demonstrated the loss of endothelial miR-126 in patients with diabetes making it a good candidate biomarker for both pre-diabetes and hyperglycemia associated vascular diseases [65].

15.3.4 miRNAs Based Therapeutics in Diabetic Vascular Diseases

The altered miRNA profile in patients with diabetes and related complications can either be a cause or effect of the disease pathogenesis. Researchers suggest that circulatory miRNAs are not just indicators of disease development but also they can be involved in the pathogenesis of disease. This assumption if proven in experimental studies will make such candidate miRNAs better targets for minimally invasive early therapeutic interventions. If miRNAs are involved in the pathogenesis of diabetes, modulating the expression of such miRNAs may be a therapeutic strategy for the management of this condition and its associated vascular complications.

Two major strategies are used for modulating miRNA activity (a) use of chemically engineered synthetic miRNA mimics or viral expression constructs producing miRNA *in vivo* to restore the regulatory function of miRNA, (b) inhibition of miRNA function by synthetic anti- miRNA oligonucleotides [66]. Currently, miRNA therapeutics is based on the use of antagomirs. *Antagomirs*, also known as anti-miRs, are chemically engineered oligonucleotides which inhibit miRNA expression *in vivo* by irreversibly binding the miRNA. Another closely related oligonucleotides are *blockmirs* [67]. Though very similar to antagomirs, they are designed to bind to mRNA sequence which serves as binding site for miRNA, thus reducing chances of miRNA-mRNA binding. Both antagomirs and blockmirs have normally only transient effects and hence repeated frequent doses are necessary in chronic diseases such as diabetes.

Homing of these oligonucleotides or tissue specific delivery is another important issue in treatment efficacy. One strategy is to conjugate them with peptides, antibodies or other bioactive molecules, which may help in the delivery of the miRNA to specific cells or tissues [68]. Alternatively, Adeno-associated virus (AAV) vectors carrying the antimiR or miRNA mimic could be used to modulate regulation *in vivo* [69]. Oligonucleotides encapsulated with lipid-based formulation are also used to increase cellular uptake of these foreign bodies. Another therapeutic approach involves artificial miRNA traps which bind endogenous miRNA to generate a loss of function of a particular miRNA. These traps are caled ‘miRNA sponges’. These oligonucleotide sponges comprise of multiple binding sites, targeted against a specific miRNA or against a whole miRNA family [70].

MicroRNA based therapeutics in diabetes and related vascular complications are at its infancy at present. Anti-miRNA oligonucleotides have been used in the past few years, for therapeutic silencing of different miRNAs in animal models to study their roles in diabetes, obesity, hyperlipidemia , and insulin resistance. Trajkovski and colleagues in 2011 demonstrated that delivery of 2′-O-methyl modified antimirs by tail-vein injection could silence both miR-103 and miR-107 in the livers and adipose of diabetic mice models for a period as long as 12 weeks. Silencing of miR-103 and miR-107 significantly reduced hyperglycemia by inducing insulin signaling in the liver and adipose tissue [71].

miR-24 has also been targeted to increase insulin sensitivity in liver and also to improve glucose-stimulated insulin secretion from pancreatic beta cells [72]. miR-33 was targeted recently in mice models of atherosclerosis (*Ldlr*$^{-/-}$) to maintain systemic cholesterol homeostasis [73]. While these studies on miRNA therapeutics are definitely encouraging, in reality a specific miRNA can target more than a few genes and hence clinical intervention can be challenging.

15.4 Long Non-coding RNAs

15.4.1 LncRNAs: Biogenesis, Function and Cellular Location

LncRNAs are large noncoding RNA molecules comprising of more than 200 nucleotides. LncRNAs do not encode proteins. Brannan et al. in 1990 discovered *H19*, the first lncRNA from human genome [74].

Biogenesis of lncRNAs resembles mRNAs transcription. They are transcribed by RNA polymerase II (Pol II) and are frequently 5′-capped and polyadenylated [75]. They differ from mRNA mostly because of the fact that they lack a translated open reading frame (ORF) or they at least lack an ORF of more than 100 amino acids. Also, lncRNAs are generally shorter than mRNAs and have fewer but longer exons. LncRNAs may be classified [76] into different subtypes according to the location and direction of transcription in relation to their target mRNAs (Fig. 15.2).

(i) Sense (overlapping) – The lncRNA sequence partly matches with the sense strand of a protein coding sequence.
(ii) Antisense (natural antisense (NATs) lncRNAs) – The lncRNA sequence overlaps with the antisense strand of a protein coding sequence.
(iii) Bidirectional – The lncRNA sequence is positioned on the opposite strand from a protein coding gene whose transcription is initiated less than 1000 nucleotides away.
(iv) Intronic – The lncRNA is derived from an intron of an mRNA.

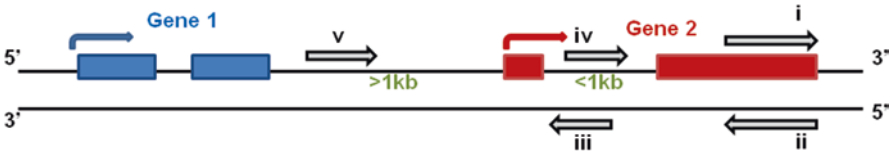

Fig. 15.2 Classification of LncRNAs based on genomic locations
(*i*) Sense lncRNAs span exons within a protein coding gene. (*ii*) Antisense lncRNAs are transcribed from the opposite strand of a coding gene and in opposite direction. (*iii*) Bidirectional lncRNAs are situated on the opposite strand but less than 1 kb of the promoter on the sense strand and direction of transcription is also opposite to coding gene. (*iv*) Intronic lncRNAs are located within one of the introns of coding gene on the sense strand. (*v*) Intergenic lncRNAs are transcripts located between two genes which have as minimum >1Kb distance

(v) Intergenic (LincRNAs) – The lncRNA sequence is strictly intergenic, as it is not located near any other protein coding genes.

Only a few lncRNAs have been properly characterized till date. Yet, it is recognized that lncRNAs are key regulators of gene expression with significant cellular and developmental functions [77]. LncRNAs modulate both gene activation and inhibition. In general, the expression level of lncRNA is at least one order of magnitude below mRNA expression [78]. This increases another level of complexity to genomic studies. The major functions of lncRNA include cell fate specification, chromatin modification to produce epigenetic changes, enhancement of transcription regulation, transcriptional repression, inactivation of X chromosome, RNA splicing, nuclear trafficking and genomic imprinting [79].

Earlier studies have shown that lncRNA expression is developmentally regulated and can be cell and tissue specific [80]. Several lncRNAs are located entirely in the nucleus, while recent studies have discovered lncRNAs which are exclusively present in the cytoplasm [80, 81]. There are also some lncRNAs which are seen both in the nucleus and the cytoplasm of cells. Studies by Cabili and colleagues using single-molecule RNA-FISH substantiate that lncRNAs display different spatial patterns ranging from distinct subnuclear location to diffuse cell cytoplasmic distribution [82].

15.4.2 LncRNAs in Diabetes Associated Vascular Diseases

Studies on lncRNA which are expressed in the vasculature and vascular diseases are actively progressing at present. Not much information is however available on the role of lncRNAs in vascular diseases associated with diabetes mellitus. Another challenge in this field of research is that unlike miRNAs, lncRNAs are not often conserved among species [83]. This feature of lncRNA makes most of the hitherto performed studies in model organisms redundant in humans. Yet, studies of lncRNA in disease models and cell models are shedding light on its mechanisms of action.

Some of the major LncRNAs that are possibly associated with diabetic vascular diseases in humans are discussed below.

1. *PVT1*: Recent studies by Alvarez et al. reveal that the lncRNA, *PVT1* (plasmacytoma variant translocation 1) is highly expressed in renal mesangial cells under high glucose conditions. *PVT1* knockdown in mesangial cells results in the downregulation of PAI-1, collagen and fibronectin genes which are related to fibrosis and diabetic nephropathy [84].
2. *MALAT1*: A lincRNA known as metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) which express in endothelial cells was recently found to increase proangiogenic effects in response to hypoxia in mouse models and *in vitro* culture. Michalik et al. demonstrated that *MALAT1* regulates endothelial migration and vascular sprouting [85]. Reports by Liu et al. in 2014 suggest that MALAT1 is involved in high glucose induced microvascular dysfunction in rats

[86]. Taken together with the finding that *MALAT1* is overexpressed in fibrovascular membranes of diabetic patients, it can be inferred that MALAT1 is linked to diabetes-induced microvascular dysfunction.

3. *MIAT*: Yan et al. in 2015 reported that myocardial infarction–associated transcript (MIAT) lncRNA was over expressed in the retinas of diabetic animals and in endothelial cells cultured in high glucose medium [87]. MIAT silencing reduced retinal microvascular dysfunction due to diabetes in rats. Knockdown of MIAT resulted in the inhibition of endothelial cell proliferation, migration, and tube formation in endothelial cell culture models under high glucose conditions.

Majority of the studies on lncRNA has been performed in animal models and *in vitro* cultures. The lack of nucleotide sequence conservation hampers extrapolation of the observations to human diseases. Contrarily, the fact that lncRNAs are conserved among various species and even in different cells, increases the potential for considering them as cell specific targets for pharmacologic modulation.

15.4.3 LncRNAs Based Diagnostics and Therapeutics in Diabetic Vascular Diseases

Considering the fact that lncRNAs have a significant role in cellular homeostasis, several research groups are focusing their studies on potential for developing diagnostics and testing the therapeutic efficacy of lncRNAs in vascular diseases. Two important lncRNAs, *ANRIL* and *LIPCAR* are being investigated currently for their significance in atherosclerosis plaque formation and cardiac remodelling in left ventricular dysfunction [88]. Information on the role of lncRNA as a biomarker for diabetes and associated diseases is scarce. The utility of lncRNA as a candidate biomarker is limited presently due to its low level expression in plasma.

Potential interventions that can augment or inhibit lncRNA expression so as to modulate angiogenesis in patients with myocardial infarction, peripheral arterial disease, or ischemic stroke are still under investigation. Several studies have utilized short antisense oligonucleotides targeted against lncRNAs successfully for various functional studies in animal and cell models [89]. The fact that many lncRNAs functionally interact with some miRNAs and other vascular factors also needs consideration during development of therapeutic interventions.

15.5 Other ncRNAs in Diabetic Vascular Complications

Recent advances in high throughput sequencing have unveiled several non-coding RNAs such as piwiRNA and snoRNA in gene expression. These RNAs either have a housekeeping role in the biosynthesis of ribosomal RNAs (snoRNA) or gene silencing (piwiRNA).

Lately, several non-canonical roles for these RNAs are being demonstrated. A noteworthy report is on the role of snoRNAs in the pathophysiological responses of pancreatic β cells. Jean Schaffer's research team from Washington University School of Medicine recently reported that snoRNAs from the *Rpl13a* locus have a regulatory role in function of pancreatic β cell under metabolic stress [90]. They also demonstrated that these snoRNAs modulate beta cell response to oxidative stress. In Rpl13a-snoless knock out models, greater insulin secretion was attained even in the presence of metabolic ROS challenge. This study indicates that snoRNAs akin to miRNA can be used in drug therapy by utilizing their antisense oligonucleotides. Coming years may witness several breakthroughs on the usefulness of these ncRNAs in diabetic vascular disease.

15.6 Future

Despite identification of many ncRNAs, their predicted target genes are not functionally characterized completely. The complexities in the pathogenesis of diabetes mellitus and vascular diseases make this effort formidable. Emerging evidence suggests that these ncRNAs indeed have a potential causative role in diabetes and related cardiovascular complications. In coming years, we can expect that these epigenetic mechanisms will be exploited to define early clinical biomarkers and targeted therapeutic interventions in the management of diabetes vascular diseases.

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Part IV
Hemostatic Factors

Chapter 16
Diabetes as a Prothrombotic State

Kanjaksha Ghosh

Abstract Both type 1 and type 2 diabetes are prothrombotic states. This state is contributed by both hyperglycaemia and hyperinsulinaemia that characterize type 2 diabetes and hyperglycaemia only in type one diabetes. All phases of blood coagulation are affected in this disease. Increase in procoagulant factors, increased tissue factor, reduced natural inhibitors of coagulation, platelet hyper reactivity, endothelial cell dysfunction, increased blood viscosity, increased red cell membrane rigidity and dysbalanced adipokines and other proinflammatory cytokines along with accelerated microparticle generation and autoimmunity in the form of anti apolipoprotein antibody contributes to acquired prothrombotic state of the disease. A proportion of such patients also have inherited thrombophilia and antiphospholipid antibodies. Acquired complications of diabetes *ie,* atherosclerosis, micro and macrovascular disease leading to end organ dysfunction also aggravate the already existent prothrombotic state in the condition. Improving diabetic control by using a combination of medicines can result in a favorable thrombohaemorrhagic profile in addition to lowering of blood sugar g and insulinomimetic properties. In addition, drugs which control dyslipidaemia and improves endothelial health and reduces platelet hyperreactivity along with blood pressure control will reduce the thrombophilic tendency and vasodegenerattve propensity in this disease. These treatments when combined with proper diet and exercise will normalise thrombohaemorrhagic balance in this disease and will holistically improve the health of the patient.

Keywords Coagulation • Thrombosis • Thrombophilia • Fibrinolysis • Diabetes • Endothelial function

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16.1 Introduction

Diabetes is recognized by a common biochemical abnormality of fasting/post prandial hyperglycaemia but it is not a single disorder. Two major types of diabetes i.e. Type I or Insulin dependent diabetes mellitus and type II i.e. maturity onset diabetes mellitus with relative hyper insulinaemia and Insulin resistant are recognized. Being a metabolic disorder it affects every aspects of metabolic machinery in the body and the major cause of death and disability in diabetes comes from vascular complications of stroke, acute myocardial infection, peripheral vascular disease affecting >80% of the patients with the disease [1, 2]. Small vessel disease and macrovascular complication along with medial degeneration in muscular arteries are also known complication of the disease [3, 4]. Venous thrombosis and pulmonary embolism too seems to increase in diabetic state [5]. Retinopathy and nephropathy characterize two very common vascular complication of diabetes. All these begs a question do these vascular changes sit side by side in diabetes with a prothrombotic state. Do the prothrombotic state in diabetes predates the vascular changes in diabetes? Or they get aggravated with the onset of vascular changes. Arterial pathology mainly atherosclerosis in diabetes is contributed by a combination of factors i.e. changes endothelial cell biology, hyperlipidaemia, blood viscosity, changes in platelet biology, hypertension and changes in flow characteristics in different places of arterial tree [6]. In addition neoangiogenesis is also an important complication in the disease.

Many of the factors leading to thrombotic tendency and vascular complication in diabetes have been discussed in this book in different chapters. Around 10% of the normal population also carry different genes for known thrombophilia (2% protein C deficiency 3% proteins S deficiency variable number 2.5–10% population with factor V Leiden and so on. If thermolabile MTH FR is included in the list then in some population more than 30% of the population have genetic thrombophilia. However thermolabile MTHFR is not regarded as a prothrombotic gene in the presence of adequate supply of folic acid). It is expected that at least some proportion of population with diabetes will in addition also carry at least the same proportion of known thrombophilic genes. Other genetic markers of atherothrombosis and endothelial pathology like Lpa level [7] and thermolabile MTHFR deficiency [8, 9] predisposing to hyper homcysteinaemia and genetic polymorphism affecting fibrinolytic system and coagulation factor genes also substantially produce a background condition on which diabetic prothrombotic state develops. The major discussion in the chapter deals with acquired changes in coagulation biology due to diabetes predisposing to a thrombophilic state [10, 11].

16.2 Thrombus Formation

Formation of thrombus inside a blood vessel is an interplay of (i) Activation of coagulation on endothelial surface or on the vessel wall due to pathology in the endothelial cells or exposure of vessel wall subendothelium and pathologic smooth muscle

cell. (ii) Deposition of activated platelets along with blood clot and growth of blood clot due to a combination of increased levels/activity of various coagulation factors and interaction with its inhibitors (Antithrombins, protein C, protein S etc.). (iii). Cross linking of the fibrin clot by factor XIII. (iv). Balance of fibrinolytic and antifibrinolytic system. All the above mechanisms together form in health a "Thrombo Haemorrhagic Balace" where minute thrombus that occurs in thousands of places in the blood vessel naturally is dynamically balanced by clearance of these minute clots by fibrinolysis. This thrombo-haemorrhagic balance is also contributed by (v) optimum viscosity and flow characteristic of the blood and dynamic nature of endothelial cells which shuttles between antithrombotic and a pre thrombotic state [12]. A schema of normal blood coagulation is shown in Fig. 1a and in various figures (Figs. 16.1a i & ii, b, 16.2, 16.3, 16.4, 16.5 and 16.6) interplay of various factors leading to prothrombotic state has been depicted. In classical thrombophilia there is a tendency for thrombus to develop at young age (under 45 years), often the thrombi are recurrent or happens in unusual location, and could be multiple and coming without anyor least provocation . Some of these patients also have family history of thrombosis. In contrast to genetic thrombophilia thrombosis in diabetic is largely due to consequences of metabolic alterations in the disease, on top of which in a given patient these thrombophilic genes may act synergistically. Table 16.1 has prevalence of various thrombophilia genes in Indian population and Table 16.2 has what thrombophilia genes or markers can be tested in a good thrombosis homeostasis laboratory. These testing should not be done in acute stage unless it is a gene study. Other thrombophilic markers should be studied 2–3 month after stoppage of antithrombotic drugs. Relatives who are not on anti thrombotic drugs could be tested at any time.

16.3 Changes in the Level and Activity of Coagulation Factors in Diabetes Mellitus

There are innumerable studies showing that several coagulation factors [10, 11, 13] are increased in diabetic patients (Table 16.3) Increase in the levels coagulation factor like fibrinogen even predates development of diabetes and may be found in the family members who has not yet developed diabetes [14] and rise in factor VII level correlates well with increased triglyceride levels in the disease and dyslipidaemia is a well known feature and complication of diabetic state [15]. In addition to these two coagulation factors, there is also increase in the tissue factor activity in the disease [16] and factor V and as well as factor XI levels are also increased. With the changes in kinins and Kininogens in the disease there is also increase in plasma factor XII levels in this condition [17]. Some of the studies did find a broad correlation with long term control of the disease as evidenced by HbA_1c levels [18] or plasma insulin levels [19]. However it is consistently shown that plasma levels of factor VIII, factor VII, Von willebrand factor and fibrinogen are higher in diabetes than non diabetics [20]. Hence increase in procoagulant factor along with tissue factor levels produce one facet of prothrombotic state in diabetes mellitus.

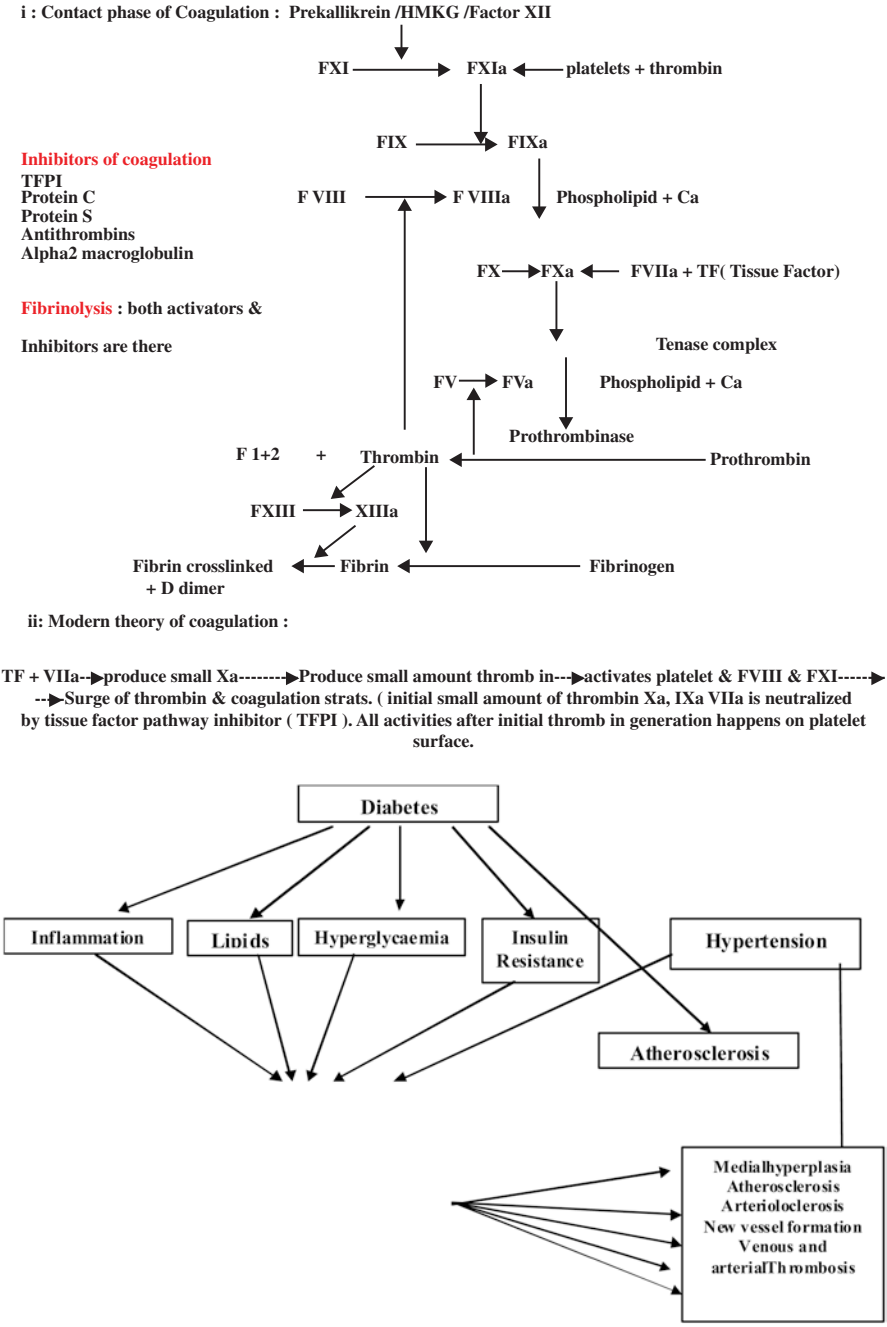

Fig. 16.1 (**a**) Modern theory of blood coagulation (*i* cascade theory, *ii* modern theory). (**b**) Genesis of endothelial dysfunction leading to vascular complication

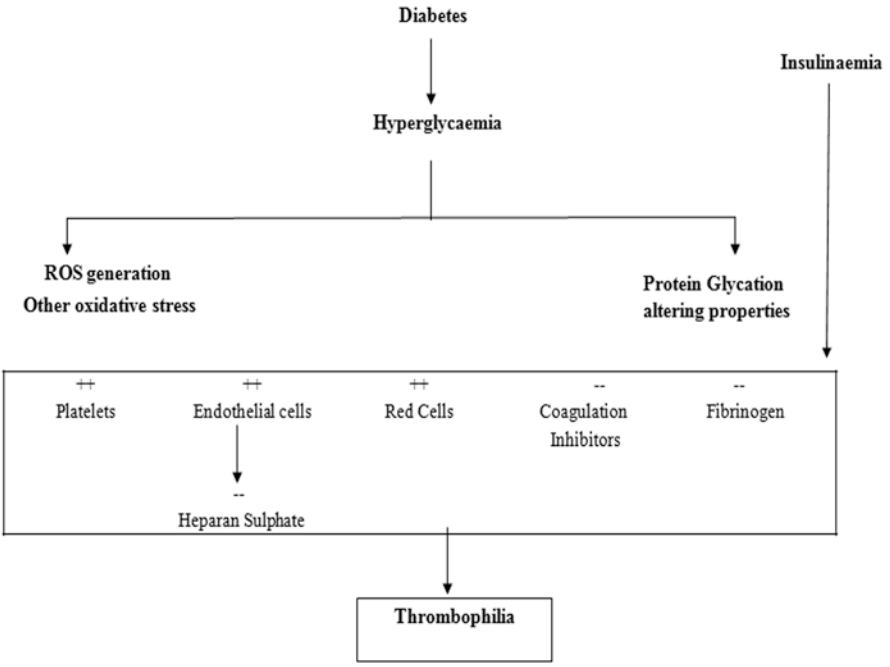

Fig. 16.2 How diabetes can cause thrombosis through hyperglycaemia and insulin resistance

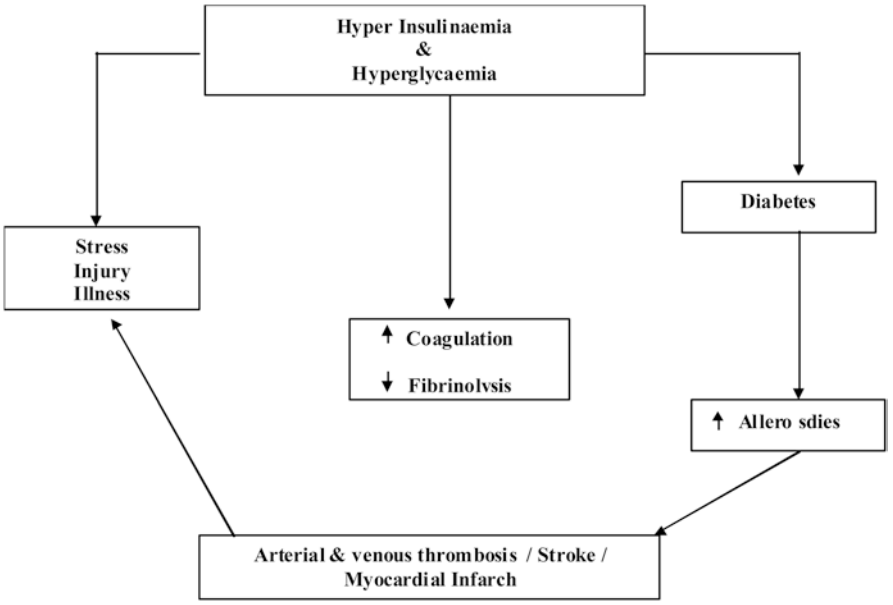

Fig. 16.3 Viscious cycle of hyperglycaemia, thrombophlia and vascular events

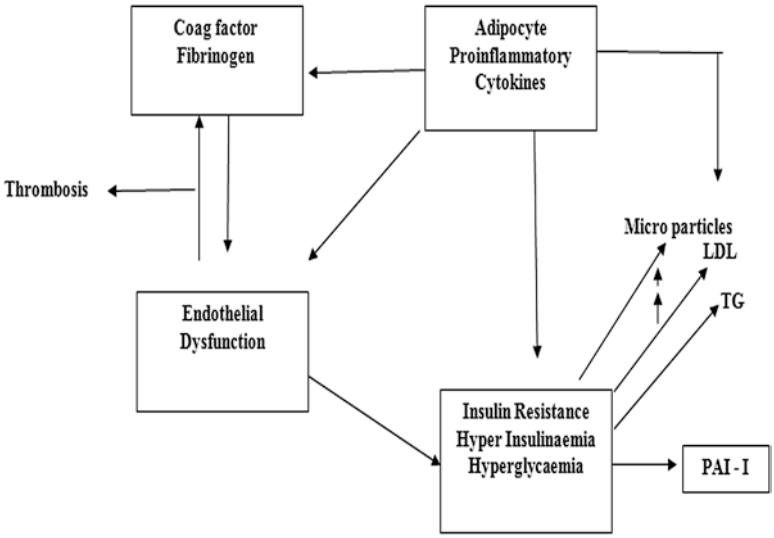

Fig. 16.4 Adipokine imbalance, endothelial cell dysfunction, insulin resistance and prothrombotic state

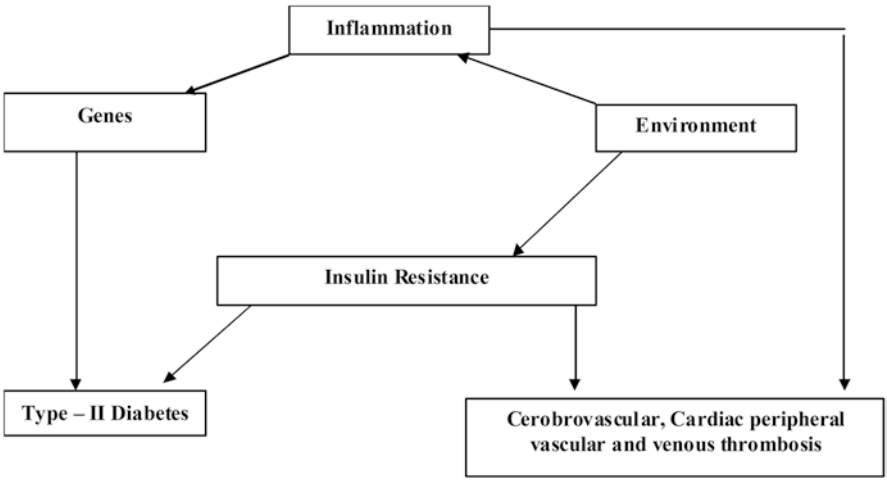

Fig. 16.5 Genes, environment and inflammation linking diabetes and vascular disorders

All the reasons for increase in some of the coagulation factor in diabetes mellitus are not clearly understood. But as increase in some of the coagulation factor level predates the diagnosis of frank diabetes mellitus as evidenced by hyperglycaemia, it stands to reason that factors other than mere hyperglycaemia i.e. insulin resistance,

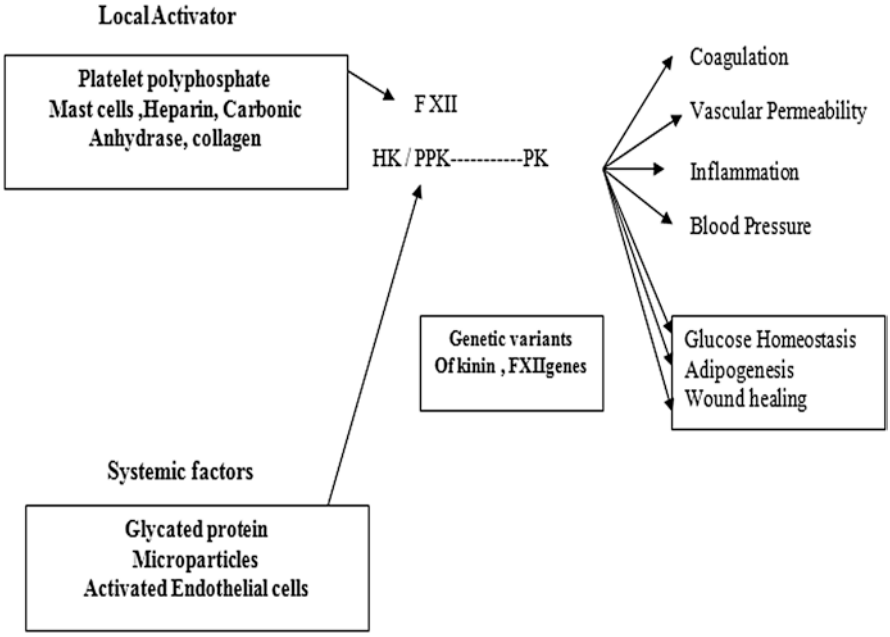

Fig. 16.6 Activation and effect of kinin system on diabetes, blood coagulation and adipogenesis

Table 16.1 Thrombophilia markers and approximate prevalence in indian population and relative risk of life time deep venous thrombosis

Marker	Prevalence	Relative risk[a]
Protein C	1%	6.5–31
Protein S	2%	2–36
Antithrombin	0.02%	5–40
Factor V Leiden	< 3.5% (1–3.5%)	2–10
Prothrombin G20210A polymorphism	Absent (extremely low)	2–6
Thermolabile MTHFR (heterozygous)	14	2–4 (if hyperhomocysteinaemia is there)
Thermolabile MTHFR (homozygous)	2%	
PAI -1 4G/5G	12%	Not known
EPCR 23 BP insertion	2–4%	Not known

From data of NIIH, Mumbai [a]Deitcher S in Hypercoagulable state. Cleveland clinic CME 2010

hyper insulinaemia, (Fig. 16.2) effects of counter regulatory condition due to associated obesity in some of these case through dis-balanced adipokine network,hypertriglyceridaemia, protein glycolsylation caused by persistent hyperglycaemia are also important factors leading to prothrombotic state [21].

Table 16.2 Thrombophilia markers usually assessed in a case of suspected condition

Activated protein C resistance	Alpha 2 macroglobulin deficiency
Anti cardiolipin antibodies	Anti thrombin deficiency
Dysfibrinogenaemia	Factor V Leiden
Factor V excess/deficiency	Factor VII excess
Factor VIII excess	Factor XI excess
Hyperfibrinogenaemia	Lupus anticoagulant
PAI-I excess	Plasminogen deficiency
Protein C deficiency	Protein S deficiency
Prothrombim G20210 A	tPA deficiency
TFPI deficiency	Thrombomodulin deficiency

16.4 Changes in the Natural Inhibitor Levels in Plasma

Prothrombotic state being a state of altered thrombohaemorrhagic balance is also influenced by changes in the level of natural inhibitors of blood coagulation. Protein C, Protein S and Antithrombin (previously known as antithrombin III) are three natural inhibitors of blood coagulation. Other protease inhibitors also contribute in neutralizing active coagulation proteins. Tissue factor pathway inhibitor (TFPI) is another specific inhibitor of activated factor x (xa) and activity of tissue factor VIIa complex. The changes in natural inhibitors in diabetes is also depicted in Table 16.3.

After a limited whiff of thrombin is produced (Fig. 16.1a i & ii) by initial action of tissue factor from injured site and factor VIIa complex,subsequent surge of thrombin occurs through platelet activation and activation of factor XI & factor VIII by thrombin . TFPI is found to increase in DM. Studies have demonstrated increased level of antithrombin antigen in the condition. However the specific activity of antithrombin i.e. antithromin activity/mg of protein is reduced in diabetes [22, 23]. However some studies showed AT III (Now called antithrombin) levels correlate with micro vascular complication and was found to be significantly elevated [20] when compared with diabetes without such complication .Protein C and protein S both were found to be either normal, increased or decreased in various studies [24–26]. Protein S circulates in blood as free and bound protein S which is bound to complement 4b binding protein. In DM complement 4b binding protein level are increased but protein S production remains steady as a result free protein S level i.e. which is active in preventing thrombosis formation tends to fall [27]. Hence all in all there is a general tendency for natural inhibitors of coagulation to decrease in diabetes and often the decrease become more pronounced with the associated diabetic complications.

16.5 Balance of Fibrinolytic and Anti Fibrinolytic System

After a blood clot is produced the size of the blood clot increases and the clot stays till it is lysed by fibrinolytic system. A mature cross linked fibrinogen clot is difficult to lyse by fibrinolytic system. In the fibrinolytic system endothelial cells secretes plasminogen activiator and in circulation additional urokinase type of inhibitor

Table 16.3 Changes in coagulation factors, inhibitors and fibrinolytic/antifibrinolytic proteins in diabetes mellitus

Tissue factor level in plasma	Increased
Fibrinogen	Increased
Prothrombin	Increased
Factor V	Nochange/increased
Factor VII	Increased
Factor VIII	Increased
Factor IX	No change
Factor X	Increased/No change
Factor XI	Increased
Factor XII	Increased
Kinins/kininogens	Increased
Factor XIII b subunit	Increased
Antithrombin	Increased/Decreased (functional activity decreased)
Protein C	Decreased
Protein S	Decreased/Unchanged (free protein S decreased because of increased binding to C4B complement binding protein).
PAI –I	Increased
tPA	Increased (but activity decreased because of complexing with PAI-I).
TFPI	Decreased.
TAFI	Increased
Viscosity of blood	Increased
Procoagulant microparticles	Increased
F1+2	Increased
D- Dimer	Increased
Platelet reactivity	Increased

sevreted from various epithelial cells and platelet is also present. The platelets also contain an inhibitor of fibrinolysis in addition to dominant PAI – I inhibitor in plasma another inhibitor called α2 anti plasmin is also present in plasma. Plasminogen attaches to cross linked fibrin through its lysine binding sites and lysine. Another protein known as Thrombin Activable Fibrmolytic Inhibitor (TAFI) which is present in plasma but is activated by thrombin prevents attachment of plasminogen to Fibrin by removing lysine residues of fibrin so that the LBS (Lysin Binding Sites) of plasminogen can not bind to fibrin and fibrinolysis is prevented.

Anti fibrinolytic activity of plasma is also associated with another protein called Lpa. In hyperglycaemia and Diabetes mellitus anti fibrinolytic activity level of plasma including PAI-1 [28], TAFI and some cases LPA levels tends to increase. On the balance diabetic plasma is more antifibrinolytic. The fibrinolytic protein levels are reduced though tissue plasminogen levels may show an increase due to increased complexing with PAI-I leading to inactive complex. A large part of anti fibrinolytic activity of blood is associated with the lipid fraction of plasma and in diabetes,

associated hyperlipidemia also contributes to increased antifibrinolytic activity in addition to independent contribution by hyper glycaemia and hyper insulinaemia with insulin resistance [29]. Table 16.3 shows changes in fibrinolytic and antifibrinolytic proteins in plasma in diabetes mellitus.

16.6 Endothelial Cell Function

In diabetes (both type 1 and type 2) the endothelial function in profoundly affected. The endothelial barrier which in normal state prevents clotting,in diabetes these cells loses may of its anti clotting behavior largely due to (i) increased apoptosis of endothelial cells [30] (ii) Increased secretion of high molecular VWF and reduced expression of thrombomodulin and protein C receptor on it there by effectively braking production of activated protein C, a potent inhibitor of blood coagulation [30]. (iii) Endothelial cells when healthy have a glycosaminoglycan (heparan sulphate) glycocalyx this glycocalyx along with antthrombin and Heparin co-factor 2 prevents blood coagulation by neutralizing various activated coagulation factors including Xa and thrombin. There is loss of glycocalyx barrier in endothelial cells in diabetes mellitus which promotes thrombus formation.

In addition to above nitric oxide production, prostacyclin production and Endothelial hyper polarisation factor (EDHF) production are all reduced in amount in diabetes mellitus compromising endothelial health [31] .Circulating endothelial progenitor cells numbers are also reduced compromising the ability endothelial cells to heal itself. Cellular and molecular basis for endothelial dysfunction is presented in Table 16.4 and Fig. 16.1b.

Table 16.4 Cellular and molecular basic for endothelial dys function in diabetes

	Molecular defects	Effect on blood vessel
1	Increased activation of PKC (Protein Kinase C)	Increased proliferation of vessels altered contraction, altered signal transfusion
2	Over expression of growth factors (Endothelin, Angiotensis II)	Increased growth and phenotypic changes of smooth muscles cells
3	Non-enzymatic glycation of protein and other molecules (DNA)	Changes in antigenicity and transcription process
4	Hyper glycemia with increased synthesis of DAG (Diacyl glycerol) and AGE (Advanced glycation ed products)	Impaired vasodialation and enhanced proliferation of VSMC
5	Impaired Insulin activation of PIP – 3 Kinase with normal MAP kinase response	Increased growth and proliferation of vessel due to hyper insulin anemia
6	Increased production PAI – I and other coagulation changes	Decreased fibrinolysis activation of coagulation
7	Oxidative stress	Decreased production of No hyper reactivity of smooth muscle vascular cells. Endothelial apoptosis, increased expression of pro inflammatory adhesion molecules (ICAM, ELAM, VCAM)

Modified from Ref. [30]

16.7 Diabetes as an Inflammatory Condition

Increasingly type 2 DM is considered as a chronic inflammatory state as evidenced by increase in serum CRP level and IL6 level. This chronic inflammatory condition is driven by hyperglycaemia through NFKB upregulation. Hyperinsulinaemia and hyperglycaemia interact and together increase NFKβ mRNA synthesis and cause profound disturbances in adipose tissue metabolism as evidenced by dysregulated adipokine levels [29, 32, 33] (Fig. 16.4). Very often type 2DM is associated with syndrome X where the chronic inflammation is also in evidence. As a part of persistent inflammatory condition coagulation, complement and kinin systems are activated [17, 34] leading to activated of factor XII and certain coagulation factors like prothrombin, fibrinogen, factor VIII, tissue factor is also elevated.

Chronic inflammatory condition also affects endothelial cells activation in addition, through toll like receptors [35]. Hence in addition to independent effects of hyperglycemia, hyper insulinaminia of diabetes mellitus additional inflammatory state tilts the thrombo haemorrhagic balance in this condition towards thrombotic state. This clearly seen as increased circulating thrombin anti thrombin complex, increased F1 + 2 levels and increased D. Dimer level [36, 49]. Multiple systemic and local factors are involved in contact system activation and in complicated in diabetes mellitus and obesity (Figs. 16.4, 16.5 and 16.6).

16.8 Prothrombotic Genes and Diabetes Mellitus

Diabetes mellitus is a common condition and thrombophilic genes are not rare in any population as a result we can at least expect genetic thrombophilia in any community will also affect their diabetic patients equally. Table 16.1 shows various thrombophilia genes and their approximate prevalence in Indian population. Factor V leiden which has been extensively studied in India show a prevalence of 3.5% in normal population in north India while in the rest of it goes down to 2.5%. In certain communities like Parsees, this prevalence could be as high as 5–10% [37, 38]. Prothrombin G 20210A polymorphism which is an important cause of thrombophilia in western population is absent in Indian people. Thromolabile MTHFR which in one of the important predisposing factor for increased plasma levels of homocystine is present in 14–18% of Indian population going upto 30% or more in some studies [9, 39]. Relative prevalence of different thrombophilia markers in a large cohort of Indian patients with venous thrombosis has been published [40]. This study show that venous thrombosis related to usual thrombophilia markers explains approximately half of the cases of venous thrombosis (Idiopathic, unusual, early) and the reason for other half needs to be worked out. Table 16.2 shows different thrombophilia markers that is tested in a good coagulation laboratory. As thrombophilia testing is costly the test may be combined with clinical findings, family and personal history then subsequently tested in stages depending on population

preponderance, ease of doing it in the laboratory, cost and likely impact of the test in future management of the patient and his relatives.

16.9 Miscellaneous Contributors of Thrombophilia in Diabetes Mellitus

In addition to hyperglycaemia,hyperinsulinaemia, obesity endothelial dysfunction and chronic inflammation many other associated complications like renal disease,congestive heart failure, chronic liver disease, nephrotic syndrome,and presence of autoantibody against apoliproproteins [11, 41] may determine the nature and depth of prothrombotic state.

Increased tendency to infections, particularly tuberculosis, increased rigidity of red cell membrane, increased viscosity of plasma, increased capillary and other tissue permeability due to activated Kinin system (Fig. 16.6), extensive microvascular and macrovascular pathology all contributes to the activation of coagulation system and development of a pro thrombotic state. One of the very important cause of acquired thrombophilia leading to global thrombophilic state is development of antiphospholipid antibody (APL). APL can be measured by various techniques and all APL is not equally thrombogenic [42]. Table 16.3 shows the changes in various coagulation factors in diabetes.

16.10 Pathobiology of Thrombophilia in Diabetes Mellitus

In addition to genetic thrombophilia which affects around 10% of Indian population, diabetes produces thrombophilia by several mechanism. Diabetes affects all phases of blood coagulation from platelet activation, endothelial dysfunction, increased coagulation factors reduced coagulation inhibitors, reduced fibrinogen and increased blood viscosity. It has been found that blood clot produced in diabetic state are stronger and difficult to lyse with fibrinolytic enzymes [43, 44] and this had been attributed to nonenzymatic glycosylation of fibrinogen. Hence in diabetes hyperglycaemia itself by non enzymatic glycosylation can change the properties of many proteins involved in thrombosis and haemostasis. At the level of gene transcription both hyperglycaemia and associated hyperinsulinaemia(Insulin resistance) work to augment transcription of many gens. Increased reactive oxygen species in this disease cause development of advanced glycation end products (AGE). AGE molecules interact with its cognate receptors ie receptors of advanced glycation end prosects (RAGE) causing [45] increased transcriptional activity of may genes including genes responsible for coagulation and thrombosis.

Table 16.5 Showing the effects of antidiabetic drugs and other ancillary drugs used in diabetics on blood coagulation

Metformin	↓ FVII, PAI-I, XIII crosslinking activity. Loose clot structure
	Assures quick fibrinolysis
Sulphonyl Urea	↓ PAI-I decreased clot permeability (possibly prothrombotic)
TZD	↓ FVII, Fibrinogen, PAI-I. ↑ Thrombomodulin
GLP-I analogues	↓ PAI-I
Insulins	↑ PAI-I, Fibrinogen (possibly thrombogenic)
Aspirin	Loose clot assist in quick fibrinolysis
Thienopyridines	↓ Fibrinogen, interfere with platelet function. Loose clot
Statins	↓ TF, FVII, PAI-I, FV & XIII activation. ↑ Thrombomodulin
	Improve tPA release, Some reduce fibrinogen levels
Fibrates	↓ Fibrinogen, TF, FVII, PAI-I
Ezetimibe	↓ PAI-I, ↑ TF (prothrombotic)
ACEI/ARB	↓ Fibrinogen, PAI-I

Modified from Ref. [13]

16.11 Effects of Antidiabetics and Related Drugs on Thrombophilia

Balance of thrombosis and haemostasis in diabetes also depends on the effects of antidiabetic and other drugs used in this condition as these drugs themselves have varied effects on this process. Hence it is possible to include a combination of drugs in diabetes favourably altering thrombohaemorrhagic balance.

There are innumerable oral and parenteral drugs which are used to treat DM. In addition diabetic hyperlipidaemia is also managed through large number of medicines .All these medicines have some effects on thrombosis and haemostatic proteins. Some of them also influence endothelial health Table 16.5 depicts effects of some of these drugs on blood coagulation.

16.12 Discussion and Conclusion

Both type1 and type 2 diabetes are associated with increased tendencies of both atherosclerotic and venous thrombotic disorders. In fact >80% deaths in type II diabetes are related to thromboembolic events [1, 2, 46–48].

This prothrombotic state in diabetes is complex hence at many place literature is confusing. The two characteristic factors of diabetes i.e. Hyperglycaemia and

hyperinsulinaemia with insulin resistance (type 2 diabetes) can directly explain most of the pathological change in coagulation (Figs. 16.2 and 16.3), anticoagulant, fibrinolytic and antifibrinolytic proteins that characterise the thrombophilic state in this condition. Hyper reactivity of platelets is also a constant companion of this hyper active coagulation compounded by endothelial dysfunction.

On top of this, obesity, inherited thrombophilia genes, dyslipidaemia, hyperviscosity of blood, associated infections, complications of generalized vasculopathy, cardiac disorders and cytokine and adipokine imbalance super imposes its additional pathobiology on the thrombosis and haemostatic process.

These pathobiology are increasingly summative and at times synergistic. Control of hyperglycaemia, hyperinsulinaemia,improving dyslipidaemia, obesity by drugs and life style changes largely reverse these thrombotic tendencies and this should be the aim of any holistic management of disease. The interaction of many of these factors are depicted in a series of figures in the chapter (Figs. 16.1a, b, 16.2, 16.3, 16.4, 16.5 and 16.6). One of the very important current development in the saga of thrombo haemorrhagic imbalance is the role of microparticles and microparticle associated micro RNA as active players in the pathogenesis of procoagulant state and endothelial dysfunction [49–51]. Activation of coagulation is a very important pathological process in diabetes state and by itself may contribute to downward spiral of ineffective management of the condition. Balancing it back to normalcy is now possible using combination of antidiabetic drugs and other measures that restrict adipokine, inflammatory cytokine imbalance and improves endothelial health.

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Chapter 17
Mechanisms of Hypercoagulation and Aberrant Clot Lyses in Type 2 Diabetes

Etheresia Pretorius

Abstract Type 2 diabetes (T2D) has attained a pandemic status with more than half a billion cases expected by 2030; and many having cardiovascular complications, with main hallmark of these events, the presence of systemic (chronic) inflammation. Systemic inflammation is in turn characterized by a changed haematological system, including a pathologic coagulation system, endothelial dysfunction and ultimately vascular complications. This chapter discusses the pathogenesis of T2D, and how it is interlinked with cardiovascular disease and inflammation. Literature is reviewed that shows the inflammatory nature of the T2D, how this inflammatory profile and pathological inflammatory markers, affects the coagulation system, and how it plays a role in the impaired vascular function, which is a fundamental characteristic of T2D. As part of the pathogenesis we discuss the considerable literature showing that both hypercoagulability and hypofibrinolysis are present in a large number of inflammatory and vascular diseases, including T2D. We discuss novel methods to monitor and study manifestations of both hypercoagulation and hypofibrinolysis in T2D. We conclude by suggesting that the multifaceted nature of the condition suggests a patient-orientated approach is followed where both traditional and novel methods should be equally explored in the monitoring of T2D patients.

Keywords Type 2 diabetes • Hypercoagulation • Hypofibrinolysis • Inflammatory markers • Cardiovascular complications • Clot structure

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17.1 Introduction

Type 2 diabetes (T2D) have reached pandemic status with more than half a billion cases expected by 2030 [1]. Comorbidities of both obesity and T2D include cardiovascular disease, cancer and neuropsychiatric disorders [2]. Cardiovascular disease in particular, is one of the most common diabetes-associated complications, as well as a leading cause for death in these patients [3]. Important cardiovascular events include myocardial infarction and stroke [4] and the main hallmark of these events are the presence of systemic (chronic) inflammation. Systemic inflammation is in turn characterized by a changed haematological system, including a pathologic coagulation system [5–9], endothelial dysfunction [10] and ultimately vascular complications.

This chapter reviews and discusses literature that shows the inflammatory nature of the condition, how this inflammatory profile affects the coagulation system, including hyercoagulation and mechanisms of impaired clot lyses; and finally shows how these changes lead to the pathological and impaired vascular function, which is a fundamental characteristic of T2D (see Fig. 17.1).

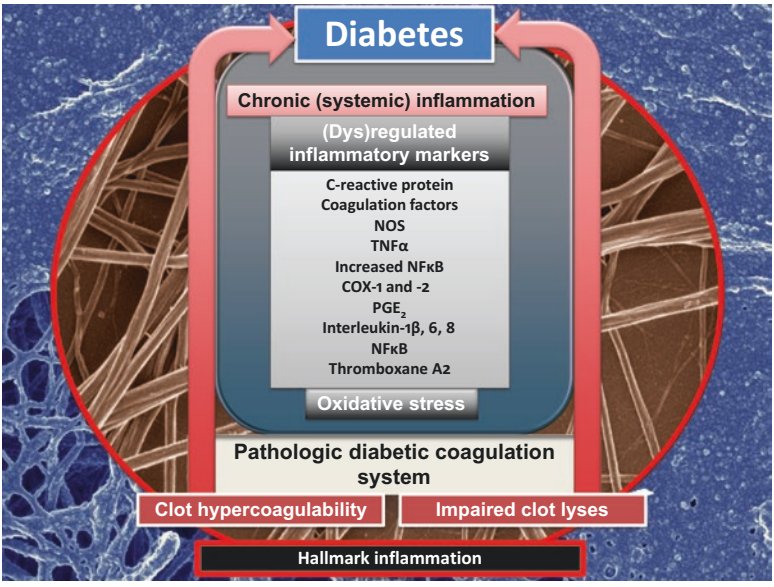

Fig. 17.1 The inflammatory nature of type 2 diabetes

17.2 Markers of Systemic Inflammation and Cardiovascular Disease (CVD)

As systemic (chronic) inflammation plays a fundamental role in many chronic conditions including T2D and CVD [11–15] and because CVD and vascular complications are a fundamental part of the ethiology of T2D, a quick review of the various dysregulated inflammatory markers in CVD follows. Dysregulated inflammatory markers like C-reactive protein (CRP), along with IL-2, IL-6, IL-8, TNF-α, NOS, PGE_2, the COX-family and thromboxane A_2 and NFκB, all belong to the cluster of general inflammation markers that are changed in systemic inflammation and CVD. In this chapter the focus will therefore be on the above-mentioned inflammatory markers, although there are others that also play important roles in inflammation.

CRP is a leading inflammatory biomarker for CVD [16–23] andis produced by the liver hepatocytesunder regulatory control from circulating cytokines, in particular IL-6 andtumour necrosis factor-α [19, 24]. Because it is increased in the presence of inflammation, it is used to screen for inflammation, particularly high-sensitivity C-reactive protein (hsCRP), adds prognostic information in CVD [19, 20, 25].

The interleukins are a cytokine group that is well-known to be upregulated in inflammation [26]. Interleukin 1 Receptor 1 (IL1R1) and its ligand, IL1β, are unregulated in CVD and infection [27]. IL-1β is also known to be present in autoimmune conditions and contributes to several chronic diseases, including atherosclerosis [28–30]. IL-6 regulates the immune response, haemopoiesis, the acute phase response, inflammation [31] and the central nervous system [31, 32]. Its expression is high and transiently unregulated in nearly all pathophysiological inflammatory conditions and also in autoimmune diseases [33, 34]. IL-8 is also a well-known circulating inflammatory cytokine [35, 36]. Macrophages and other cell types such as epithelial cells, airway smooth muscle cells and endothelial cells produce IL-8.

Tumour necrosis factor- α (TNF-α) is a cell signalling cytokine involved in inflammation and is one of the cytokines that make up the acute phase reaction, and its primary function is to regulate immune cells [37–40].TNF-α dysregulation plays an important role in the development of metabolic syndrome features, including dyslipidaemia and altered glucose tolerance, and is therefore is an important cytokine in the development and maintenance of systemic inflammation [39]. Vascular endothelial cells also respond to TNF-α by undergoing pro-inflammatory changes, which ultimately promote thrombosis [41, 42].

Another important marker of inflammation is the nitric oxide synthases (NOS) family. They are synthesized by many cell types involved in immunity and is also well known for its role in systemic inflammation and cardiovascular disease [43–46]. It is also crucial in maintaining cardiovascular homeostasis [45] and a modulator of vascular disease [47]. In CVD, endothelium damage induced by atherosclerosis leads to the reduction in bioactivity of endothelial NO synthase (eNOS) with subsequent impaired release of NO and ultimately leads to a cascade of oxidation-sensitive

mechanisms in the arterial wall [47, 48]. In a comprehensive review, Costa and coworkers discussed the 3 NOS isoforms, neuronal NOS (nNOS or NOS 1), endothelial NOS (eNOS or NOS 3), and an inducible NOS (iNOS or NOS 2). eNOS is considered the main isoform involved in the control of the vascular function, however, the role of nNOS in vascular homeostasis andcardiovascular disorders such as hypertension and atherosclerosis has recently come to light [43].

Prostaglandins (PGs) have two derivatives, namely prostacyclins and thromboxanes and are critical mediators of inflammation [49–54] Cyclooxygenases (COXs) are the biosynthetic enzymes of PGs.PGE_2 (which inhibits platelet activation and is also an effective vasodilator), and thromboxane (Tx)A2 (TXA_2); and is synthesized via three sequential enzymatic reactions: The first step being arachidonic acid (AA) release from membrane phospholipids by phospholipase A_2 ($cPLA_2$); then, AA is converted into the unstable endoperoxide intermediates PGG_2 and PGH_2 by cyclooxygenase-1 (COX-1) or COX-2 [55]. Markers like COX-1 and -2 and prostaglandin E_2 are all closely connected and also play a prominent role in inflammation and CVD [56]. As mentioned before, TXA_2 is also a product from COX [51, 57] and is a vasoconstrictor, and a potent hypertensive agent that also facilitates platelet aggregation. Both PGE_2 and TXA_2 are therefore key role-players in inflammation and CVD.

NF-κB is a protein complex that is activated by pro-inflammatory cytokines such as interleukin 1 (IL-1) and TNFα [58] and the chronic activation or dysregulation of NF-κB signalling is the central to the pathogenesis of many diseases, including CVD [59, 60]. The activity of NF-κB in the canonical pathway results in upregulation of pro-inflammatory (TNFα, IL-6 and IL-8) and pro-thrombotic [MMPs and TF (tissue factor)] mediators, which are known to be pro-atherogenic [60].

Central to the dysregulation of the mentioned (and other) markers of inflammation is the resulting oxidative stress and ROS generation, which plays crucial roles in both inflammation and CVD [61–63]. In CVD there is an imbalance between the antioxidant defence mechanism and ROS production and this leads to oxidative stress [63–65]. Ultimately, oxidative stress, is a strong pro-thrombotic factor [66], and the hallmark of inflammation is a prothrombotic prevalence and this translates to hypercoagulation. Inflammation causes hypercoagulation (which is a prothrombotic state) because of an elevated expression of the above-mentioned markers, and also elevated expression of the prothrombotic molecules like plasminogen activator inhibitor-1, tissue factor (TF) and increased platelet activation [67–70]. TF is the main trigger of the coagulation cascade; by binding Factor VIIa it activates Factor IX and Factor X, thereby resulting in fibrin formation [71, 72]. Increased fibrinogen and pathological fibrin formation are key in the development of a hypercoagulable state during inflammation.

If we take a closer look at the pathology in T2D, we see that the primary cause of death in T2D patients, is CVD and it is 2–4× times higher in people with T2D compared with those who are non-diabetic [73]. It is thus noteworthy that patients with T2D have an increased risk of atherothrombotic events [74]. Also, T2D can be classified as an inflammatory condition, due to upregulation of different inflammatory markers [18].

17.3 Type 2 Diabetes and Its Relation with Cardiovascular Disease

The pathogenesis of T2D, and how it is interlinked with CVD and inflammation, is summarized below:

- There is an intimate relationship between inflammation and metabolism, including glucose, fat and cholesterol metabolism [75].
- T2D is known to be one common risk factors for CVD [63], and both obesity and T2D are associated with a state of chronic low-level inflammation [18, 76, 77] and cardiovascular complications [78, 79].
- Patients with CVD and T2D have increased circulating inflammatory markers [80] and a number of systematic reviews have shown the association between inflammatory markers, such as CRP, IL-1β, IL-6, TNF-α, IL-4, or IL-10, and cardio-metabolic diseases (e.g. T2D) [15, 81–86]. TNF-α e.g. has emerged as a key cytokine that influences intermediary metabolism [39].
- Oxidative stress plays an important role in T2D and it has a critical impact on the development and progression of vascular pathologies, including atherosclerosis and diabetic vasculopathy [64].
- Endothelial dysfunction is implicated in the pathogenesis of vascular disease seen in T2D [10]; and central to this dysfunction is microvascular complications which are related to oxidative stress, and inflammation, all factors traditionally associated with the pathogenesis of vascular damage seen in CVD [87].
- In T2D there is a decreased fibrinolysis, increased thrombin generation, and platelet hyperactivity.
- In T2D there is elevated levels of circulating TF and this is a biomarker for the severity of microvascular disease in these individuals [67, 72, 88].

17.4 Hypercoagulability and Hypofibrinolysis in Type 2 Diabetes

Recently, we have reviewed in great detail the considerable literature showing that both hypercoagulability and hypofibrinolysis are present in a large number of inflammatory and vascular diseases [89] (e.g. [90–123]). We have also shown that in T2D, fibrin structure is fundamentally changed, and that both erythrocytes and platelets are affected by oxidative stress and circulating up regulated inflammatory markers [6–9, 124–127]. Also see Table 17.1 for selected references for the co-occurrence of hypercoagulation and hypofibrinolysis in diabetes; adjusted from [89].

Because T2D is associated with both a hypofibrinolytic and hypercoagulable state, both these pathologies are of crucial importance in the overarching mechanism for increased cardiovascular risk in this population. This forms the basis of the pathology related to, and involved in atherothrombotic complications, which are the

Table 17.1 Selected references for the co-occurrence of hypercoagulation and hypofibrinolysis in type 2 diabetes

Type 2 Diabetes	Some references showing blood hypercoagulability	Some references showing reduced clot permeability or decreased susceptibility of clot to (fibrino) lysis
	[118, 150–156]	[128, 131, 132, 151, 154, 156–163]

Adapted from [89]

main cause of mortality in T2D. This inflammatory state in T2D presents itself as premature atherosclerosis, increased platelet reactivity and activation of coagulation factors, with associated hypofibrinolysis. Ultimately all of these pathologies together contribute to increased cardiovascular risk in this population [128].

Except for the pathological levels of inflammatory markers in T2D leading to ROS generation and oxidative stress that we discussed in the previous paragraphs, a number of factors have been implicated in impaired fibrin clot lyses are:

- Altered structure of the fibrin (ogen), including glycation and oxidation, resulting in a more compact clot with thinner fibres and increased branching that are more difficult to lyse [74, 129, 130].
- Increased incorporation of antifibrinolytic proteins (e.g. plasminogen inhibitor and complement C3 into the clot [131, 132] with both proteins having antifibrinolytic activities [74].
- Higher levels of plasminogen activator inhibitor-1 (PAI-1), which causes a pathological fibrinolytic process, because of a decreased plasmin generation [128]. PAI-1 has been found in blood from patients with T2D and in other conditions associated with insulin resistance [133–135]. IncreasedPAI-1 in blood is also associated with a tendency toward venous thrombosis and pulmonary embolism [135], and is associated with a decreased fibrinolytic activity or hypofibrinolysis [136]. This hypofibrinolysis are also related to insulin resistance [137]. Schneider and co-workers in 2004 already suggested that an increase in PAI-1 in vessel walls might predispose to acceleration of atherosclerosis and development of plaques with specific characteristics rendering them vulnerable to rupture [138]. Glycation of plasminogen in T2D also directly affects fibrinolysis by decreasing plasmin generation and reducing protein-specific activity [74].
- Elevated glucose levels result in increased plasminogen glycation, which affects protein clearance [139]. Tissue plasminogen activator (tPA) mediates plasminogen conversion to plasmin. Binding of tPA to fibrin typically increases the catalytic conversion of plasminogen to plasmin while simultaneously localizing plasmin generation to the site of thrombus formation, thus preventing systemic plasmin generation [74]. Therefore, hypofibrinolysis in T2D is also the result of glycation of plasminogen leads to both decreased plasmin generation and lower catalytic efficiency of plasmin activity [74].

All of the above, result in an inhibition of the fibrinolytic process and together with the known hypercoagulability contribute to the development of (specially ischemic) cardiovascular disease in T2D [74].

Two of the more novel methods to study clot structure in inflammatory conditions, including T2D, is thromboelastography (TEG) that shows both clot formation and clot

lyses, as well as scanning electron microscopy (SEM) that gives visual information regarding the structure of the actual clot. These two techniques are grouped under the general term, visco-elastic techniques, and together with inflammatory marker analysis, can give valuable information in an individualized patient-orientated approach, when treating individuals with T2D. For a background on the technique, see various publications of Vance Nielsen's group [140–146]. Table 17.2 shows the typical parameters that show clot formation and lyses with TEG, and Fig. 17.2 shows examples of healthy and aberrant T2D fibrin clot structures. In a typical healthy individual, we see a spaghetti-like fibrin network with elongated fibrin fibres (for additional examples of healthy fibrin fibres (see https://1drv.ms/f/s!AgoCOmY3bkKHgkFy7q1sVsxRv_2s) [147]. In T2D, plasma with added thrombin forms a clot with finer fibre structure and areas of thick matted areas [6, 9, 124, 126, 148, 149]. Such a pathologic finer fibrin structure might be the cause of the known hypofibrinolytic clot in T2D, where the denser clot areas, together with the netted areas may also lead to the characteristic a hypercoagulable state in T2D. We have also previously found that in T2D, the TEG results vary considerably, depending of the individual clot parameters. This condition is extremely complex, and therefore we have suggested a individualized approach, using not only traditional pathology tests, but also novel methods like SEM and TEG to monitor patient wellness [125].

Table 17.2 TEG parameters typically generated for whole blood and platelet poor plasma

Parmeters	Explanation
R value: reaction time measured in minutes	Time of latency from start of test to initial fibrin formation (amplitude of 2 mm); i.e. initiation time
K: kinetics measured in minutes	Time taken to achieve a certain level of clot strength (amplitude of 20 mm); i.e. amplification
A (Alpha): Angle (slope between the traces represented by R and K) Angle is measured in degrees	The angle measures the speed at which fibrin build up and cross linking takes place, hence assesses the rate of clot formation; i.e. thrombin burst
MA: Maximal Amplitude measured in mm	Maximum strength/stiffness of clot. Reflects the ultimate strength of the fibrin clot, i.e. overall stability of the clot
Maximum Rate of Thrombus Generation (MRTG) measured in $Dyn.cm^{-2}.s^{-1}$	The maximum velocity of clot growth observed or maximum rate of thrombus generation using G, where G is the elastic modulus strength of the thrombus in dynes per cm^{-2}
Time to Maximum Rate of Thrombus Generation (TMRTG) measured in minutes	The time interval observed before the maximum speed of the clot growth
Total Thrombus Generation (TTG) measured in $Dyn.cm^{-2}$	The clot strength: the amount of total resistance (to movement of the cup and pin) generated during clot formation. This is the total area under the velocity curve during clot growth, representing the amount of clot strength generated during clot growth
Lysis time (LY30)	% Percentage lysis obtained 30 min after MA

Adapted from [164]

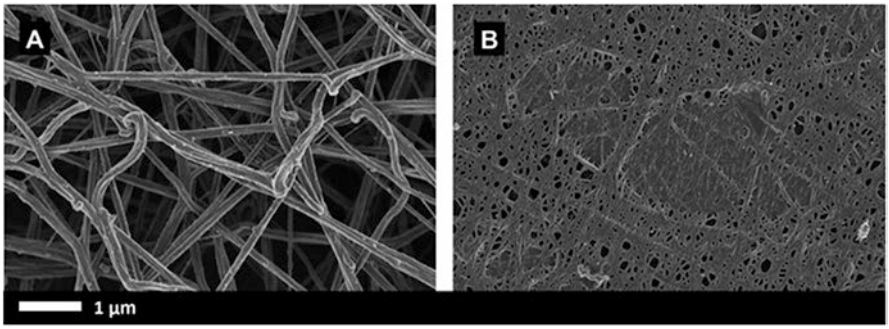

Fig. 17.2 (**a**) Fibrin clot from plasma of a healthy individual; (**b**) Fibrin clot from plasma of a patient with type 2 diabetes. Clots were created by adding thrombin to plasma

17.5 Conclusion

T2D is probably one of the most complex inflammatory conditions that clinicians need to treat, particularly due to the complex cardiovascular involvement. The mechanisms of both hypercoagulation and aberrant clot lyses in T2D are of great importance in the treatment of the condition. Furthermore, the multifaceted nature of the condition suggests that we follow a patient-orientated approach and educate clinicians to use e.g. TEG as an additional method for disease monitoring. Only by closely following each individual patient's progress with a variety of research and traditional laboratory pathology methods will we ensure the healthiness of this vulnerable population. The most important strategy is to manage systemic inflammation, and the resulting cardiovascular pathology; only then will we be able to reduce the T2D pandemic.

Ethical Approval Disclosure Ethical approval was granted at the University of Pretoria (UP) (Human Ethics Committee: Faculty of Health Sciences): E Pretorius. (EP was previously employed at UP).

Conflict of Interest The author has nothing to disclose.

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Chapter 18
Platelet Dysfunction in Type-2 Diabetes Mellitus

Gundu H.R. Rao

Abstract According to the Diabetes Atlas of the World, published by the International Diabetes Federation (IDF Diabetes Atlas, 7th edn, 2015), India has currently, over 70 million subjects with type-2 diabetes and China, 110 million subjects. The number of adults estimated to be living with diabetes has reached 422 million worldwide, nearly four-fold increase from 1980 figures, according to a World Health Organization (WHO) report (2014). Non-communicable Disease Risk Factor Task Force in their article in Lancet (April 2016) summarize, that if the year 2000 trends in prevalence of diabetes continues, It will not be possible to reach the Millennium Goals (www.un.org/millenniumgoals) of keeping the incidence of type-2 diabetes in 2025, at the 2010 level. The collective prediction of this study group has already come true. Patients with type-2 diabetes carry an equivalent or greater cardiovascular risk to that of a non-diabetic, who has already experienced a coronary event. The risk for acute coronary event in this population seems to be 2–3 times higher than non-diabetic subjects. It is a potentially fatal, chronic disease, whose risks can be prevented by better management of known risks and lifestyle changes. Inflammation, oxidative stress, hyperglycemia, endothelial dysfunction, altered hemorheology and hyper-platelet and coagulation activation pathways, seem to contribute significantly to the clinical complications of type-2 diabetes. In this article, we provide a brief overview on, vascular dysfunction, platelet biochemistry, physiology and altered function, as it relates to the clinical complications of adult on-set diabetes.

Keywords Diabetes • Hyperglycemia • Vascular dysfunction • Platelets physiology • Platelet dysfunction

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18.1 Introduction

Number of adults with type-2 diabetes has reached 450 million worldwide and has quadrupled from 1980 to 2014. A pooled analysis of 751 population-based studies by NCD Risk Factor Collaboration (NCD-RisC) concludes, that with increasing population growth and ageing, if post 2000 trends in incidence of diabetes continue, the probability of meeting the global target of halting the rise in the prevalence of diabetes by 2025–2010 levels worldwide, is lower than 1% [The Lancet 387:1513–30, 2016]. It is a pretty dim conclusion, but is based on extensive population-based comparative studies worldwide. Most populous nations like India and China (China already has achieved that position) are competing for the number one position in terms of incidence of type-2 diabetes. According to International Diabetes Federation (www.diabetesatlas.org), India has an estimated 70 million diabetics and an equal or larger number of pre-diabetics [1, 2]. Diabetes related clinical complications includes, coronary artery disease, cerebrovascular disease, diabetic nephropathy, neuropathy and retinopathy. In our opinion, all of these clinical complications are caused by altered vascular function, including the endothelial dysfunction as well as dysfunction of circulating blood cells [3–11]. In this overview, we discuss altered function of platelets in type-2 diabetic subjects and their role in the progression of clinical complications leading to acute vascular events or end organ failure.

Over 100 years ago, Professor Bizzozero from Turin University, Italy, described the function of circulating platelets. He observed them in circulating blood of living animals and in the blood removed from the blood vessels. In well-planned experiments, he demonstrated that they were the first components of the blood, to adhere to injured blood vessel*in vivo* and *in vitro* [12]. Since that time considerable progress has been made in our understanding of how platelet works in hemostasis and thrombosis. In spite of this collective knowledge, there still exist gaps in our understanding of platelet function or dysfunction in cardiometabolic diseases. Platelets circulating in blood interact with a variety of soluble agonists, such as Adenosine diphosphate (ADP), Epinephrine, many insoluble cell matrix components, including fibronectin, collagen, laminin, and biomaterials used for the construction of implantable medical devices [2, 13]. They play a critical role in the recognition of vascular injury, formation of effective hemostatic plugs, retraction of clots and wound healing. When hyperactive, they can initiate events leading to many clinical complications associated with acute cardiovascular and cerebrovascular events.

18.2 Platelet Activation

Although they circulate as single entities, they can interact and form aggregates with the slightest stimulation. The degree of activation depends on the strength of the activating stimuli and the nature of the surface or the information available (sequence of amino acids) on the surface of interaction. For instance, Laminin and

type 1 V collagen are major components of tumors. Platelets form a monolayer on type 1 V collagen, whereas they just anchor on laminin with minimal activation. On the other hand, metastatic tumor cells seem to be rich with fibronectin, which elicits spreading of cells. Triple-helical type-3 collagen facilitates aggregation as well as secretion of granule contents. Circulating blood has large quantities of fibrinogen, yet the platelets do not interact with soluble fibrinogen. They do not recognize the characteristic RGD (arginine, glycine, aspartic acid) sequence of amino acids on fibrinogen. However, once the glycoprotein (GP) 11b/111a receptor is activated, platelets can bind to the RGD sequence and form aggregates. On the other hand, if the fibrinogen is bound on a surface, then the RGD sequences are exposed and are available for the GP11b/111a receptor for interaction. Similarly, at low shear rate for instance in venous circulation, thrombus will be fibrin-rich, whereas, in arterial circulation at high shear the thrombus will be platelet-rich. It is important to understand these subtle differences in the activation mechanisms, in order to develop effective anti-platelet or anti-thrombotic therapies.

Since the time O'Brien and Born described some 50 years ago, light transmission aggregometry remains the reference method for measurement of platelet function [14, 15]. Four distinct phases of activation are recognized: (1) Development of stickiness; (2) shape change; (3) contraction and secretion of granule contents; (4) irreversible aggregation. The exact biochemical mechanisms involved in the first two phases of platelet activation (development of stickiness and shape change) are not known.

Major biochemical events associated with ligand binding to specific membrane associated receptors, activation of receptors, trans-membrane signal transduction, formation of second messengers, cytosolic calcium mobilization, release of arachidonic acid, generation of thromboxanes, assembly of filamentous actin, contraction, secretion of granule contents and irreversible aggregation have been described by several researchers [16–25]. Vessel wall injury brings similar response in platelets. In order to arrest bleeding, platelets undergo shape change, mobilize calcium, assemble actin, and cover the injured surface and form an effective hemostatic plug (Fig. 18.1).

18.3 Platelet Morphology and Biochemistry

Platelets have a discoid form in their resting state. This shape helps them to circulate close to the vicinity of the vessel wall and detect areas of vascular injury. James G White a pioneer of platelet ultrastructure biology from University of Minnesota, has divided the platelet structure and anatomy into distinct zones. The peripheral zone consists of membranes and closely associated structures like receptors, and an exterior coat of glycocalyx, which is rich in glycoproteins. The middle layer of the peripheral zone is rich in phospholipids. More than 15% of the dry weight of platelets is lipid of which 80% is phospholipid. Major lipids include: cholesterol (30.8%), phosphatidylcholine (26.3%0 phosphatidyl ethanolamine (8.6%), sphingomyelin

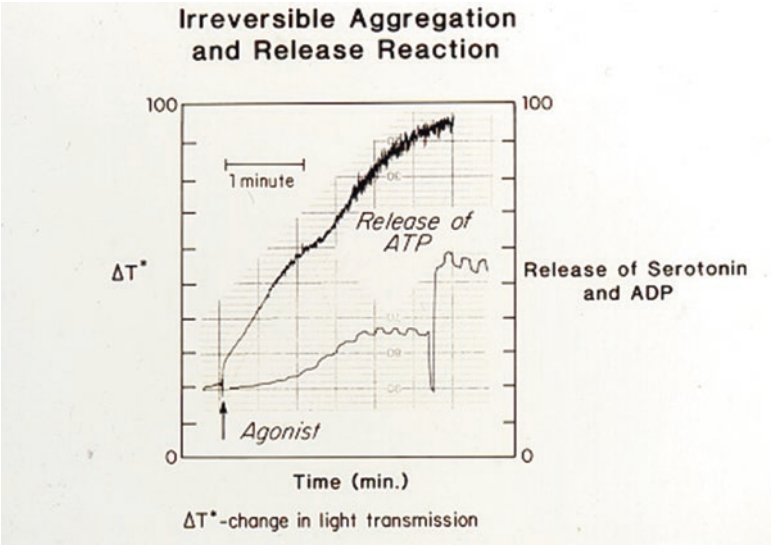

Fig. 18.1 Agonist induced irreversible aggregation and release of atp and serotonin (an example of cell-cell interaction)

(11.6%), phosphatidyl serine (6.6%0 and phosphatidyl inositol (2.7%). In a recent study researchers at the University of Cardiff, UK, have identified over 8000 species of lipids in platelets [26]. Agonist mediated activation of platelets is associated with changes in membrane lipids, and formation of bioactive lipids (second messengers), which play an essential role in hemostasis and thrombosis.

The membrane system plays a major role in platelet physiology and function. The dense tubular system (DTS) has been shown to be the site of releasable calcium store, an important modulator of platelet activation. The DTS is also the site where enzymes involved in prostaglandin synthesis are localized [27]. The surface connected canalicular system provides access to the interior for plasma borne substances and serves as a conduit for products secreted during the release reaction [28].

Platelet plasma membranes contain trans membrane proteins as well as glycoprotein-rich domains. Glycoproteins are embedded in the lipid bilayer. Platelets contain integrin as well as non-integrin domains [29]. Integrins are trans membraneglycoproteins with alpha and beta subunits coupled non-covalently (GP11b/111a, GP1a/11a) GP1c/11a). They participate in both cell–cell and cell-matrix interactions (Fig. 18.2). Platelets also havenon-integrin domains capable of binding other proteins such as collagen and von Willebrand Factor (GP1V, GP1b1X).

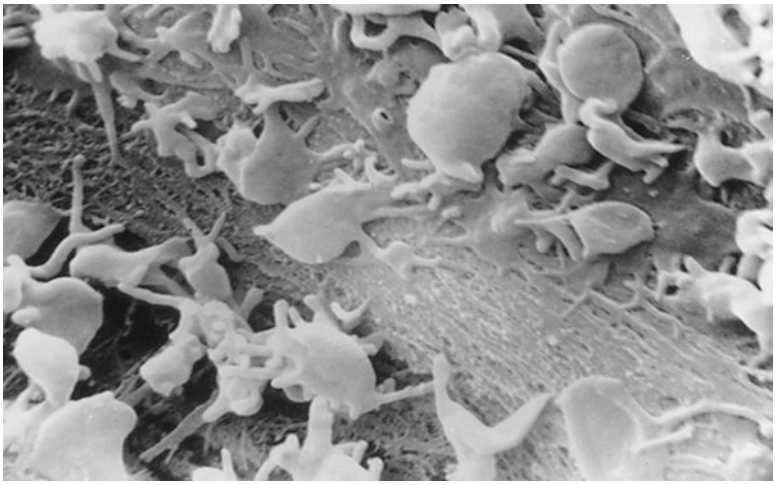

Fig. 18.2 Platelet interaction with blood vessel wall (cell-surface interaction) (Courtesy: James G. White)

18.4 Platelet Physiology and Function

Circulating platelets interact with a variety of soluble agonists as well as cell matrix components exposed at vessel wall injury sites. These interactions stimulate specific membrane receptors and glycoprotein-rich domains (integrin and non-integrin) on the plasma membrane and lead to the activation of intracellular enzymes [24]. The majority of cellular and molecular regulatory events seem to require participation of ionized calcium. Studies from our laboratory at the University of Minnesota, using calcium specific flourophores (Quin-2 AM, Fura-2), demonstrated the role of ionized calcium and formation of assembled actin, in platelet activation, contraction and release reaction [30–35]. Major enzymes that regulate the free cytosolic calcium levels via second messengers include, Phospholipase C (PLC), phospholipase A_2, and phospholipase D, together with adenylyl and guanylyl cyclases (Fig. 18.3).

Agonist interaction with the receptor results in the activation of PLC via trans membrane signaling through hydrolysis of GTP to GDP. Platelets contain monomeric low molecular weight G proteins as well as heteromeric membrane associated G-proteins. GTP binding to the alpha subunit of G proteins facilitates the interaction with effector enzymes. Activation of PLC results in the hydrolysis of phosphatidyl inositol 4,5 bisphosphate (PIP_2) and formation of second messengers, 1, 2- diacyl glycerol (DAG) and 1,4,5 -inositol trisphosphate (IP_3). Diglyceride activates protein kinase C (PKC), induces translocation of cytosolic PKC to membranes, whereas IP_3 mobilizes calcium from internal stores [32]. Elevated cytosolic calcium is also essential for the assembly of filamentous actin from its native soluble form. It also plays a major role in contraction and secretion of granule contents.

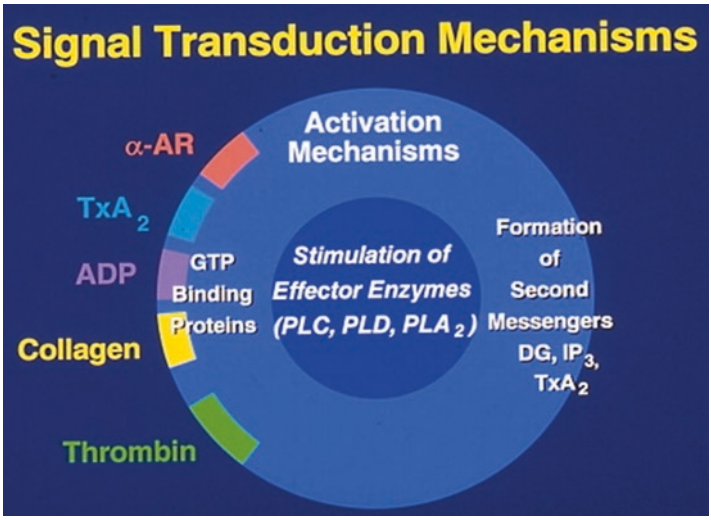

Fig. 18.3 Signal transduction and platelet activation mechanisms

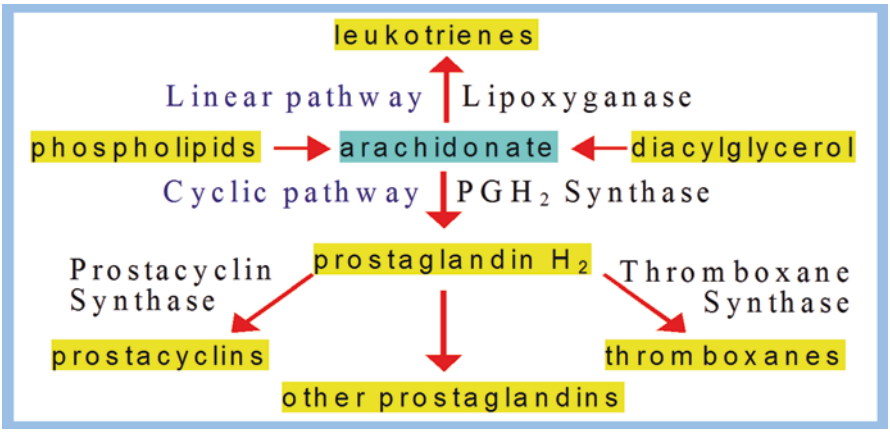

Fig. 18.4 Arachidonic acid metabolism by cyclooxygenase and lipoxygenase enzymes

Elevation of cytosolic calcium activates phospholipase $A_2$and liberates arachidonic acid (AA) from membrane phospholipids (Fig. 18.4). Free arachidonic acid is transformed by cyclooxygenase (COX) to prostaglandin (PG) endoperoxides $PGG_2$and PGH_2 (Fig. 18.5). These transient metabolites are further transformed to thromboxane A_2in platelets. Thromboxane is the major metabolite of this pathway and plays a significant role in platelet recruitment, granule mobilization, and secretion. Cyclic endoperoxides also are potent vasoconstrictors and potent platelet agonists.

Similar to this metabolic pathway, the endothelial cells also generate vasoactive compounds from substrate arachidonic acid. Unlike platelets, the EC produces

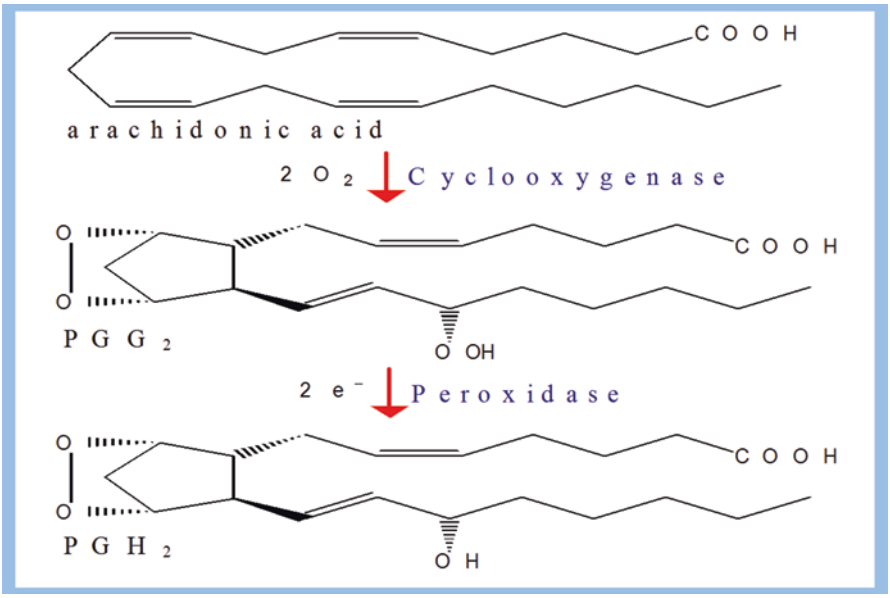

Fig. 18.5 Arachidonic acid metabolism by cyclooxygenase enzymes

vasodilatory prostacyclins (PGI_2) from PG endoperoxides. These transient bioactive metabolites of AA can be utilized by platelets as well as vessel wall ECs to generate, thromboxane or prostacyclin. In view of this possibility, earlier studies focused on this aspect of cellular AA metabolism, to manage platelet hyper function, hoping that they could preferentially lower the production of pro-aggregatory thromboxanes and increase the generation of vascular prostacyclin. Use of low dose aspirin and transdermal aspirin to achieve these goals, did not yield successful results.

Majority of the platelet agonists, initiate platelet activation via specific receptor mediated stimulation. Having said that, we should point out, that platelets have multiple mechanisms for activation. For instance, thrombin a protease cleaves a part of the thrombin receptor, generates thrombin receptor activation protein (TRAP), a potent agonist of platelet aggregation and promoter of release reaction. Similarly, various cell matrix components interact with specific domains on the membrane, induce platelet activation. It is generally believed, that in spite of the multiple activating mechanisms, the sequence of events that follow platelet stimulation, are common and are aimed at achieving GP11b/111a activation, fibrinogen binding, calcium mobilization, actin assembly, contraction and release of granule contents. Secretory granules contain a variety of growth factors, mitogens, and inflammatory mediators. Secretion of granules, promote expression of adhesion molecules (P-selectin) on the platelet membranes. Platelet activation also promotes expression of acidic lipids and tissue factor on the membranes, thus making these cells procoagulant. Platelet activation and changes in membrane composition promotes stimulation of pro-thrombinase and formation of thrombin. Fully activated platelets can activate coagulation path-

ways; modulate the function of other circulating blood cells such as leukocytes, monocytes, and macrophages as well as endothelial cells. Agonist induced stimulation of platelets promote the expression of an epitope on GP11b/111a receptors. Activation of this receptor is essential for the binding of circulating fibrinogen, which promotes aggregation, thrombus formation and growth [36]. von Willebrand Factor (vWF) binds platelet GP1b1X complex only at high shear rate. Unlike GP11b/111a receptor, GP1b1X receptor does not need activation to bind vWF, the globular protein changes its confirmation at high shear and binds the GP1b1X complex. Up-regulation in signaling pathways will increase the risk for clinical complications associated with acute coronary events. Down-regulation of signaling pathways may precipitate bleeding diathesis or promote hemorrhagic stroke.

18.5 Vascular Dysfunction

As mentioned earlier, we feel strongly that altered vessel wall pathology and that of blood cells play a significant role in the major clinical complications associated with the progression of type-2 diabetes. Therefore, it is important to consider not only altered blood cell physiology and function, but also the impact of vasculardysfunction. Functional and structural changes in the arterial wall precede the development of atherosclerosis, obstructive coronary artery disease, as well as serve as an early marker for hypertensive disease. Function and structural changes of vascular endothelial cells (ECs) are modulated by a variety of thrombogenic factors as well as anti-thrombogenic factors. Some of the vasoactive compounds, released by the ECs include adenosine, prostacyclin, and nitric oxide and vaso-constrictory molecules such as cyclooxygenase derived PG endoperoxides, endothelium dependent constriction factor (EDRF), hypoxia-induced endothelium dependent constriction factor. In addition, lipid peroxides, oxidized lipids, and lipoproteins promote the formation of vasoconstrictors from circulating platelets. These lipid peroxides inhibit enzymes that promote the formation of vasodilators by the healthy endothelium and lower the production of vasodilators. Alterations in the balance between platelet associated vasoconstrictors and EC-derived vasodilators result in vascular dysfunction [4]. This is probably the earliest stage at which one can detect the manifestation of the arterial dysfunction, hypertension and atherosclerosis. One can use acetylcholine, L-arginine, or nitric oxide synthetase inhibitor LNNMA and monitor the flow response to determine the degree of EC dysfunction. Alternately, one can use CV Profilor (DO-2020: Hypertension Diagnostics, Eagan Minnesota) or Periscope (Genesis Medicals Systems, Hyderabad) or the system by Endothelix Inc., (Houston, Texas) for monitoring arterial stiffness. There are numerous developments in the use of Ultrasound scanning technology for monitoring the progression and management of atherosclerosis [37–39].

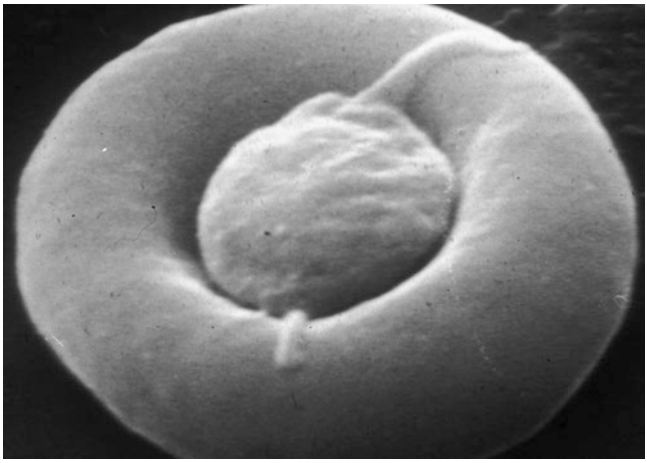

Fig. 18.6 Electron photomicrograph of a platelet and a red cell (Courtesy: James G White)

18.6 Hemorheology and Vascular Dysfunction

Blood flow velocity and pressure in large arteries are largely influenced by the deformability of the vessel as well as the deformability of the blood cells. In addition to variety of blood constituents in the plasma (soluble and suspended), the cellular components as well as their rheological properties play major role as determinants of blood fluidity and viscosity. Red blood cells (RBCs) are major determinants of this effect. Considering the size difference in platelets and red cells (Fig. 18.6), any changes in membrane fluidity of the RBCs will cause obstruction in the flow of blood in the microvasculature. Therefore, deformation and orientation are the primary cellular factors affecting blood viscosity and flow velocity at high shear rates. Studies from our laboratory have developed a novel method to follow cellular deformability [40]. We have demonstrated that common drug, aspirin affects the deformability of platelet membranes and promotes adhesion of platelets to the vessel walls whereas, epinephrine reverses the effect of aspirin on platelet membranes. It is well known that the red blood cells change their shape to a parachute like shape to move through the capillaries. Any alteration of the fluidity of cell membranes will hinder the flow of blood in microcirculation.

18.7 Altered Circulating Blood Components

When we say that vascular pathology contributes significantly to the clinical complications of type-2 diabetes, it not only means the vascular dysfunction, but also includes, altered state of the circulating blood and its components. Known alterations include, inflammation, oxidative stress, elevated insulin levels, insulin

resistance, elevated blood glucose, glycated or glycosylated proteins, altered vessel wall compliance, altered rheology of blood cells, imbalance in cellular activation mechanisms and coagulation pathways, changes in flow dynamics, loss of blood supply, nerve and tissue damage. Although these alterations have been reported as contributing factors to observed vascular and blood cell dysfunctions, exact role of many of these risk factors have not been validated.

Many investigators have attempted to correlate *in vitro* functional response of platelets to clinical manifestations of thrombotic or bleeding episodes. Yet, it remains a difficult task to establish a clear relationship between specific functional responses and their role in hemostasis and thrombosis. However, the presence of functional glycoprotein receptors, and the ability of the platelets to undergo shape change, become sticky, spread, irreversibly aggregate and release granule contents, are considered essential for normal hemostatic function.

Blood flow velocity and flow dynamics is a complex process, combines the fluid shearing in both the plasma and interior of red cells with elastic deformation of blood's other solid elements. As we have mentioned earlier, red cell membranesare the major contributors for this altered flow dynamics, however even platelets and other white cells also contribute to the altered flow [41, 42]. Elevated blood glucose has been shown to contribute significantly to the whole blood viscosity, erythrocyte deformability and aggregation [43]. Several researchers have demonstrated a role for altered blood and blood cell rheology in the progress of diabetic microangiopathy. They also have noted alterations in whole blood viscosity, plasma viscosity, increase in red cell 2, 3-DPG levels, elevated platelet adhesion and aggregation [44].

18.8 Infection, Inflammation and Oxidative Stress

Elevated blood glucose is the hallmark of progressive diabetic condition and this condition also supports the growth of opportunistic bacteria. It has been shown that *Mycobacterium avium* can induce apoptosis and leak of lysosomal contents and thus initiate inflammation and oxidative stress [45]. Various inflammatory diseases and soft tissue pathologies in oral cavities are associated with diabetes mellitus [46]. Studies at the University of Minnesota have demonstrated that platelet activation;aggregation and thrombosis can be initiated by platelet-associated activation protein (PAAP) expressed on oral plaque bacteria, including *Streptococcus sanguis* and *Poyphyromans gingivalis* [47, 48]. Diabetes-induced changes in immune cell function produce an inflammatory immune cell phenotype (up-regulation of pro-inflammatory cytokines from monocytes, leukocytes and down regulation of growth factors from macrophages). This predisposes to chronic inflammation, progressive tissue breakdown, and diminished tissue repair capacity [48–50]. Twin epidemics of diabetes and coronary artery disease fit the hypothesis of "common soil", when it comes to the role of low-grade inflammation [51–53]. In view of the fact that excess weight and obesity is associated with low-grade inflammation; there is considerable interest in the role of inflammation in the pathophysiology of diabetes.

Contrary to these observations, a US study of adults did not show any support to the hypotheses, that inflammation is an etiologic factor for diabetes [53]. Similar to the above mentioned hypothesis, oxidative stress has been implicated as a unifying hypothesis linking various molecular disorders of type-2 diabetes [54, 55]. Studies with antioxidants like vitamin E have failed to demonstrate convincing beneficial effects of antioxidants [56]. Effect of various antioxidants needs further exploration.

18.9 Hyperglycemia and Clinical Complications of Diabetes

Chronic hyperglycemia leads to long-term macro vascular and micro vascular complications. Glycosylation refers to the covalent bonding of blood glucose to the hemoglobin of red cells [57]. Increased amount and duration of glucose in the blood allows glycosylation of not only hemoglobin, but also with other important proteins having reactive amino groups. Such glycosylation of proteins can affect cell function and structure. This condition seems to target tissues that are not dependent on insulin for their absorption of glucose (Kidneys, blood vessels, peripheral nerves and lenses of the eye). Elevated glucose levels have been shown to enhance platelet activation [58]. Researchers used 5, 15 and 30 mmol/l glucose and conducted *in vitro* studies. Platelet activation was monitored by whole blood flow cytometry. Elevated levels of glucose enhanced ADP and TRAP induced expression of P-selectin as well as fibrinogen binding to platelets. Blockade of cyclooxygenase, phosphatidylinositol-3 (PI3) kinase, or nitric oxide synthetase did not influence the effect of hyperglycemia. Using an ex*vivo* extracorporeal perfusion protocol, researchers monitored platelet-dependent thrombosis (PDT) in 42 patients with stable CAD [59]. Similar to the earlier studies, flow cytometry was used to monitor platelet activation. They found that PDT was significantly greater in patients with elevated blood glucose. There are several studies pointing to the ill effects of hyperglycemia and a variety of dysfunctions caused by this imbalance, however what is not clear is the exact role of hyperglycemia in the alteration of various cellular activation mechanisms and the entire coagulation pathway.

Insulin on the other hand, seems to sensitize the platelets to PGI_2 and enhance the generation of PGI_2 and Nitric Oxide [60]. Hyperglycemia and insulin resistance inhibit production of Nitric oxide (NO) by blocking endothelial cell nitric oxide synthetase (eNOS) in ECs, thereby impairing NO-mediated vasodilation, increasing production of reactive oxygen species (ROS) especially superoxide anions (O^{2-}). Superoxide quenches NO by forming toxic peroxynitrile ion, which uncouples eNOS. In addition to these mechanisms, vasoconstriction is promoted by production of angiotensin, which stimulates the generation of ROS and leads to endothelial dysfunction and inflammation.

Endothelial cells also are exposed to altered concentrations of circulating metabolites as well as glucose and therefore, are likely to be involved in the precipitation of chronic complications of the disease [61]. There is some speculation that

hyperglycemia- induced polyol pathway hyperactivity associated with nerve sorbitol accumulation and myo-inositol depletion, may play a part in the genesis of diabetic neuropathy [62]. When discussing hyperglycemia mediated dysfunctions of vascular endothelium, platelets or red cells, one should take into account that most data available are derived from experiments done in *in vitro* studies and are carried out in conditions not closely related to what is seen in *in vivo* conditions, and as such the results reported may be quite contradictory. Although we talk about glycation, glycosylation and glycosylated proteins, we do not know very much about how this process modifies the structure and function of proteins, vascular ECs and circulating blood cells. There is a great need to study the effect of hyperglycemia and hyperinsulinemia on initiation and progression of diabetes pathophysiology.

18.10 Altered Platelet Physiology and Function

Researchers from several laboratories have demonstrated hyper aggregability of platelets in response to agonists in subjects with diabetes with or without vascular disease [63–77]. The increased sensitivity to agonists is attributed to elevated levels of von Willebrand Factor (vWF). Platelets of patients with diabetes are more responsive to the arachidonic acid (AA) stimulation than platelets from normal subjects. Aspirin abolishes the increased response of platelet to AA, suggesting that cyclooxygenase metabolites of AA are responsible for increased aggregation response of platelets in diabetic subjects. They concluded that platelets of patients with diabetes have increased prostaglandin synthetase activity and a PGE_2-like material was responsible for hyperactivity of platelets [63]. Gensini and associates showed that changes of platelet function in diabetics existed even in pre-diabetic conditions [66]. In view of the fact that thrombin and arachidonic acid stimulation of platelets resulted in elevated levels of malondialdehyde, they speculated increased endoperoxides-thromboxane forming activity in platelets of subjects with diabetes. They also found hypercoagulable condition in diabetics. Professor Barry Coller and associates studied diabetics with and without retinopathy and found that hemoglobin A1c was elevated in all diabetic patients [67]. Whereas fibrinogen was elevated, only in diabetic subjects with retinopathy. They concluded from their studies that elevated levels of fibrinogen and vWF (promoters of venous and arterial thrombosis respectively), recognized plasma cofactors of platelet function, are associated with proliferative diabetic retinopathy. Eldor et al. developed a rat model for diabetes with streptozotocin and demonstrated altered platelet function and its reversal by washing the plasma off the platelet suspensions, suggesting that the components responsiblefor platelethyper function were plasma factors [65].

18.11 Altered Eicosanoid Metabolism in Diabetes

Studies from our laboratories at the University of Minnesota demonstrated increased prostaglandin production by stimulated platelets from streptozotocin treated rats. On the other hand, the vessel wall production of prostacyclin was significantly reduced in these rats. This imbalance in the production of platelet thromboxanes and vessel wall prostacyclin was normalized by islet cell transplantation [4]. Di Mino et al. have suggested on the basis of their work that increased fibrinogen binding and aggregation of platelets from diabetic subjects in response to agonists is mediated by increased formation of PGH_2 and thromboxane [71]. In spite of the fact several studies have demonstrated such altered arachidonic acid metabolism in diabetic subjects, a study from Alessandrini et al. could not find increased urinary metabolites of thromboxane B_2 in diabetic patients (type-1) with or without retinopathy [78]. Davi et al. did similar studies with type-2 diabetic subjects and as in earlier studies, did not find significant difference in urinary metabolites of Thromboxane B_2 between diabetics and normal subjects. They concluded that in type-2 diabetes, increased urinary 11-dehydro-thromboxane B_2 excretion reflects enhanced biosynthesis of thromboxane A_2 by platelets, rather than a shift in its metabolic disposition [75]. Contrary to this observation, in patients with coronary artery or cerebral artery disease, researchers have found significant correlation between excess urinary metabolites of TXB_2 and risk for acute vascular events [79]. Professor Carlo Patrono and associates from Italy have described the presence of aspirin insensitive thromboxane biosynthesis under oxidant stress in severe unstable angina [80]. Since diabetics also are supposed to be under chronic oxidative stress, availability of aspirin insensitive thromboxane synthetase cannot be ruled out. Several studies have reported hyperglycemia induced oxidative stress and increased production of superoxide by blood cells [82, 83].

18.12 Platelet Hypersensitivity in Diabetes

Over three decades of studies on diabetic subjects have documented a hyper aggregable state of platelets and red cells in patients with chronic diabetes mellitus. However, all attempts to make a case for infection, inflammation, oxidative stress, altered blood cell rheology, vascular dysfunction, elevated arachidonic acid metabolism, altered calcium homeostasis, expression of excess integrin receptors like GP 11b/111a and GP1b1X, to explain altered platelet function and elevated thrombotic status of blood, have failed to impress the clinicians, who have to manage this chronic disease and its clinical complications. Having said that, if we look at the way clinicians by and large treat patients with this disease, it is more or less confined to the management of blood glucose levels and in some cases hemoglobin A1c (HbA1c). There is still a window of opportunity to look at better management of each and every one of these risk factors. At the time of this writing, we still do not

fully understand as to how exactly increased levels of blood glucose, glycation and glycosylation induce so many dysfunctions related to diabetes condition. The relationship between macroangiopathy and fasting plasma glucose or HbA1c is weaker than that observed with microangiopathy. Plasma glucose or HbA1c alone are unable to thoroughly explain hyperglycemia-mediated disorders of diabetes [81–83]. Several studies have reported relevance of post-prandial glucose levels as well as hyperglycemia on free radical generation and oxidative stress [81, 82]. Lipid peroxidation is an oxidative process, which occurs at relatively low levels in cells and tissues. Generation of free radicals also is a normal physiological process. To some extent these processes are regulated by the endogenous enzymatic and non-enzymatic antioxidants. Hyperglycemia mediated complications as they relate to microangiopathy may be explained to some extent by the known effect of increased blood glucose level on hemorheology. The results of Diabetes Control and Complications Trial (DCCT) in which tight control of blood glucose was one of the primary goals, reveals the beneficial effect of tight glycemic control on micro vascular health. A 10-year follow up of over 1000 individuals, demonstrated 76% reduction in retinopathy, 50% reduction in nephropathy and 60%reduction in neuropathy [84]. In a comparative study of type-1 and type-2 diabetic patients, researchers demonstrated a relationship between the antioxidant statuses of the platelets with elevation or otherwise of eicosanoid metabolism. They found that basal thromboxane levels significantly increased in both type-1 and type 2 diabetic subjects, while malondialdehyde was increased only in type-2 subjects. Vitamin E and glutathione peroxidase activities were lower in patients with diabetes [85]. They concluded that platelet hyper activation was detectable in well-controlled diabetic patients without any clinical complications. Researchers from Belgium have studied blood levels of antioxidants, peroxides and malondialdehyde (MDA) of diabetic subjects as well as age matched healthy control subjects. In their studies they found that diabetic subjects had lower platelet glutathione and higher MDA [86]. Following this logic, Hill et al. from the University of Minnesota, studied the role of glutathione in platelet function and reported platelet hypersensitivity induced by 1-chloro-2,4-dinitrobenzene, hydroperoxides, inhibitors of lipoxygenase and glutathione depleting agents [87–89]. They further demonstrated, that the glutathione deficient platelets upon stimulation by arachidonic acid produce increased quantities of thromboxane and therefore, are hyperactive [89] Radha and associates from the same group described a circadian rhythm in platelet glutathione levels [90]. Studies from the University of Minnesota on the role of glutathionein inducing platelet dysfunction demonstrate, that lower antioxidant status in platelets, predisposes them to hypersensitivity to the action of arachidonic acid and promotes generation of increased quantities of PG endoperoxides. Similar studies on platelets of diabetic subjects also have shown increased level of basal Ca^{2+} (an indicator of activation), well as alteration in calcium homeostasis [91, 92].

Hyperglycemia, to a large extent exists together with hyperinsulinemia and insulin resistance in type-2 diabetes. Effect of insulin on platelet function is poorly understood. In age matched insulin resistant individuals, researchers have found increased platelet activity, suggesting that defects in insulin signal cause platelet

hypersensitivity and altered calcium homeostasis [92]. Insulin is known to inhibit agonist mediated cytosolic calcium mobilization. On the other hand, epinephrine is known to enhance the sensitivity of platelet to activating agents by reducing the levels of cAMP and thereby antagonizing the effect of insulin. Elevation of plasma glucose is the main trigger for the pulsatile release of insulin to the blood. Other signals of signaling pathways that increase cytosolic calcium also release insulin. For instance the receptors coupled to heterotrimeric GTP binding proteins that stimulate PLC to produce second messengers DAG and IP3 in platelets, also can release insulin. As mentioned before, the insulin story as it relates to platelet dysfunction is poorly understood.

Now that we have briefly covered hyperglycemia, role of insulin, altered eicosanoid metabolism and platelet dysfunction, we need to discuss mechanisms independent of these established pathways of platelet activation. Studies from our laboratory using platelets devoid of dense granules, demonstrated that platelet aggregation could be achieved independent of released ADP [93, 94]. Furthermore, using platelets from patients devoid of cyclooxygenase, we demonstrated that platelet aggregation could be achieved independent of ADP or prostaglandin synthesis [95]. In view of these observations further studies were conducted to demonstrate that irreversible aggregation could be achieved in aspirin treated platelets with epinephrine as the potentiator of agonist action [96, 97, 98]. Our studies also demonstrated that epinephrine potentiates the action of all agonists in drug-induced refractory platelets by a mechanism, described as “membrane modulation” [98]. We were able to demonstrate in a series of studies, that platelet aggregation depends upon the availability of activated GP11b/111a receptor and fibrinogen binding. We further demonstrated that so called, irreversibly aggregated platelets, could be disaggregated by using agents, which elevate AMP or cGMP and lowered cytosolic calcium [99]. These disaggregated cells could be again reaggregated, by using a combination of agents such as epinephrine and AA or epinephrine and ADP. These cycles of aggregation were accomplished by promoting fibrinogen binding to GP11b/111a receptors, phosphorylation of cytoskeletal proteins, whereas disaggregation was followed by dissociation of bound fibrinogen, dephosphorylation of cytoskeletal proteins [100].

We have earlier discussed the subtle nuances related to platelet interaction with fibrinogen. Platelets need activation of GP11b/111a receptor to find the RGD sequence and bind fibrinogen in suspension. They do not need this receptor activation, to find this sequence on fibrinogen, which is bound on a surface or on injured vessel wall. We were able to demonstrate this phenomenon in a series of experiments using denuded rabbit aorta exposed to circulating human blood. Our studies demonstrated that common anti platelet drugs inhibit cell-cell interaction (aggregate formation and thrombus growth) but not cell-surface interactions or in other words platelet vessel wall interactions [101, 102]. These studies further emphasize the complexities of platelet activation mechanisms and the difficulties one encounters in the management of platelet hyper function.

18.13 Wound Healing and Platelet Function

Platelets of type-2 diabetic subjects have been shown to be hyper sensitive to the action of agonists. Increased platelet reactivity has been attributed, to hyperglycemia, increased levels of insulin, insulin resistance, insulin deficiency, altered blood rheology, chronic inflammation, oxidative stress, endothelial dysfunction, increased expression of integrin receptors, altered arachidonic acid metabolism and platelet hyperfunction [103]. In view of these observations, explaining the role of platelets in wound healing especially in diabetic foot ulcers (DFU) becomes complicated. Studies by Knighton and associates at the University of Minnesota for the first time demonstrated, a role for platelets and platelet derived growth factor in wound healing [104, 105]. They used autologous platelet derived wound healing factors (PDWHF) from healthy subjects with nonhealable wounds and diabetic subjects with foot ulcers. In these studies, they demonstrated 100% healing of wounds in about 10 weeks time. Since they demonstrated that locally acting growth factors promote the healing process, these findings suggest unavailability of these factors at the site of the non-healing wound. These studies also suggest that the platelets of patients with diabetes do contain releasable platelet derived growth factors. Based on the results of these studies, one can conclude that diabetic foot ulcers are non healable because of poor circulation and lack of platelets at the wound healing sites. Indirect evidence from studies in which, ability of trans membrane delivery of nitric oxide have been shown to accelerate the healing process, also suggests the beneficial effect of improved circulation in wound healing. Portable NO generators have been developed for therapeutic purposes (Sci. Transl. Med. 2015, 7:294). Yet another evidence that supports this hypothesis is the use of hyperbaric oxygen therapy for healing diabetic foot ulcers. Hyperbaric oxygen therapy is known to increase regional blood flow. Since the role of NO is well established, it is worth trying various NO generating mechanisms such as use of substrate L-arginine, or platinum nanoparticles or herbal products for wound healing applications [106, 107].

18.14 Management of Platelet Dysfunction in Diabetics

One of the major factors contributing to the increased activity of platelets in diabetics is the elevated production of platelet thromboxane. Studies of Patrignani et al. demonstrated selective cumulative inhibition of thromboxane production by low dose aspirin in healthy subjects [108]. They also showed that this cumulative inhibition of the platelet enzyme did not inhibit the enzymes of renal PGI_2 producing cells. Similar studies by Davi et al. did not show any such effect in the platelets of diabetics [77]. During the early years of eicosanoid research, there was considerable interest in the use of altered eicosanoid synthesis in the management of platelet hyper function. It was believed that one could preferentially facilitate the production of increased PGI_2 from ECs and lower the production of platelet TXA_2. Earlier

studies had demonstrated that the transient endoperoxides PGG_2 and PGH_2 could be used by platelets to make TXA_2whereas; the vessel wall ECs could use them to make PGI_2. It was speculated that a low dose of aspirin would preferentially suppress platelet production of TXA_2 and spare the COX enzymes in ECs to produce normal amounts of PGI_2.

In addition to this concept, use of Eicosapentaenoic (EPA) acid and Docosahexaenoic acid (DHA) also were recommended for the management of platelet hyper function. The idea behind this recommendation was that these fatty acids upon conversion were supposed to generate triene-Thromboxane (TXA_3) and tetraene-Thromboxane (TXA_4), instead of diene-TXA_2. Furthermore, it was speculated at that time, that the TXA of the 3 and 4 series were less potent stimulators of platelets, compared to TXA_2. Furthermore, it was speculated that the triene-PGI_3 and tetraene-PGI_4were biologically as active as PGI_2. Studies from our laboratory demonstrated that AA is the preferred substrate for platelet COX enzymes and not much conversion of EPA and DHA occurs from platelet COX enzymes [109, 110]. Nagakawa et al. on the other hand, administered 2 g/per day of EPA and found increased ratio of EPA to AA in plasma and platelet phospholipids. They found a decrease in platelet aggregation following EPA consumption [111]. Terano et al. used purified EPA in healthy subjects and found improvement in erythrocyte deformability as well as platelet function [112]. Woodman et al. found highly purified DHA to be more effective anti-thrombotic agent than EPA [113]. Studies by Phang et al. demonstrated a differential effect of these Omega-three fatty acids on men and women tested [114]. In their studies they found that both in Men and Women EPA and DHA reduced platelet aggregation relative to placebo. In subgroup analyses in men, only EPA treatment reduced platelet aggregation. In contrast in women only the DHA treatment reduced platelet aggregation. In spite of these observations, clinicians do not use Omega three fatty acids as one of the anti platelet or antithrombotic therapeutic modality.

18.15 Antiplatelet Therapies

Aspirin seems to be the drug of choice for antiplatelet therapy, for both primary and secondary prevention of acute vascular events [115–117]. Those individuals who have undergone interventional procedures, such as angioplasty or coronary bypass surgery, may need dual antiplatelettherapy (combination of Aspirin and Clopidogrel). American Diabetes Association (ADA) has a position statement on “Aspirin Therapy in Diabetes [118]. According to ADA recommendations, low dose aspirin therapy should be prescribed as a secondary prevention strategy. However, they also recommend low dose aspirin for primary as well as secondary prevention in men and women with diabetes, who are at high risk for cardiovascular events. In the UK-guidelines, recommendation for diabetic patients includes treatment with aspirin (75 mg daily) or Clopidogrel 75 mg/per day). Professor Belch and associates from the UK, studied the effect of aspirin and antioxidants (200 mg Alpha

Tocopherol, 100 mg ascorbic acid, 25 mg pyridoxine hydrochloride- with small quantities of zinc sulphate, nicotinamide, selenium and lecithin) for the primary prevention in the progress of arterial disease in diabetic subjects [119]. They did not find any evidence to support the use of aspirin or antioxidants in primary prevention of acute cardiovascular events. Prof Eric Topol and associates from the USA studied the effect of aspirin alone or with Clopidogrel under CAPRIE clinical trial protocol [120]. Bhat et al. in their report concluded (CAPRIE Trial), that the dual antiplatelet therapy was not significantly different from that of aspirin alone. Although some studies have shown that Prasugrel may be better than Clopidogrel, all dual anti platelet therapies have reported increased bleeding episodes in the trial participants [120–123]. There are some reports recommending increased doses of aspirin or Clopidogrel [123]. Aspirin has a very limited life in circulation. Once it is hydrolyzed to salicylic acid, metabolite salicylic acid has no inhibitory effect on COX enzymes. On the other hand, new platelets are continuously introduced into the circulation by the megakaryocytes. These newly added platelets have sufficient COX activity to generate thromboxanes capable of activating aspirin-treated-platelets [94]. In view of these observations there are some reports suggesting the use of multiple doses of low dose aspirin to lower the platelet activity. Similarly, studies have suggested increased doses of Clopidogrel as well, in the management of platelet hyper activity [123]. Kokoska and associates from USA, in their most recent (2016) meta-analysis, conclude that, “It remains unclear whether aspirin may reduce the occurrence of a first atherosclerotic event or mortality in patients with diabetes” [124]. Having concluded their findings with a negative note, they suggest that more research on the use of aspirin in patients with diabetes is required. We agree with this suggestion and encourage not only further studies on aspirin use, but also the development of newer antagonists for GP11b/111a receptors.

Diabetic patients have an increased risk for atherothrombotic events as well as for end organ failure, due to the progress of microangiopathy, loss of circulation and regional ischemia. Although currently approved anti platelet and antithrombotic therapies have proven useful in improving the outcomes, diabetic patients continue to have much higher risk for acute cardiovascular and cerebrovascular events. In spite of the fact that studies after studies have suggested, that the contributing factors for diabetes mediated clinical complications are many, the clinicians by and large concentrate on the management of blood glucose levels alone or at the most, provide minimal anti platelet therapies. Novel methods of management of this complex chronic disease should include, early detection of the risks and lowering all the well-known risk factors associated with diabetes-related clinical complications [6–11]. Major land mark trials of glycemic therapies like DCCT of USA and UK Prospective Diabetes (UKPD) study, have demonstrated the beneficial effects of risk management. Studies headed by Professor Robert Turner from Oxford, unlike other clinical trials, broke many rules of clinical trial design by constant addition of further interventions and analyses. An excellent summary of this study has been provided by ADA [125]. Diabetes is a major epidemic worldwide and its clinical complications are too many to be neglected. In view of these observations an all out effort should be made, to come up with novel risk diagnosis, risk management and prevention strategies [5–11].

18.16 Conclusions

Increase in the incidence of type-2 diabetes has reached epidemic proportions worldwide, exceeding all estimations. Major contributing factors for diabetes related clinical complications include hyperglycemia, blood insulin levels, insulin resistance, inflammation, oxidative stress, changes in hemorheology, endothelial dysfunction, and platelet hyperactivity. To a great extent, all of these events are interrelated. Progression of the macrovascular disease results in increased occurrence of acute coronary or cerebrovascular events. In addition, dysfunction of the microvascular flow results in poor regional circulation or loss of circulation, ischemia and end organ failure (peripheral neuropathy, nephropathy and retinopathy). As the title of this chapter implies, platelet dysfunction plays a major role in the diabetes mediated clinical complications. Having said that, just anti platelet therapies alone, cannot solve all the problems associated with diabetes. In view of the observed clinical complications, prevention of all the major risks is the primary choice or a better choice, followed by early detection and effective management of all the known risk factors.

As part of the 2020 impact goals, the American Heart Association (AHA) has set out seven ideal health goals; not smoking, maintaining normal weight, increased physical activity, a healthy diet, normal blood lipid levels, normal blood pressure and a normal fasting glucose. An analysis of the National Health and Nutritional Examination Survey (NHANES) showed, that individuals who met five of the seven ideal metrics of AHA, had a 78% reduction in the hazard ratio for all cause-mortality [126]. From the INTERHEART study, which included 52 countries, it is estimated that modifiable risk factors account for 90% of the population attributable risk for heart disease in Men and 94% of the risk in Women [127]. In view of these observations, goals of our professional society, South Asian Society on Atherosclerosis and Thrombosis (SASAT), has always been early diagnosis of the risks, effective management of the risks and prevention [128–130]. Finally, I would like to close this overview, with a statement from Professor David Katz, director of Yale University Prevention Research Center and president of the American College of Life Style Medicine, " There is no pill, and there never will be any pill, that can reduce burden of chronic disease in the way that a healthy lifestyle can"(Lifestyle Interventions. Medscape Apr 22, 2105).

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Part V
Metabolic Factors

Chapter 19
Advanced Glycation End Products (AGEs) in Diabetic Complications

Shweta Bhat, Sheon Mary, Ashok P. Giri, and Mahesh J. Kulkarni

Abstract Hyperglycemic condition in diabetes accelerates formation of advanced glycation end products (AGEs) that are formed as a result of series of reaction between reducing sugars and proteins. Accumulation of AGEs has been implicated in development of insulin resistance as well as in the pathogenesis of diabetic complications. The principal mechanism by which AGEs render harmful effects is through interaction with cell bound receptors. Certain receptors like AGE-R1 are involved in degradation of AGEs, while certain other receptors like receptor for AGE (RAGE) bring about counter effects exacerbating the situation. Accumulation of diverse AGEs, synergistically down regulate AGE-R1 while up regulate RAGE causing vicious cycle leading to enhanced formation and further accumulation of AGEs. In this article we discuss the formation of heterogeneous AGEs, importance of detection and quantification of AGEs, biological degradation of AGEs via different receptors, AGE-RAGE and its role in proinflammatory signaling, AGE mediated diabetic vascular complications such as nephropathy, retinopathy, neuropathy, cardiovascular and cerebrovascular diseases and finally the biological inhibition of AGEs is discussed along with chemical inhibitors for AGEs and natural products in AGE inhibition as a measure for the prevention of diabetic complications.

Keywords Advanced glycation end products • Nephropathy • Retinopathy • Neuropathy • Cardiovascular diseases • Cerebrovascular diseases • Receptor for AGE (RAGE) • Aminoguanidine

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19.1 Introduction

Diabetes mellitus is a chronic disorder, believed to be caused by both genetic and environmental factors. Broadly, diabetes mellitus is classified into 2 main types: (i) Type 1 diabetes or insulin dependent diabetes (IDDM), resulting from the failure in insulin secretion due to autoimmune destruction of β-cells of the pancreatic islets [1], and (ii) Type 2 diabetes or non-insulin dependent diabetes (NIDDM) caused by the insulin resistance, often combined with reduced insulin secretion [2]. Type 2 diabetes accounts for about 90% of the cases of diabetes worldwide whereas type 1 diabetes accounts for about 5–10%. The prevalence of diabetes is increasing at an alarming rate, owing mainly to high sugar and fat rich diet along with reduced physical activity. Nearly 8% of adults in high-income countries and over 10% of adults in upper-middle and middle-income countries have diabetes. Approximately 180,000 people died in the United States, 983,203 in India and it was 1,133,918 in China during 2011 due to diabetes. Perhaps, these numbers may not precisely represent enormity of diabetes burden, since a large number of people are unaware of the fact that they have diabetes because they have not been diagnosed [3].

19.2 Advanced Glycation End Products (AGEs)

Irrespective of type of diabetes, hallmark of the disease is persistent hyperglycemia and consequent abnormalities in carbohydrate, fat and protein metabolism [4]. The inevitable consequence of hyperglycemia is enhanced glycation eventually forming advanced glycation end products (AGEs). Glycation is a non-enzymatic reaction between reducing sugars such as glucose and/or its auto-oxidation products with amino groups of nucleic acids, lipids, peptides and proteins. This reaction was described for the first time by Louis Camille Maillard, who noticed the characteristic brown color when the mixture of amino acid and reducing sugars were heated together [5], therefore it is also called Maillard's reaction or Browning.

19.2.1 Formation of AGEs

Glycation reaction commences when reducing sugars react reversibly with ε-amino groups or N-terminal groups of the proteins (or free amine-containing lipids or DNA) to form Schiff's base, which spontaneously rearranges to form a relatively stable entity called Amadori product [4]. The Amadori product undergoes series of reactions to form highly reactive α-dicarbonyls and oxoaldehydes, such as 3-deoxyglucosone (3-DG), glyoxal (GO), and methylglyoxal (MGO), which are major AGE precursors [5, 6]. Yet another route of formation of AGEs also known as autoxidative glycation or glycoxidation occurs in nature. Monosaccharides are known to exist in equilibrium

with enediol. This enediol in presence of metal ions undergoes autoxidation forming enediol radical, which in turn can reduce molecular oxygen to the superoxide radical and itself gets oxidized to a dicarbonyl ketoaldehyde [7, 8]. These ketoimines and dicarbonyls generated via Amadori rearrangements, then indiscriminately react with lysine and arginine functional groups of proteins, yielding a wide variety of irreversible and stable modifications. In addition, oxidation of polyunsaturated fatty acids and arachidonic acid involving metal ion-catalyzed reactions, which are intermediates of lipid metabolism, can also lead to the modification of proteins. These protein adduct are called advanced lipoperoxidation end products (ALEs). Both advanced glycation and lipoperoxidation end products are collectively termed as AGEs and exhibit physiochemical characteristics such as fluorescence, brown color, and intra or intermolecular cross-linking [5, 9–12]. Predominant AGEs, well characterized for their presence in diabetic complications include mainly, fructosyllysine (FL), carboxymethyl lysine (CML), carboxy ethyl lysine (CEL), pentosidine, argpyrimidine, pyralline, methylglyoxal deerived hydroimidazolone MG-H1 [6, 13–16]. In addition to the formation *in vivo*, AGEs can also accumulate in the body by consumption of dietary AGEs and are known to be involved in the development of complications [17]. In diabetes, accumulation of AGEs is accelerated and they are invariably associated with micro and macrovascular complications of diabetes namely nephropathy, retinopathy, neuropathy, coronary artery disease and cerebrovascular diseases [18]. The figure depicting formation of different AGEs is shown in Fig. 19.1.

19.2.2 Degradation and Clearance of AGEs

AGE catabolism and turnover of the biomolecules is facilitated by cell surface-bound AGE receptors (AGE-Rs) mediated by endocytosis and degradation. Even though AGE detoxification system is predominant in mononuclear/macrophages, it also exists in endothelial, mesangial, neuronal and other mesenchymal cells.The cell surface receptor binding to AGEs formerly termed oligosaccharyltransferase complex-48 (OST-48) or AGE-R1 is mainly involved in binding to AGEs, endocytosis, active turnover and negative regulation of inflammatory response mediated by AGEs [19, 20]. Low expression of AGE-R1 in mononuclear cells was reported in type 1 diabetic patients with complications which also correlated with increased level of serum AGEs [21]. Along with its involvement in AGE removal, AGE-R1 was also shown to negatively regulate the AGE-induced proinflammatory signals and hence exhibiting the protective role in murine mesangial cells [20]. Another receptor which was co-purified with AGE-R1 was termed AGE-R2 [22]. Further, one more receptor Galectin-3, associated with R2 was identified, which was found to be readily translocated from cytoplasm to cell membrane on exposure to AGEs and able to bind to the AGEs with high affinity, it was given the name AGE-R3 [23]. The mice deficient with AGE-R3 exhibited enhanced AGE deposition and development of severe renal disease, confirming the role of this receptor in AGE

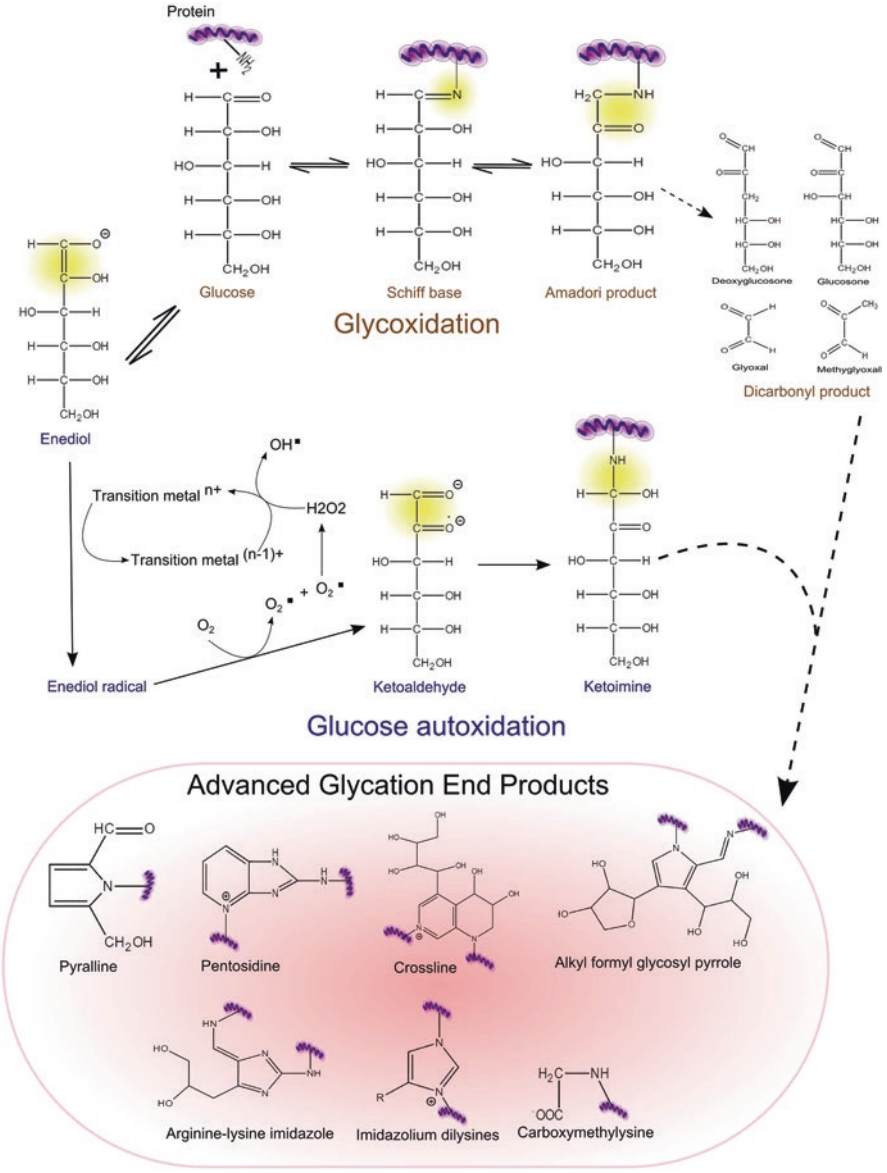

Fig. 19.1 The schematic flowchart represents the formation of AGEs via glycoxidation (*brown label*) and glucose autoxidation (*blue label*) process. The *oval box* represents a few types of AGEs

degradation and maintenance of tissue integrity [24]. These 3 receptors together are termed as AGE receptor complex. AGE modification of protein acts as a specific signal for recognition and subsequent degradation of the macromolecules by mouse peritoneal macrophages and it was hypothesized that incomplete removal and

accumulation of AGE-modified biomolecules could be the associated with molecular events of normal ageing [25–27]. Macrophage-associated receptors, that recognize AGE are termed as scavenger receptors (SR). There are two types of this receptor, SR-A and SR-B. SR-A is a key molecule in endocytic uptake of AGE ligands with high affinity for glycoaldehyde modified bovine serum albumin (BSA) than methylglyoxal or glyoxal modified BSA [28, 29]. SR-B1 has been shown to selectively mediate hepatic uptake of high-density lipoprotein cholesteryl ester (HDL-CE) without endocytic uptake of HDL apolipoproteins [30], and efflux of cholesterol from peripheral cells to HDL proteins [31, 32]. SR-B1 was also demonstrated to bind AGE-BSA, facilitating endocytosis and further, lysosomal degradation in CHO cells overexpressing hamster SR-BI. Binding of AGE-BSA was shown to inhibit selective uptake of HDL-CE and also cholesterol efflux from the cells to HDL was markedly inhibited by AGE-BSA [33]. SR-B1 plays a major role aiding in atheroprotective function of HDL [34]. CD-36 is another receptor belonging to SR-B family binds to AGEs and leads to intracellular degradation [35]. Even though these receptors are involved in AGE catabolism and turnover, their expression is regulated depending on the type of the tissue or cell and metabolic conditions. They can lead to reactive oxygen species (ROS) production, the release of pro-inflammatory molecules such as cytokines and growth factors causing cell activation and cell proliferation [36]. Yet another vastly studied receptor is a receptor for AGE or RAGE, which is known to be involved in pathways eliciting chronic cellular oxidant stress by binding not only to AGEs but the diverse spectrum of ligands including amyloid beta, amphoterin, components of the S100/calgranulin family to name a few. RAGE has been shown to be involved in intracellular signal transduction but not in endocytosis and turnover of the AGE-modified proteins [37–39]. Also, it is important to note that part of the tissue derived or dietary AGE degradation products are excreted in urine, which is hampered during diabetes and renal dysfunction causing retention of AGEs in tissues and circulation [40, 41]. The AGEs and pathological involvement of the RAGE are discussed in the specific complications.

19.2.3 Receptor for Advanced Glycation End Products (RAGE)

RAGE is a pattern recognition, multiligand receptor belonging to immunoglobulin (Ig) superfamily [42, 43]. For the first time, RAGE was identified as a receptor binding to AGEs [44]. Human RAGE is reported to be comprising of 404 amino acids and molecular weight of 42.8 kDa. The full-length RAGE (fl-RAGE) protein consists of a large extracellular domain, a single transmembrane-spanning helix, and a short cytoplasmic domain. This intracellular domain of fl-RAGE was reported to be essential for downstream signaling [45]. AGEs such as CML are reported to modulate and increase the expression of RAGE [46]. RAGE is known to express on many different cell types such as endothelial cells [47], smooth muscle cells [48], neuronal cells, glial cells [49], monocytes/macrophages [50, 51], fibroblasts [52]. A large number of diverse ligands such as amyloid β peptide, S100/calgranulins, amphoterin or

high mobility group protein B1 (HMGB1) binds to RAGE along with classical AGE-binding [53]. AGE interaction with RAGE leads to activation of transcription factor NF-kB (preferentially p50/p65 heterodimer) and Sp1 leading to production of ROS *via* numerous intracellular signaling pathways including ERK1/2 MAPK, JAK/STAT, SAPK/JNK MAPK, Akt, rho GTPase, PI3kinase, caspase-3/7, TGF- β -Smad, p21waf, p21ras, and cdc42/rac which further lead to different pathological responses [46, 54, 55]. Soluble RAGE (sRAGE), a splice variant of RAGE, lacking C-terminal domain, but containing all of the immunoglobulin domains of fl-RAGE is also reported [56]. sRAGE acts as a decoy of fl-RAGE and prevents interaction of ligands with the receptors [57]. In addition, fl-RAGE is also known to undergo proteolytic cleavage through the metalloproteinase ADAM10 and matrix metalloproteinase 9 (MMP9) to release sRAGE [58]. Several studies have indicated upregulation of RAGE in hyperglycemia driven accumulation of AGEs during diabetes and that AGE-RAGE axis is predominantly involved with the progression of vascular complications of diabetes [59, 60]. The implications of AGE-RAGE interactions are further discussed in the specific cell types involved in the complications of diabetes.

19.2.4 Detection and Quantification of AGEs

Detection and quantification of AGEs in clinical condition are promising strategy for assessment of the severity of pathogenesis in diabetes since they are found freely in the circulation as well as accumulate in the tissues. The levels of AGEs vary depending on the disease state and the metabolic conditions. AGEs have been proposed as markers for the prediction of development of micro and macrovascular complications of diabetes [61]. The classic example is the glycated hemoglobin or HbA1c, which is a marker for assessment of integrated glycemic status over a period of 1–3 months. Monitoring HbA1c was used as a predictive measure for the development of diabetic complications in long-term studies such as Diabetes Control and Complications Trial (DCCT) and the UK Prospective Diabetes Study (UKPDS) [62, 63]**.** Fluorescence or Browning is a characteristic property exhibited by certain AGEs, which is exploited in their detection and quantitative measurement using visible and fluorescence spectrometry**.** Skin content of the AGEs can be measured using fluorescence phenomena and it has been shown that auto fluorescence of the skin positively correlates with the aging and also diabetic complications [64–66]. Serum AGEs are measured by ELISA. Phenylboronate affinity chromatography is used as a preparative technique for the enrichment of glycated proteins for the enhanced detection and the same principle is also used in thedetection of AGEs using phenylboronate derivatized with methyl acrylamide has been used in SDS-PAGE (mPAGE:methacrylamidophenylboronate acrylamide gel electrophoresis) [67].

Proteomics and mass spectromtertic studies are extensively used in detection and characterization of AGE-modified proteins [68–71] based on neutral loss and elec-

tron transfer dissociation (ETD)-based MS techniques for characterization of AGE modifications [72, 73]. Isotope labeling with C-13 or $^{13}C_6$-reducing sugars has been used for the analysis of glycated proteins from human plasma [74, 75]. We reported comprehensive MS/MS fragment ion library for Amadori modified lysine (AML), carboxy methyl lysine (CML) and carboxy ethyl lysine (CEL) modifications of human serum albumin (HSA) and also this was used as reference for HSA-AGE-peptide quantification in plasma of healthy, prediabetic, diabetic, and microalbuminuria patients [76]. Recently we have also reported fragment ion library for the quantification of N-1-(carboxymethyl) valine (CMV) and N-1-(carboxyethyl) valine (CEV) peptides of β-hemoglobin, which can be a prospective marker along with measurement of HbA1c for better assessment of glycemic status in diabetes management.

19.3 AGEs in Diabetic Vascular Complications

19.3.1 Nephropathy

Diabetic nephropathy is largest single cause for the end stage renal disease eventually resulting in renal failure, termed aptly as a medical catastrophe worldwide [77]. After the onset of type 1 or type 2 diabetes, within 20–25 years, about 25–40% of patients are reported to develop diabetic nephropathy [78]. The diabetic nephropathy progresses through identifiable steps from subclinical condition to an early stage of nephropathic symptoms, microalbuminuria (MIC) with persistent albumin excretion in urine (30–300 mg/24 h), to macroalbuminuria or diabetic nephropathy with excretion of urine albumin >300 mg/24 h. Hyperglycemia mediated formation of AGEs has long been linked to diabetic nephropathy, which is characterized by progressive glomerular and tubular basement membrane thickening, microvascular damage, mesangial extracellular matrix expansion. “AGE burden” in the kidney is increased by the accumulation of AGE-modified proteins, not only from the kidney but also from the circulation since filtration of AGEs take place in the kidney [79, 80]. Monoclonal anti-AGE antibodies were shown to react with the mesangial area in renal biopsy samples from diabetic nephropathy patients and also reactivity increased with the degree of mesangial expansion [81]. In early stages of nephropathy, AGEs such as CML, pentosidine, malondialdehyde-lysine have been found to accumulate in the expanded mesangial matrix and thickened glomerular basement membrane and in advanced stages of the disease, these AGEs have been detected in nodular lesions [82]. Previously we identified increased AGE-modified protease resistant proteins belonging to metabolic pathways, oxidative stress, cell signaling, and transport in the kidneys from streptozotocin-induced diabetic rats by using 2D electrophoresis, western blotting with anti-AGE antibodies and MS analysis [83, 84]. Chronic accumulation of AGEs leads to a progressive alteration in the renal architecture and eventually loss of renal function. Various events have been reported

to be involved in this process such as cross-linking of the modified matrix proteins such as cross β-sheets, deranged matrix-matrix and cell-matrix interactions and activation of downstream signaling [12, 85].

Serum albumin is a most abundant plasma protein, which is the principal target for glycation in circulation and competitively protects low abundant plasma proteins from glycation [86]. While low albumin levels are associated with increased plasma protein glycation [87]. AGE modification of the albumin leads to structural changes and hence loss of its innate biochemical properties [88]. This altered protein structure can lead to the generation of antibodies and form complex with AGE-modified protein called circulating immune complex (CIC). The immune complex containing AGE-modified proteins have been shown to get deposited in the glomerular basement membrane leading to progression of diabetic nephropathy [89]. Recently in our study, we have observed AGE-modified, elevated levels of serum albumin in the CICs from microalbuminuria patients [90]. The release of transforming growth factor-β (TGFβ) was reported in cultured glomerular endothelial cells on exposure to glycated albumin, indicating the chronic effect of circulating AGEs in the renal cells eventually contributing to renal dysfunction [91]. Also, AGE-modified matrix proteins become resistant to proteolytic digestion by metalloproteinases causing basement membrane thickening and mesangial expansion and also can trap extravasated plasma proteins such as lipoproteins leading to severe glomerulosclerosis [92, 93]. AGEs can induce apoptotic cell death, overexpression of vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1) in mesangial cells, which are central structural units and facilitate glomerular filtration due to their smooth muscle like activity [94, 95]. This, in turn, can lead to glomerular hyperfiltration. Antibodies against VEGF, alleviated the symptoms of hyperfiltration and albuminuria in experimental diabetes [96]. AGEs have also been shown to induce insulin-like growth factor-I, −II, platelet-derived growth factor and TGFβ in mesangial cells, mediating production of type IV collagen, laminin, and fibronectin [97]. One long-standing notion is involvement of AGE-RAGE axis in the development of diabetic nephropathy, which has a considerable amount of evidence so far. RAGE is a signal transducing receptor, which mediates inflammatory reactions on binding to AGEs eliciting the generation of reactive oxidative stress (ROS) [98–100]. ROS generation in mesangial and renal tubulointerstitial cells is cytotoxic in nature and activates different inflammatory pathways such as mitogen-activated protein kinase (MAPK), nuclear factor kappa-B (NFκB) and/or protein kinase C (PKC) pathways stimulating the production of TGFβ and connective tissue growth factor (CTGF) which in turn serve as are pro-sclerotic [5, 95, 101, 102]. An increased level of NFκBp65 due to AGE-RAGE signaling leads to sustained activation of NFκB, which overcomes even endogenous negative feedback mechanism, causing persistent kidney damage [103]. RAGE overexpression is also reported in podocytes and mesangial cells of diabetic patients with nephropathy [104, 105]. Further, many studies using animal models have given a considerable amount of evidence for the involvement of RAGE axis in diabetic nephropathy. Administration of RAGE neutralizing antibody was reported to inhibit the renal changes such as glomerular hypertrophy, glomerular basement membrane thickening,

mesangial matrix expansion, CTGF overexpression, and NFκB activation in *db/db* or streptozotocin-induced diabetic mice [106, 107]. RAGE overexpressing diabetic mice have been reported to show rapid glomerulosclerosis and renal dysfunction compared to that of diabetic mice lacking this RAGE transgene [108]. In addition, it was shown that diabetic homozygous RAGE null mice did not develop mesangial matrix expansion or thickening of the glomerular basement membrane, confirming the involvement of RAGE in the development of diabetic nephropathy [109].

19.3.2 Retinopathy

Diabetic retinopathy is a severe microvascular complication among many ocular complications of diabetes, which is largely asymptomatic at the beginning eventually having a debilitating impact on the clarity of vision, leading to blindness in most of the cases [110, 111]. In case of type 1 diabetes, the prevalence of retinopathy is about 80% in the first 10 years of disease, which increases to 95% in about 20 years of disease onset [112]. Whereas, in case of type 2 diabetes, the evidences suggest that onset of retinopathy takes place at least 7 years before the clinical diagnosis of the disease [113]. About 80% and 50% of T2D patients whether they are dependent on exogenous insulin or not, will develop retinopathy in about 20 years of time [114, 115].

Retinopathy begins with mild structural abnormalities, which are characterized by increased vascular permeability progressing to non-proliferative diabetic retinopathy (NPDR) and then to proliferative diabetic retinopathy (PDR) marked by the abnormal growth of new blood vessels in the hypoxic retina and posterior surface of vitreous [110]. Diabetic hyperglycemia acts as a key link in the development of retinopathy. Several pathways mediated by hyperglycemia such as polyol accumulation, AGEs, oxidative stress and activation of PKC are thought to be involved in the pathogenesis of retinopathy. Increased intracellular sorbitol through polyol or hexosamine pathway causes osmotic damage in vascular cells [116]. A distinct accumulation of AGEs and co-localization of AGEs with their receptors AGE-R1 and AGE-R2 have been reported in the retina from diabetic rats [117, 118]. AGEs increase the expression VEGF, a potent mitogen, which upregulates the expression of intercellular adhesion molecule-1 (ICAM-1) leading to increased adhesion of monocytes to retinal endothelial cells [119]. Increased VEGF expression is also reported to stimulate angiogenesis and neovascularization which are the key events of proliferative retinopathy [120]. Among different AGEs, CML has been shown to be elevated in the cells of the retina and vascular walls of diabetic humans and diabetic rats [117, 121]. Elevated levels of CML in type 2 diabetic patients have been reported to act as key modulators in NPDR and PDR [122]. Glyoxalase 1 mediated detoxification of MGO reduces the formation of AGEs and has been shown to prevent retinal neuroglial and vascular lesions [123]. The AGE-RAGE signaling leads to sustained inflammation causing neurodegeneration and microvascular

dysfunction *via* increased oxidative stress. This has been found to play a central role in diabetic retinopathy. AGEs are thought to be inducing basic fibroblast growth factor (bFGF) in retinal Muller cells [124]. RAGE expression is also reported to be increased with AGEs in pericytes and microvascular endothelial cells [125]. The early events of diabetic retinopathy are the apoptotic death of pericytes [95]. The AGE-RAGE signaling along with inhibition of integrin-mediated protein kinase B/Akt phosphorylation, and also decreased survival signaling by platelet-derived growth factor (PDGF) have been reported to be involved in the process of pericyte loss [126, 127].

19.3.3 Neuropathy

Diabetic neuropathy comprises of clinical syndromes affecting the peripheral or autonomous nerve system. Peripheral neuropathy is the most common and well characterized diabetic neuropathy, with the prevalence of 8% in newly diagnosed patients and 50% in patients with more than 25 years of diabetes [128]. The common manifestation is a distal symmetric polyneuropathy (DSP), in which patients have tingling, pain, numbness and weakness that begin in the feet and spread in a length-dependent fashion [129]. Diabetic foot ulcers are seen in one in four patients during their lifetime [130]. Immunoelectron microscopy of diabetic human peripheral nerve revealed the presence of CML deposition as irregular aggregates in the cytoplasm of vascular endothelial cells, axoplasm, pericytes and Schwann cells of both myelinated and unmyelinated fibers [131].

Hyperglycemia-induced damage to the nerve *via* polyol and hexosamine pathways, excess PKC activation, AGE accumulation and a hyperglycemia-induced decrease in neurovascular blood flow *via* oxidative stress and ischemia, are known to be the major pathogenic reasons for diabetic neuropathy [132]. Two different mechanisms by which glycation damages nerves are: (i) glycation of nerve protein that leads to functional loss and thereby affecting the nerve activity, (ii) binding of AGE to nerve cell surfaces RAGE triggering inflammation response causing further damage of neurons [133]. Axonal cytoskeleton proteins such as tubulin, actin, and neurofilament undergo glycation that leads to axonal dysfunction [134–136]. The ability of Schwann cells to guide regeneration of nerve fibers are affected due to glycated laminin, a major constituent of basal laminae [137]. AGE-adduct formation on Po-protein of peripheral nerve myelin in diabetic rats have been reported to promote recognition and scavenging by macrophages, this might contribute to the segmental demyelination in diabetes [138].

AGE formation on extracellular matrix (ECM) protein such as collagen produces cross linkages that alter the physical property of ECM leading to regenerative failure and creates a physical barrier for axonal mobility due to less digestibility by metalloproteases [139]. AGE modification on ECM also alters the electric charge and causes thickening of the basement membrane by increasing the permeability of blood vessel and quenching of vasodilators such as NO [140]. This reduces the

blood flow to the micro vessels in the peripheral nerve and induces hypoxia. Moreover, the RAGE expressed on the endothelial cell surface of peri- and endoneurial blood vessels interacts with the AGE, thus activating transcriptional factors NF-κB that regulates inflammation and apoptosis [141]. RAGE expression on neuronal cells such as dorsal root ganglia has shown to induce oxidative stress and cellular injury *via* caspase activation and nuclear DNA degradation [142].

19.3.4 Cardiovascular and Cerebrovascular Complications

Diabetes increases the cardiovascular mortality risk by 2-fold in comparison to non-diabetic patients [143]. The diabetic cerebrovascular disease is the major cause of mortality in patients with diabetes mellitus, almost 20–40% patients suffer from this intracranial large or small vessel disease [144]. Stroke, cerebral small vessel disease, cerebral atherosclerosis and acute cerebral vascular disease are some of the clinical manifestations seen in these patients.

Elevated serum levels of AGEs, CML and glycated serum albumin are proposed to be used as predictors of presence CAD in patients with T2DM [145, 146]. Glycation of lipoproteins and other proteins are one of the major factors responsible for accelerated atherosclerosis in diabetes. AGE-modified ECM proteins result in crosslinking and hardening of artery walls, which was shown to be reduced with aminoguanidine (AMG) an AGE inhibitor [147]. Glycated low-density lipoproteins (LDL) are the most extensively studied in relation to atherosclerosis in diabetes. AGE modification on LDL apolipoprotein B impairs its uptake by the LDL-receptor, increasing it's *in vivo* accumulation [148]. These glycated LDL are recognized by a non-specific (scavenger) receptor present on the fibroblasts, monocytes derived macrophages, and human aortic intimal cells, this consecutively increases the intracellular accumulation of cholesteryl esters and promote atherosclerosis [149, 150]. Moreover, these glycated LDL is more susceptible to LDL oxidation contributing to atherogenicity [151]. Glycated collagen also traps LDL that accelerate development of atherosclerosis in diabetic patients [152]. Elevated levels of apolipoprotein A1 glycation and reduced activity of HDL-associated paraoxonase 1 and 3, and their interaction is shown in the case of severe CAD in T2DM patients [153].

Expression of RAGE and enRAGE is significantly increased in the coronary atherosclerotic lesion in diabetic patients [154]. Binding of AGE to RAGE in the endothelial cells, smooth muscle cells, and monocytes enhances coronary dysfunction. For example, interaction of AGE with RAGE on endothelial cell surfaces induces oxidative stress, activates NF-κB [155] and VCAM-1 [156], this results in increased endothelial permeability that leads to increased lipid entry into subendothelium and transendothelial migration of monocytes [155, 157, 158].

The involvement of various AGEs in different complications of diabetes is shown in Fig. 19.2.

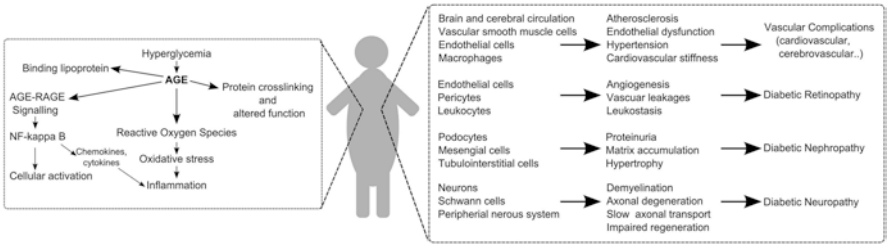

Fig. 19.2 The influence of AGEs in various diabetic complications

19.4 AGE Inhibition as a Preventive Measure in Diabetic Complications

The involvement of AGEs in the development of diabetic complications has long been targeted for the prevention of the AGE-mediated secondary complications of diabetes. *In vivo* cells have evolved biochemical mechanisms to inhibit the formation of AGEs by various enzymatic machinery and to prevent the AGE-induced burden to a certain extent. These can be exploited for the development of preventive strategies for AGE-mediated diabetic complications. Further, the synthetic chemical AGE inhibitors have also been developed as an intervention strategy to inhibit the formation of AGEs at various steps of glycation. In addition, there are natural compounds which have been exploited for their efficacy in the prevention of AGE formation and hence in the micro and macrovascular complications of diabetes. Different measures to prevent formation of AGEs at different stages of glycation are shown in Fig. 19.3.

19.4.1 Biochemical Intervention of AGEs In Vivo

The biological intervention mechanisms for reducing AGEs, which living systems have evolved can be exploited for the development of newer strategies. Formation of Schiff's base or Amadori products is intrinsically reversible in nature. The rate of formation of Amadori product is faster compared to its reversible reaction and hence during hyperglycemic conditions it accumulates [159]. Cells have evolved deglycation mechanism for the reversal of this reaction by class of enzymes collectively known as "Amadoriase" which include fructosylamine oxidase, fructoselysine 3-phosphokinase, and fructoselysine-6-phosphate deglycase. These enzymes are known to occur in all the living systems starting from prokaryotes, fungi to mammalian cells [160, 161]. Few small molecules like taurine, glutathione, carnosine and anserine are capable of causing non-enzymatic deglycation *in vivo* called transglycation [160, 162]. The reactive dicarbonyl intermediates such as GO, MGO

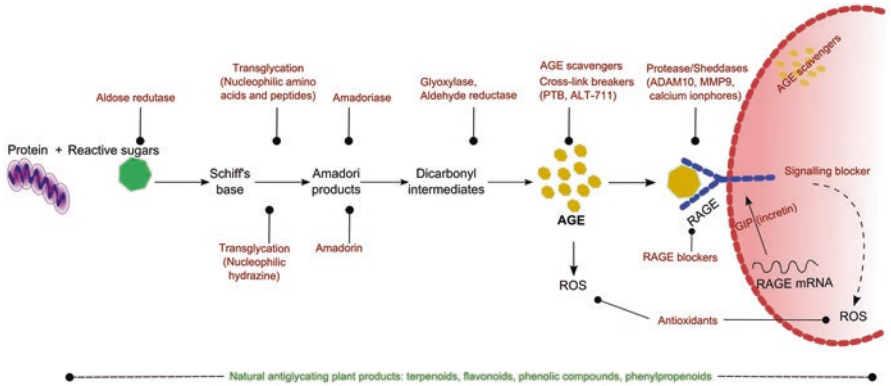

Fig. 19.3 Examples of various chemical, biological (*red*) and natural (*green*) glycation inhibitors at various stages of AGE formation and signalling (*black labels*)

and 3-DG are known to be cleared off by the family of enzymes glyoxalases and aldo-keto reductases [163]. Cleaved portion of membrane-bound RAGE or sRAGE binds to the circulating AGEs and involved in the inhibition of binding of AGEs to membrane-bound RAGE and thus preventing downstream inflammatory signaling [164]. The shedding of membrane-bound RAGE to sRAGE has been a promising intervention technique in preventing AGE-RAGE mediated complications. This process of shedding is known to be catalyzed by ADAM10 (a disintegrin and metalloproteinase 10), MMP9, and gamma-secretase [58, 165]. Calcium ionophores such as calcimycin and phorbol esters (e.g., PMA: phorbol 12-myristate 13-acetate, APMA: 4aminophenylmercuric acetate) are also known to induce the shedding of sRAGE *via* calcium-dependent PKC [58, 165]. In addition, glucose-dependent insulinotropic polypeptide (GIP), an incretin was shown to reduce RAGE mRNA and protein levels in endothelial cells and hence protecting from the deleterious effects of AGEs. N-acetylserine is another antioxidant mimicking the role of GIP and also inhibits RAGE signaling in endothelial cells [166], which can be a promising therapeutic target.

19.4.2 Chemical AGE Inhibitors

AMG, a nucleophilic hydrazine compound that traps the reactive carbonyl intermediates of glycation, was the first ever molecule to be identified as AGE inhibitor, suggesting AGE inhibition can prevent diabetic complications [147]. Since then extensive studies have been conducted using AMG which provided the strong basis for the development of new molecules that scavenge reactive carbonyl species and hence prevent the formation of AGEs. However, AMG was withdrawn from clinical usage in phase III trial due to adverse effects on human diabetics. Nevertheless,

AMG continues to be used as a prototype AGE inhibitor in development of new molecules with AGE inhibition properties [167]. Strategies for the prevention of AGE formation involve inhibition of glycation at various steps. The first step of glycation i.e. Schiff's base or Amadori product formation can be blocked by using various molecules such as amines, polyamines and small peptides [168–170]. Chemical deglycation or transglycation is another promising strategy to AGE inhibition. Nucleophilic hydrazine compounds act as potential deglycating agents. We have observed Hydralazine, a vasodilator and antihypertensive drug to act as a potent transglycating agent in experimental diabetic mice studies [171]. Certain chemical agents modifying amino acid side chains and preventing glycation can also be potential therapeutic agents. One such example is acetylsalicylic acid, which acetylates lysine residues blocking the reaction with glucose [172, 173].

Amodorins are the class of molecules that prevent the conversion of Amadori products to AGEs [174]. The first Amadorin to be identified was vitamin B6 or pyridoxamine (PM) which was used in the treatment of diabetic nephropathy [175]. Reactive dicarbonyl scavenging is a predominantly promising strategy for the inhibition of AGEs. Few examples of compounds with dicarbonyl trapping function are AMG, OPB-9195 [(±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-yl acetanilide] etc. Another popular drug being used for its efficacy as an anti-hyperglycemic agent is Metformin, an analog of AMG that brings about AGE inhibition by scavenging the dicarbonyls [176, 177]. Targeting AGEs by AGE crosslink breakers such as N-phenacyl thiazolinium bromide (PTB) and N-phenacyl-4, 5-dimethylthiazolium chloride (ALT-711/alagebrium), is also a strategy in reducing the AGE burden in the system [178]. Compounds that decrease the RAGE expression and/or block AGE-RAGE interaction are also promising in reducing the deleterious effects of AGEs. RAGE fusion protein competitively binding to RAGE ligands, thereby negatively regulating membrane-bound RAGE signaling was found to prevent the development of lesions of retinopathy in diabetic mice [179]. Neutralization of RAGE by using RAGE antibody in db/db and in streptozotocin-induced diabetic rats showed improvement in the renal status. Administration of recombinant sRAGE is also reported to suppress atherosclerosis in diabetes [180]. Yet another effective route of prevention of AGE formation is by the removal of ROS in the system. Even though dietary antioxidants to certain extent alleviate harmful effects of AGEs, they do not provide extensive protection against the development of complications [181]. Angiotensin-converting enzyme (ACE) inhibitors such as ramipril, temocaprilat, enlaprilat, captopril and perindprilat block the rennin-angiotensin system and angiotensin receptor blockers (ARBs) valsartan, olmesartan, candesartan etc. also provide protection by chelating transition metals and scavenging ROS, thereby blocking the formation of AGEs [182]. In addition ARBs also downregulate the RAGE expression *via* activation of PPAR-γ and thus suppress AGE-RAGE axis associated oxidative stress and inflammation [183].

19.4.3 Natural Products with AGE Inhibitory Properties

There has been a tremendous effort in the development of alternative medicine for the prevention of AGE-mediated complications. Natural products have been proven to be relatively safe for clinical use. Some known natural products inhibiting Amadori product formation include gallic acid, catechin and quercetin [184]. Plant extracts containing high triterpenoids and phenolic compounds, generally known to possess antioxidant and antiglycation activity have been used in thetreatment of diabetes and related complications [185]. *In vitro* prevention of AGEs by scavenging reactive dicarbonyl MGO is reported by natural compounds such as 2,3,5,4′-tetrahydroxystilbene 2-O-β-D-glucoside (THSG) and 4,6,7-trihydroxy-2-methoxy-8-(methylbut-2-enylphenanthren-1-1′-4′,6′,7trihydroxy-2′-methoxy-8′-(methylbut-2′-enyl)-phenanthrene isolated from *Polygonum multiflorum* [186] and *Prosthechea michuacana* [187] respectively. Natural flavonoids like luteolin, apigenin,myrcetin and silymarin have also been reported to exhibit carbonyl trapping properties and hence protect against AGEs [188–190].

Triterpenoids for example Oleanolic acid, along with two phenolic acids, caffeic acid, and chlorogenic acid from *Ilex paraguariensis,* were reported to show antiglycation activity more potent than that of AMG, a standard antiglycating agent [191]. Oleanolic acid has also been shown to exhibit many protective properties in experimental diabetic mice such as lowering blood glucose level, reducing the weight, inhibiting oxidative stress-induced insulin resistance and antiglycation in the kidney [192, 193]. β-carotene, a tetraterpenoid and a precursor for the synthesis of vitamin A, has been reported to prevent AGE formation in BSA [194]. Quercetin is a flavonol shown to inhibit AGE formation at different stages of glycation [195].

Along with terpenoids and flavonoids, many other phytochemicals such as phenylpropanoids, phenolic compounds, some polysaccharides and plant extracts, in general, have been reported for their antiglycation activity and a detailed review of plant products with antiglycation activity can be read from [185]. This field of research for the finding of alternative medicines from natural compounds continues to be explored tremendously for the AGE inhibition properties and prevention of AGE-mediated complications of diabetes.

19.5 Summary

Extensive research has provided considerable insights in the field of AGEs and consequence of their involvement in development of diabetic vascular complications. Here in this article we have discussed the mechanism of formation of AGEs in biological system during hyperglycemia, RAGE protein biology and its involvement in mediating pathogenesis is given special emphasis as this receptor is known to be involved in development of all the micro and macrovascular complications of diabetes as a whole. Further different cell bound receptors are discussed, which are

mainly involved in turnover of AGEs, however their neutralizing AGE effect is regulated and override by the increased levels of AGEs rendering them to participate in overt disease progression. AGEs play central role in the diabetic micro and macrovascular complications by perturbing the homeostasis of the tissues as in case of diabetic nephropathy, important of all diabetic ocular derangements, the retinopathy, devastating diabetic neuropathy, and systemic cardiovascular and cerebrovascular diseases. Last but not the least, inhibition of formation of AGEs is a promising strategy which has long been explored in attempt to prevent the development of complications. Even though cells have evolved mechanisms to keep the levels of AGEs at check, it is deranged during diabetic AGE burden. Hence finding chemical agents which can mimic the action of some biological molecule to curb the harmful effect of AGEs was attempted in clinical research and many molecules with such properties have been discovered and the area is still a hot spot of clinical research. Further natural products are historically known to have beneficial effects by their inherent antioxidant properties which proves to be beneficial in reducing ROS, reactive dicarbonyl quenching and also they are reported for their anti glycation properties. Several lines of intriguing evidences have indicated that natural compounds can be very useful in preventing and treatment of diabetic complications and are in clinical trials to confirm their efficacy in human beings.

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Chapter 20
Atherogenic Dyslipoproteinemia in Type 2 Diabetes Mellitus

Arungovind G, Kamalanathan A.S, and Krishnan Venkataraman

Abstract There has been an increased incidence of Type II Diabetes Mellitus worldwide and in an epidemic proportion especially in the developing countries. It has already caused considerable health, psychological and socio-economic burden to the individuals of the family and to the health care system. Diabetic patients (type II) are often associated with secondary complications affecting heart, kidney, brain, eyes, etc. Most of the deaths in diabetic patients occur due to the derangements in the cardiovascular system with the progression towards atherosclerosis. Diabetic atherosclerosis is considered a chronic inflammatory disease involving multiple risk factors such as hypercholesterolemia, insulin resistance, dyslipidemia, obesity, hypertension, smoking, etc., all of which contributes to the progression of the disease. This review focuses on understanding some of the diabetic complications leading to derangement in lipid and lipoprotein metabolism, formation of advanced glycation end products with special reference to lipoproteins, lipotoxicity and endothelial dysfunction contributing to the progression of atherosclerosis subsequently leading to cardiovascular diseases in diabetic patients.

Keywords Atherosclerosis • Advanced Glycation End products (AGE) • Cardiovascular disease risk • Diabetes dyslipidemia • Dyslipoproteinemia • Endothelial dysfunction • Lipids • Lipoproteins • Lipotoxicity

20.1 Introduction

Diabetes mellitus is one of the most prominent metabolic diseases afflicting human societies [1]. According to the 2016 World Health Organization report, globally about 422 million adults are known to be diabetic [2] and among the Indian population about 62 million (globally second largest diabetic populated country) are

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surviving with this disorder. The prevalence of type II diabetes or Non-Insulin Dependent Diabetes Mellitus (NIDDM) contributes a significant proportion in any given population. In spite of efficient disease management with oral drug(s) and/or insulin(s) treatment regimes, the associated complications are often detrimental. Major complications include myocardial infarction, stroke, kidney failure, vision loss and nerve damage. These complications arise due to the persistent higher than optimal blood glucose levels, insulin resistance and abnormalities in metabolism (alternations in the biochemical or metabolic pathways and biomolecules therein). Eventually, these complications lead to an overall increase in the risk factors and mortality. Diabetes caused 1.5 million deaths during the year 2012 [2]. Most of these deaths were due to the complications arising out of the cardiovascular system.

Cardiovascular atherosclerosis is one of the major risk factors leading to morbidity and mortality in diabetic population- type I (Insulin dependent diabetes mellitus) and type II diabetes [3–5]. This risk factor is about two to four fold higher in type II diabetics compared to non-diabetics. In addition, complications due to obesity also contribute significantly in diabetic patients who are prone to cardiovascular diseases (CVD). A common overlap between diabetes, obesity and CVD is that they are categorized as metabolic disorder or lifestyle diseases. All these three metabolic disorders appear to share certain common inflammatory pathways, which in turn links all the risks associated with them. Recently, there has been a tremendous improvement in understanding the various metabolic and signalling pathways that underpin the disease mechanisms which is providing better insights for controlling the disease. Most of the studies have put forward, insulin resistance, hyperglycemia, dyslipidemia (changes in lipid and lipoprotein metabolism), lipotoxicity (excessive accumulation of fatty acids and certain bioactive lipids), endothelial dysfunction *etc.,* are the major causative factors for cardiovascular atherosclerosis (Fig. 20.1) [4, 6]. The consequences of these metabolic abnormalities are known to have great impact and disarray on vascular tissues, leading to diabetic complications [7, 8]. Since a number of studies have established dyslipidemia as one of the major causative factors, we first discuss about lipids and lipoproteins, their metabolism and changes occurring in the course of pathogenesis of atherosclerosis and its consequences in diabetes.

20.2 Lipids and Lipoproteins

Lipids generally known as fats occupy a central role in the energy metabolism of the body. Being a rich source of energy due to their composition of mostly carbon and hydrogen they are body's stores of excess calories and their unique physical property of fluidness allow them to play a vital structural role in cellular membranes. Lipids are classified into simple lipids (oils, fats and waxes), compound lipids (phospholipids, glycolipids etc.) and derived lipids (fatty acids, glycerol, cholesterol etc.). They are insoluble in water and so in the body, these lipids are carried and transported by a special transportation system wherein the lipids are associated

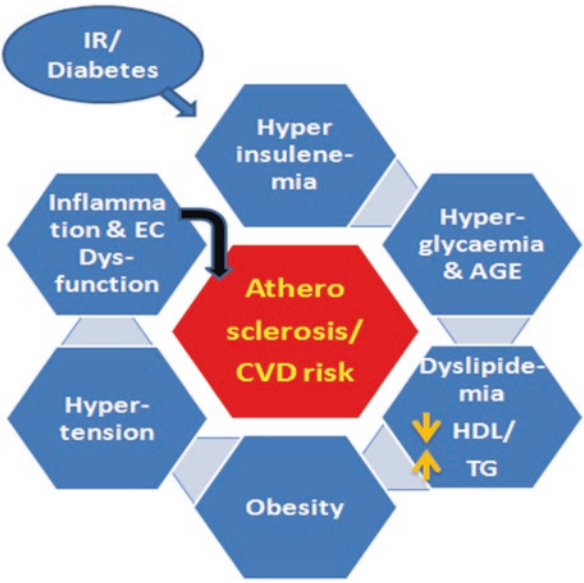

Fig. 20.1 Schematic representation of events that leads to atherosclerosis in patients with type 2 diabetes mellitus

Normally, insulin promotes glucose uptake in muscles, triglyceride synthesis in liver and adipose. But in obesity and type 2 diabetes, insulin is dysfunctional and there is development of insulin resistance (IR). Due to this, insulin levels raises leading to hyperinsulinemia. This also causes glucose levels to increase, thereby leading to hyperglycemia. On the other hand insulin resistance also alters lipid metabolism in the body where levels of triglycerides (TG) shoots up and subsequently levels of high density lipoprotein (HDL) drop down leading to a condition, termed dyslipidemia. This when combined with hypertension and injury to the blood vessel endothelium leads to endothelial cell dysfunction, thus triggering a cascade of inflammatory events that culminates into atherosclerosis and cardiovascular disease

with proteins and such lipid conjugated protein complexes are known as lipoproteins (Fig. 20.2). These lipoproteins are miscible in water and they contain mainly triglycerides, phospholipids, cholesterol and cholesterol esters. The protein component is known as apolipoprotein and one or more different types of apolipoproteins are present in each lipoprotein classes. Lipoproteins exist as spherical particles ranging in size from 10–1200 nm and they have a single monolayer of phospholipids and cholesterol on the surface. Inside these particles, triglycerides and cholesterol esters together form a central core. These particles are delivered to various tissues of the body for energy metabolism. This whole spherical lipid assembly is structurally stabilized by apolipoproteins, which is located on the surface and they also serve as ligands for cell surface receptors and activators or inhibitors of enzymes that will modify these lipoprotein particles (Fig. 20.2) [9–11]. Lipoprotein particles are classified based on their physico-chemical properties and the apolipoproteins present in them. Variations in the lipid composition alter the lipoprotein particle's density and size. Lipoprotein particle with high triglyceride and cholesterol content

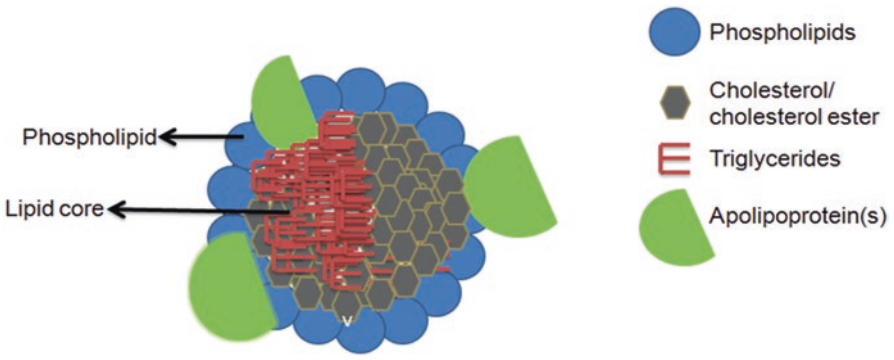

Fig. 20.2 Schematic representation of a lipoprotein particle
Lipoprotein particle comprises of a single phospholipid layer in which high amounts of cholesterol and triglycerides are packed to be transported to various tissues of the body. The lipoprotein particles are stabilized by apolipoproteins. In addition, lipoprotein particles carry certain bioactive lipids, enzymes/proteins involved in lipid metabolism

will be larger in size and less dense when compared with the lipoprotein that has less triglyceride and cholesterol. Here, the quantity of apolipoproteins present also changes the density of the particles. The different lipoprotein particles isolated using density gradient ultracentrifugation are chylomicrons (density: < 0.93gm/ml and diameter: 80–1200 nm), very low density lipoprotein (VLDL: density: 0.95–1.006gm/ml and diameter: 30–90 nm), intermediate density lipoprotein (IDL: density: 1.006–1.019gm/ml), low density lipoprotein (LDL: density: 1.019–1.063 gm/ml, diameter: 18–22 nm) and high density lipoprotein (HDL: density: 1.063–1.210gm/ml, diameter: 5–12 nm).

The different lipoproteins are also classified based on their apolipoprotein composition. Apolipoproteins are labelled by a series of letters from A-E and are abbreviated as apo A-I, apo C-III, etc. and currently nine major human apolipoproteins have been identified, namely A-I, A-II, A-IV, B-100 & B-48, C-I, C-II, C-III, D and E respectively. So the different lipoprotein particles contain different apolipoproteins and are classified accordingly. HDL along with apolipoprotein A-I (apo A-I) as its major apolipoprotein [12] also contains apo A-II, apo A-IV, apo C-I, C-II, C-III, apo D and apo E. Apo B-100 is the major apolipoprotein found on VLDL and LDL [13] and apo B-48 is found exclusively in chylomicrons [14]. Apart from apo B-100, VLDL also contain apo C-I, C-II, C-III & apo E which are also found in chylomicrons along with apo A-IV. Lipoprotein (a) or Lp (a) is another type of lipoprotein that is found in blood, which is a plasminogen like protein [15].

These apolipoproteins have several functions ranging from providing structural integrity to the lipoprotein assembly to being activators and inhibitors of various enzymes involved in the metabolic processes such as *lecithin: cholesterol acyltransferase* (LCAT), *lipoprotein lipase* (LPL), etc. They also help in activating cell surface receptors like low density lipoprotein receptor (LDL-R) and cellular transporters like ATP binding cassette class A type 1 (ABCA1), which are involved in cholesterol metabolism.

20.2.1 Overview of Lipid and Lipoprotein Metabolism

During lipid metabolism, chylomicrons synthesized by intestine carry triglycerides and cholesterol, which are released during digestion of dietary fat to the liver and peripheral tissue for usage as a source of energy (Fig. 20.3); [14, 16, 17]. After supplying triglycerides to all the cells they reach the liver as remnant particles due to the action of *lipoprotein lipase* (LPL), which is also known as exogenous pathway [18]. The liver utilizes this triglyceride to synthesize VLDL and releases them into circulation. VLDL carries this lipid load to all parts of the body, particularly, to adipose tissue and skeletal muscles for energy requirements and again by the action of LPL, VLDL gets converted into LDL [19, 20]. LDL, being rich in cholesterol with minimum amount of triglycerides further continues the lipid supply to the cells [21, 22]. LDL can also exchange its cholesterol with HDL through a protein called cholesterol ester transfer protein (CETP) for triglycerides [23]. In the cells, LDL is readily taken up through LDL-R [21] and also due to the small size, it readily infiltrates the extracellular space in the blood vessel wall where macrophages takes it up through scavenger receptors [24]. In this way, LDL regulates nearly 70% of the body cholesterol which is considered as the endogenous pathway of regulating cholesterol in the circulation (Fig. 20.3).

High density lipoprotein being the smallest of all lipoproteins is synthesized in liver and intestine [12, 25] and plays a major role in transporting accumulated cholesterol in the peripheral tissues back to the liver where it will be either reused or removed through excretion [26, 27]. HDL also participates in exchanging triglycerides for cholesterol with LDL, which further adds to the benefit of removing excess cholesterol from the body [23]. Thereby, these different lipoproteins regulate and metabolize various lipids obtained from diet in the body (Fig. 20.3).

20.3 Dyslipidemia

Abnormal concentrations of various lipids and lipoproteins in the plasma lead to several pathological conditions known as dyslipidemias. It has been extensively studied and noted that dyslipidemia cause change, either by increasing or decreasing the concentrations of various lipoproteins. Dyslipidemia occurs either due to genetic abnormalities or environmental-lifestyle imbalances [28, 29]. Two major categories exist in dyslipidemia namely (i) hyperlipoproteinemia, wherein lipoprotein levels increases and is again subdivided based on the levels of triglycerides and cholesterol into three categories, namely hypercholesterolemia, hypertriglyceridemia and combined hyperlipidemia; (ii) hypolipoproteinemia, wherein lipoprotein levels decreases and is further divided into hypoalphalipoproteinemia with low levels of LDL cholesterol (LDL-C) and hypobetalipoproteinemia with low levels of HDL cholesterol (HDL-C) [30–32]. In hypercholesterolemia, cholesterol levels in the blood are increased due to increase in LDL-C. This disorder is caused due to a

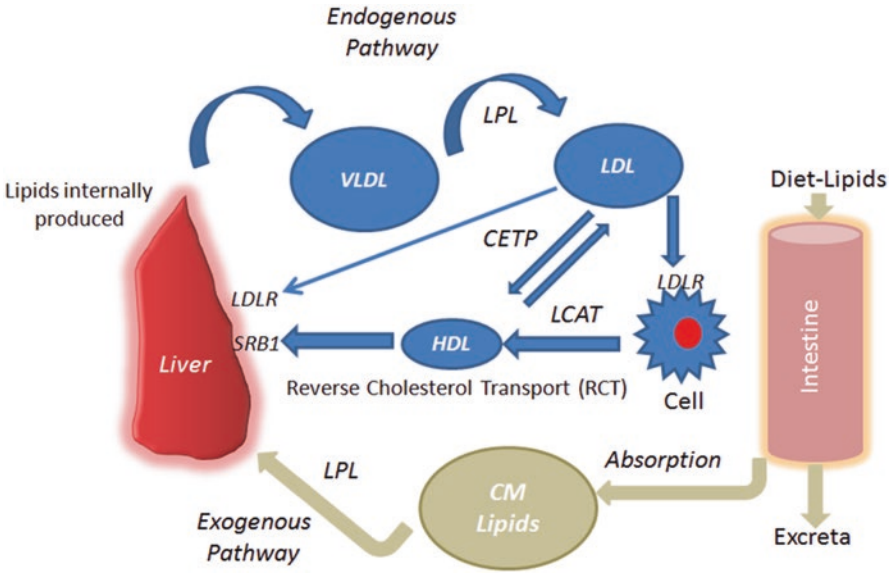

Fig. 20.3 Schematic representation of major lipid metabolic pathways
Lipids from the diet is broken down and absorbed by intestine, which releases it into circulation in the form of chylomicrons (CM). Chylomicrons reach the liver as chylomicron remnant particles after the action of LPL and this is the *exogenous pathway*. Liver synthesizes VLDL particles using lipids obtained from the diet and releases it into circulation, which gets converted to LDL by the action of LPL. This LDL can return back to the liver or taken up by body cells through LDLR for utilization in energy production which is known as *endogenous pathway*. It can also exchange its lipid load with HDL through CETP. HDL interacts with body cells to remove excess lipids with the help of LCAT. Now HDL carries the excess lipids obtained from both LDL and body cells and gives it to the liver through SRB1 and this is known as reverse cholesterol transport (RCT) pathway. *LPL* lipoprotein lipase, *VLDL* very low density lipoprotein, *LDL* low density lipoprotein, *LDLR* LDL receptor, *HDL* high density lipoprotein, *CETP* cholesterol ester transfer protein, *LCAT* lecithin cholesterol acyl transferase and *SRB1* scavenger receptor class B type 1

genetic abnormality, which is termed as familial hypercholesterolemia (FH). Studies have shown that both homozygote and heterozygote individuals synthesize intracellular cholesterol normally, but they lack enough number of active LDL-R resulting in increased LDL-C levels in circulation and deprived cellular cholesterol uptake. Statin treatment in heterozygote individuals showed decreased intracellular cholesterol synthesis and increased production of LDL-R, thereby helping in lowering LDL-C levels in the circulation. In the homozygotic individuals, however, this benefit is not observed due to fact that they lack enough functional LDL-R for cellular uptake of cholesterol [33–35].

Hypertriglyceridemia occurs as a result of imbalance in the synthesis and the clearance of VLDL in circulation [36, 37]. It can occur either due to genetic predisposition or due to hormonal abnormalities in pituitary, adrenal glands and pancreas. Hormones from these organs control blood triglyceride levels by influencing hormone sensitive lipase [38] and in case of severe hypertriglyceridemia it is poten-

tially life threatening due to recurrent acute pancreatitis [31, 39]. Treatment involves low fat diet, use of fish oil and triglyceride lowering drugs primarily fibric acid derivatives [40, 41]. Deficiency of LPL or apo C-II can also lead to hypertriglyceridemia by affecting chylomicrons and VLDL metabolism [42].

Combined hyperlipidemia or hyperlipoproteinemia is characterized by the presence of elevated levels of both triglycerides and total cholesterol. This disease is a result of defective catabolism of chylomicrons remnants and accumulation of cholesterol rich VLDL particles. Genetics also plays an important role and several rare genetic forms of this disease have been identified like familial dysbetalipoproteinemia or type III hyperlipoproteinemia and the presence of a rare form of apo E called apo E2/2 is observed. These patients can be treated with niacin, gemfibrozil, HMG-CoA reductase inhibitors or just low fat diet.

20.3.1 Dyslipidemia and Diabetes Mellitus

Dyslipidemia is associated with several metabolic disorders like diabetes, insulin resistance syndrome, obesity, and coronary heart disease (CHD) particularly atherosclerosis and CVD. Dyslipidemia functions as a connecting bridge in the above mentioned metabolic disorders and those individuals affected with one kind of disorder are susceptible for another one. It has been well documented that patients suffering from type 2 diabetes is more prone for cardiovascular atherosclerosis and these patients exhibit a particular pattern of lipid and lipoprotein abnormality in them wherein elevated levels of small dense LDL particles, hypertriglyceridemia and decreased levels of HDL cholesterol (HDL-C), a condition termed as "atherogenic lipid triad", is observed [43]. In addition, inflammation represents a common link in the above said disorders. Furthermore, insulin resistance, lipotoxicity and hypertension also contribute significantly to the progression of these disorders [44] (Fig. 20.1). Combined effects contribute to the risk of atherogenesis and subsequent myocardial infarction.

One of the hallmark and key pathophysiological feature of type 2 diabetes mellitus and obesity is resistance to the action of insulin in liver, skeletal muscles and adipose tissue [45]. The chronic insulin resistance in diabetic patients often leads to hyperinsulinemia, hyperglycemia, dyslipidemia, changes in lipid and lipoprotein metabolism, hypertension, thereby contributing to chronic inflammation and endothelial dysfunction, thus promoting atherogenesis with increased CVD risks (Fig. 20.1) [45].

Insulin resistance drastically alters lipid metabolism and causes free fatty acid efflux to increase from adipose tissue and it also impairs skeletal muscle uptake thus channelling the excess fatty acids to the liver. This free fatty acid in the form of triglycerides gets deposited in muscles, liver, heart and pancreas [46, 47]. Concentration and size of various lipoprotein subclass particles like VLDL, LDL and HDL are strikingly affected by insulin resistance [48, 49]. Here, activity of lipid metabolizing enzymes like *hepatic lipase* and *lipoprotein lipase* are significantly

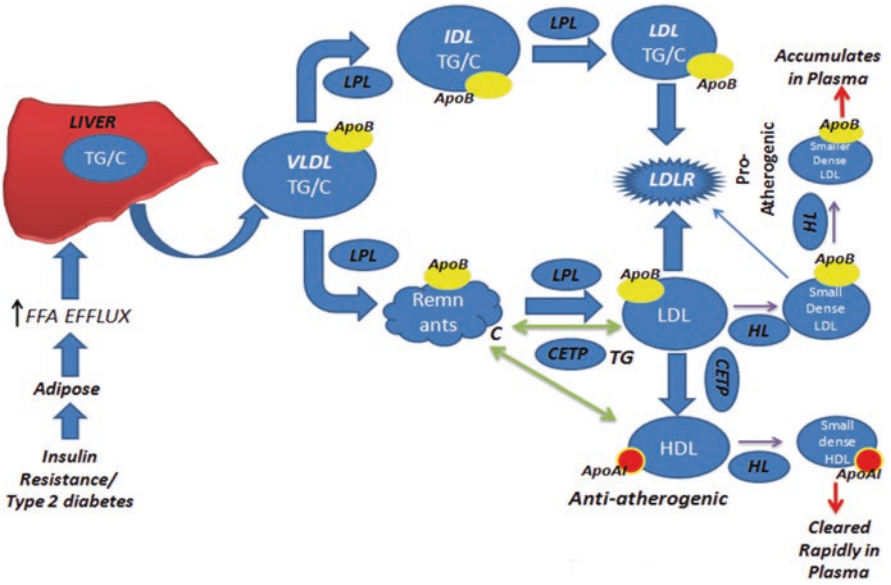

Fig. 20.4 Schematic representation of altered lipoprotein metabolism caused by insulin resistance leading to atherogenic dyslipidemia
Insulin resistance in type 2 diabetes causes increased release of free fatty acid (FFA) from adipose tissue, which is channelized to the liver where it is converted to triglycerides (TG). The liver uses this to produce VLDL along with cholesterol (C). VLDL is metabolized by LPL either to IDL or VLDL remnant accordingly based on TG content which is further converted to LDL by LPL. This LDL is taken up by cells through the LDL receptor (LDLR) for usage in energy requirement. LDL derived from VLDL remnant is large in size and they are further metabolized into small dense and smaller dense LDL particles by *hepatic lipase* (HL). These smaller, denser LDL particles accumulate in plasma due to poor recognition and impaired removal by LDL receptor and become pro-atherogenic. HDL, which is anti-atherogenic in nature constantly exchanges cholesterol for TG through CETP with VLDL remnant and LDL and this TG rich HDL is converted to small, dense HDL by *hepatic lipase* which are rapidly cleared from circulation. *VLDL* very low density lipoprotein, *IDL* intermediate density lipoprotein, *LPL* lipoprotein lipase, *LDL* low density lipoprotein, *CETP* cholesterol ester transfer protein, *ApoB* apolipoprotein B, *ApoA1* apolipoprotein A1 and *HDL* high density lipoprotein

increased eventually hydrolyzing phospholipids in LDL and HDL leading to the formation of small and dense LDL particles (sdLDL). These particles accumulates in blood due to impaired LDL-R recognition and also decreases HDL particles, which are rapidly cleared from circulation [50–52]. These small and dense LDL-particles accelerate the pro-atherogenesis. In contrast, the small and dense HDL-particles which are anti-atherogenic, are cleared from the blood through the renal system (Fig. 20.4). The crucial pathophysiology of the atherogenic dyslipidemia in diabetes is the alteration in triglyceride- rich lipoprotein metabolism like VLDL and chylomicrons. The alteration involves (a) increased hepatic secretion, (b) impaired clearance of VLDL and (c) intestinally derived chylomicron as partially lipolyzed remnant particles; contribute for their prolonged retention in circulation. The

lipolyzed remnants mainly include cholesterol rich IDLs that act as precursors for subsequent production of LDL and sdLDL [53, 54]. Diverse LDL lipoprotein particles (LDL-1, 2a, 2b, 3a, 3b, 4a and 4b) differ in their metabolic behaviour and pathological roles for atherogenesis [55]. In LDL, precursor triglyceride enrichment through the action of CETP and hydrolysis of triglycerides and phospholipids by *hepatic lipase* leads to increased production of sdLDL which are poorly recognized by LDL-R, thereby increasing their plasma residence time. On the other hand, HDL particles that are heterogeneous (HDL- 2a, 2b, 3a, 3b, and 3c) [56] being atheroprotective are decreased in number and their levels inversely correlate with LDL size and density particularly the HDL_2 subclass [57]. HDL level reductions in diabetes and insulin resistance are multifactorial, but a major cause appears to be increased cholesterol transfer for triglycerides from HDL to other lipoproteins which seems to be the reversal of CETP function. Now, these triglyceride rich HDL particles undergo hydrolysis by *hepatic lipase* and are rapidly catabolized in liver and cleared from circulation. In type 2 diabetes the reduced HDL levels represent the levels of HDL_{2b} subspecies which is the key for atheroprotection and relative increase in smaller, denser HDL_{3b} and HDL_{3c} subspecies that are not much involved in the cardioprotection (Fig. 20.4); [58, 59].

The small, dense LDL particles can also permeate through the endothelial cell barrier and can get accumulated in the vascular bed and these LDL particles get oxidized. In addition, the phospholipids present in it also get oxidized. These oxidized phospholipids trigger the activation of the vascular endothelium to produce various cell proinflmmatory adhesion molecules such as VCAM1, ICAM1, E-selectin and recruits leukocytes to the site and promotes atherogenesis. This oxidation is a result of increased levels of the *myeloperoxidase* enzyme secreted by the activated macrophages and neutrophils. This defensive enzyme is known to oxidize cell wall proteins to fight bacterial infection, which here can also oxidize both HDL and LDL particles. As a consequence the oxidized HDL particle is not only rendered dysfunctional but also converted into proatherogenic and proinflammatory particles. These oxidized HDL particles does not remove or transport excess cholesterol accumulated in the peripheral tissue or arteries [60]. In contrast, the oxidized LDL particles promote the formation of foam cells by the accumulation of cholesterol in the macrophages [61]. These foam cells promote inflammation in the vasculature and further worsen the vascular homeostasis [62]. The repeat cascade of events involving the oxidization of both HDL and LDL particles triggers the progression of atherosclerosis resulting in ischemia, myocardial infarction/stroke. These risks are much higher in type II diabetic patients, resulting in death due to selective insulin resistance.

20.3.2 Hyperglycemia and Lipoprotein Glycation

Largely, relationship of hyperglycemia and atherosclerosis remains a topic of debate for both medical and basic science investigators. Despite enormous clinical and research data, the impact of hyperglycemia alone for the acceleration of

atherosclerosis in diabetes remains a challenge. This has been mainly due to the difficulties in differentiating the effects of hyperglycemia and the lipid abnormalities (dyslipidemia), contributing to the pathology of atherosclerosis. Nevertheless, the studies on vascular biology and hyperglycemia reveal a positive correlation [7, 8]. Perhaps, a complex network of events promotes atherosclerosis in diabetics. From the molecular view, hyperglycemia is proposed to work, either directly or in concert with other events which include the formation of Advanced Glycation End products (AGE), oxidative stress and protein kinase C activation [63]. It has been conceptualized that the adverse metabolic events and the changes in the hemodynamic pathway driven by chronic hyperglycemia results in the atherosclerosis event. From above said mechanisms, the formation of glycated products or AGE plays a crucial role in the episode of diabetic complications.

Advanced Glycation is an irreversible modification of proteins, lipids and nucleic acids due to increased levels of glucose circulation in the body. Here, a non-enzymatic reaction (chemical) occurs between an excess of glucose and proteins or lipoproteins, from an initially subtle and reversible reaction to stable Amadori products (also known as AGE). However, the Amadori product further undergoes various modifications with the changes in the redox potential, metal ions and other catalysts, substantially increasing AGE formation. Chronologically, following few factors contribute to the AGE formation and its accumulation *in vivo*; (1) glucose metabolism and accumulation of glucose derived intermediate products, (2) time factor and (3) impaired detoxification of the products. AGE modification occurs in proteins that are generally rich with surface accessible lysine and arginine residues. These two amino acids are the most susceptible site for the modification, resulting in the production of heterogeneous AGE compounds. In general, both conformation and function are challenged, if such modifications occur in the protein. Similarly, AGE-modified lipoproteins alter the topology of lipid particles, resulting in the perturbance of the cardiovascular system [64–66]. Further, the degree of the glycation determines the changes in the lipoprotein's structure-function and the extent of its deleterious effects [63, 67, 68].

AGE affect lipoproteins, modifying them into proatherogenic particles and promotes dyslipidemia. AGE affects both apo B and phospholipids of the LDL. Glycation of the apo B occurs mainly on the lysine residues in the protein. Depending on the degree of glycation, the functions of the LDL particle is altered [69]. Eventually, the modified molecule looses its ability to bind with the LDL-R, due to modification at a putative site. This results in accumulation of defective LDL in the circulation.

For decades, HDL remained an intriguing and interesting molecule due to its diverse role in biological processes, and it is found to be mostly protective in nature [70]. The major protein constituent of HDL is apoAI and generally it is well accepted that it is atheroprotective. However, it is found that the levels of circulating HDL are generally low in diabetics and more over it undergoes variety of modifications in its primary structure [71]. More often, the modified HDL is dysfunctional, becomes proinflmmatory and proatherogenic. Thus, the quantitative and qualitative changes of HDL are gaining importance in understanding the CVD events.

Recent studies have indicated that HDL-apoAI is susceptible to glycation resulting in the loss of its structure-function [71, 72]. Modification of both lipid-free apoAI and lipid bound apoAI with methylglyoxal, one of the AGE products, led to alternation of the structure and function of the apoAI molecule. Report by Nobecourt et al. [73]; describe that arginine, lysine and tryptophan residues are preferably modified by methylglyoxal. The glycation modification of HDL-apoAI is both time and concentration dependent process. Some of the modified sites were found to be the binding site of cholesterol/lipid to apoAI and the activation of LCAT enzyme. Glycated apoAI with carboxy methyl lysine impaired the cholesterol efflux (70%) in THP-1 macrophages and human monocytes through ABCA1 dependent pathway [74]. Yet, another data describes that the apoAI glycation with moderate excess of glucose or reactive glycoaldehydes prevents apoAI binding to phospholipids but does not affect the cholesterol efflux from macrophage cells [75]. Nevertheless, AGE modified apoAI's involvement in plaque progression was observed in a population case study, wherein the modified apoAI was measured in the plaques by densitometry and the activation of serum LCAT [76]. An extensive study on modification of HDL particle and its physiological function and prediction of CVD risk from normal and type 2 diabetes is reported by Godfrey et al. [77]. The authors have isolated HDL, HDL_2 and HDL_3 particles from plasma and quantified for methylglyoxal and dicarbonyl modifications at the molecular level by using the mass spectrometry. The findings explain that the arginine at site 27, 123 and 149 in apoAI is modified and a significant increase in methylglyoxal and dicarbonyl adduct HDL particles were noted in diabetic plasma. This accumulation of adducted HDL is due to obscured metabolism and the formation of discoidal reconstituted HDL particles. Both, AGE and oxidative modification of apoAI, HDL and intermediate HDL particle, changes the overall particle density and size. Thus, the HDL cardio-protective function is impaired and it is converted into pro-atherogenic particles.

20.4 Lipotoxicity

Insulin resistance causes over supply of lipids such as triacylglycerol which are stored in peripheral tissues. The stored triacylglycerols are susceptible for degradation to produce free fatty acids, which enters into circulation. These circulating fatty acids are known to cause lipotoxicity of insulin responsive tissues such as liver, skeletal muscle and adipocytes [78]. In addition, these circulating free fatty acids affect the functioning of endothelium, pancreatic islet cells, cardiomyocytes etc. [79]. The circulating free fatty acids serve as substrates of many other lipids including glycerolipids (monoacyl glycerol, diacylglycerol etc.); phospholipids (membrane lipids such as phosphotidyl choline, phosphotidyl serine, phosphatidyl ethanolamine etc.) and sphingolipids (ceramides, sphingomyelin and glycosphinoglipids). The saturated free fatty acids are often channeled into the synthesis of both glycerolipid diacylglycerol (DAG) and sphingolipid ceramides respectively.

Sphingolipid ceramides are more potent in inhibiting insulin mediated signalling than DAG or by free fatty acids [79, 80].

Ceramides are found to be present in LDL particles [81]. The circulating ceramides are known to impair insulin signalling in insulin sensitive tissues, such as liver, skeletal muscle or adipocytes. Depletion of ceramides packaging in LDL particles has reversed the ill-effects of ceramides on insulin signaling. Ceramides, essentially, prevents the phosphorylation of AKT and the translocation of Glucose Transporter (Glut4) and prevents the uptake of glucose in muscles, liver and adipocytes. In addition Ceramides may also prevent the activation of *endothelial Nitric Oxide Synthase* (eNOS) due to the inhibition of AKT.

Ceramides have been documented to be cardiotoxic and they induce free radical generation. This increases the mitochondrial membrane permeability and decreases ATP production, resulting in dysfunctional mitochondria and ultimately affecting the functioning of cardiomyocytes [82]. Ceramides, may also contribute to cardiac dysfunction in both obesity and diabetic related cardiomyopathy through promotion of apoptosis, ER stress and insulin mediated signalling [83].

20.5 Endothelial Dysfunction

Vascular endothelium, which supplies blood, oxygen, and various essential requirements for normal cellular homeostasis is a dynamic tissue by itself. It releases a number of bioactive molecules such as nitric oxide [84] cardio protective lipids such as sphingosine 1-phosphate [85], which are involved in vascular homeostasis. In addition, they secrete vasoconstrictors such as endothelin, angiotensin II and generate ROS in response to inflammation. Now, it has been well accepted that the dynamic balance between vasoactive molecules which could be either vasoconstrictors or vasodilators determines the fate of vascular homeostasis between healthy *vs* atherogenesis and its pathophysiological consequences.

Nitric oxide produced by vascular endothelium during conversion of L-arginine to L-cirtulline, releases gaseous molecule involved in the relaxation of smooth muscles and maintains the vascular tone and elasticity of the vascular endothelium. Nitric oxide produced by vascular endothelial cells in response to various signalling events and blood flow is considered as beneficial for the cardio-vascular system. Nitric oxide produced by vascular endothelium is known to be cardioprotective and acts as a vasorelaxant. Additionally, it contributes to the anti-inflammatory, antioxidant and anti-proliferative effects. It is also known to prevent the coagulation of blood leading to thrombosis [86].

Similar to the unusual accumulation of the lipids in peripheral tissues, the blood vessel, especially arteries also exhibit lipid accumulation. The building up of excessive lipids leads to the formation of plaques and the continued accumulation of lipids with cells such as monocytes and macrophages elicits inflammatory responses in vascular endothelium. In addition, there is generation of oxidized lipids and oxidized lipoproteins which further worsens the proinflammatory responses

from vascular endothelium with a concomitant reduction in the availability of nitric oxide produced by vascular endothelium. The increased generation of FFA and its elevation in the circulation also activates NADPH oxidase and impairs mitochondrial electron transport chain to produce excessive ROS which could essentially trigger the cascade of events promoting atherogenesis. The increased FFA levels also impair NO production by blunting phosphorylation of *endothelial Nitric Oxide Synthase* (eNOS). As described earlier, ceramides and DAG may also directly interfere with functioning of IRS, AKT signaling which is involved in the phosphorylation of eNOS to generate a burst in the NO. Thus, this provides a basis for the inflammation and vascular dysfunction. Moreover, free fatty acid also promotes proinflammatory TLR-4 signalling which further promotes the inflammation of vascular endothelium, insulin resistance and impaired NO signalling, together contributes to the progression of atherosclerosis. This sequence of events severely abrogates the beneficial effects of nitric oxide with the formation and progression of atheroma leading to thrombosis, blood clotting, and myocardial infarction/stroke.

During the onset of atherosclerosis, which arises as a consequence of lipid deposition, not only attracts macrophages to site of deposit, but also triggers chronic inflammation both from vascular endothelium and from macrophages. This inflammatory reaction leads to the vicious cycle of production of various proinflammatory cytokines such as TNFα, IL-6 which promotes the progression of atherosclerosis by building plaques. In addition, the generation of free radicals in turn decreases either the generation or its bioavailability. All these factors contribute collectively to the progression of the atherosclerotic plaque, pathogenesis, vasoconstriction, leukocyte adhesion, platelet activation, mitogenesis, oxidation, thrombosis, and blood coagulation leading ultimately to CAD and further leading to either severe morbidity or mortality in individuals [84].

20.6 Conclusions and Future Perspective

Based on the large body data emanating from various basic science and clinical laboratories, this review has focused on providing the mechanistic basis for the increased incidence of CVD risks in diabetic patients with special reference to dyslipidemia i.e., changes in lipids and lipoprotein metabolism. Furthermore, how these changes increase the vulnerability of diabetic patients for CVD risks is discussed. For instance, how (i) accumulation of pro-atherogenic small dense LDL particles rich in cholesterol (ii) clearance of anti-atherogenic HDL particles (iii) accumulation of triglycerides in peripheral tissues (iv) lipotoxicity due to increased levels of circulating free fatty acids and its effect on insulin resistance and insulin signalling (v) formation and accumulation of dysfunctional lipoprotein particles as a result of non-enzymatic glycation of LDL-apoB and HDL-apoAI through advanced glycation end products (vi) oxidation of phospholipids and HDL particles, triggers proinflmmatory responses leading to endothelial dysfunction. Although, there is lot of literature, here we could provide only a bird's eye view on the

burgeoning problem of diabetes. Certainly there has been increased understanding of the basis of CVD risks in diabetic patients, however much more studies are needed for better understanding the qualitative changes occurring in lipids and lipoproteins and its consequences. Use of modern technologies such as mass spectrometry, imaging technologies coupled with cell based assays and *in vivo* experiments in animal models and correlation with human clinical data should further aid in better understanding the pathogenesis of CVD in diabetes. Importantly, these studies should pave way for developing cost effective, high throughput diagnostic and prognostic tools for use in clinical settings and identify newer targets to provide better treatment options for patients who suffer due to diabetes, obesity and CVD.

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Chapter 21
Vascular Remodeling: Homocysteine and Diabetes

Pankaj Chaturvedi

Abstract Remodeling of the vascular wall contributes to hypertension in humans owing to changes in vessel diameter and thickening of intimal and medial layers of the vascular wall promoting total peripheral resistance. This chapter describes the mechanisms that contribute to the pathogenesis of arterial remodeling. It also highlights endothelial dysfunction observed in arteries of hypertensive subjects and role of elevated homocysteine in facilitating peripheral vascular remodeling. The chapter also describes metabolism of homocysteine and how it functions as an independent risk factor of atherothrombotic complications of vascular disease.

Keywords Vascular remodeling • Homocysteine • Hypertension • Atherosclerosis • Thrombosis • Vascular disease • Endothelial dysfunction

21.1 Introduction

Arterial remodeling is the form of vessel wall adaptation to mechanical and hemodynamic stimuli, characterized by structural and functional changes of the vascular wall mediated by different mechanisms (hyperplasia of the arterial intima and media, redistribution of extracellular matrix components such as collagen and elastin, fibrosis, arterial calcification and endothelial dysfunction). Structural reorganization of the vascular wall architecture facilitates elevation of resistance to blood flow, contributing to amplification of total peripheral resistance that is the hallmark of hypertension [1]. Arterial remodeling is classified based on changes in luminal diameter: outward remodeling (increased vessel diameter) and inward remodeling (decreased vessel diameter), and based on changes in wall thickness: hypertrophic (thickening of the vessel wall), eutrophic (constant wall thickness) and hypotrophic

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(thinning of the vascular wall) [2]. Outward hypertrophic remodeling is characterized by increased vessel diameter and thickening of intimal and medial layers of the vascular wall promoting stiffness of the large central elastic arteries that occurs in hypertension [1]. However, muscular peripheral arteries develop one of two types of arterial remodeling: inward eutrophic or inward hypertrophic remodeling. In the first type of remodeling, that is attributed to essential hypertension in humans and is also found in spontaneously hypertensive rat model, the lumen is reduced and the wall thickness is constant and similar to that of normotensive individuals [1]. The second type or inward hypertrophic remodeling is developed in secondary hypertension (renovascular hypertension, pheochromocytoma, diabetic, salt-dependent and mineralocorticoid hypertension) and is characterized by reduced lumen and enlarged media cross-sectional area [1].

21.2 Pathogenesis of Arterial Remodeling

Arterial remodeling occurs due to the complex of mechanisms (smooth muscle cell proliferation and differentiation, elastin degradation and collagen deposition, arterial calcification and endothelial dysfunction) that mediate reorganization of all layers of the vascular wall [2]. Tunica media that is comprised of smooth muscle cells (SMC) plays a major role in regulation of vascular tone and diameter through smooth muscle contraction and relaxation. SMC content in the tunica media raises up to 85% with decrease in vessel diameter. In contrast to large central elastic arteries, SMC of the small arteries circumferentially arranged with a pitch angle smaller than 2°. Such an arrangement of SMC allows optimal resistance against vessel distention [3]. Under normal condition, SMC have contractile phenotype and express respective SMC proteins: smooth muscle 22- alpha (SM22α), alpha- smooth muscle actin (αSMa) and smoothelin [4, 5]. However, studies have shown that in vascular injury or stress contractile SMC undergo phenotype switching, where SMC differentiate into synthetic phenotype that further gives two classes of SMC (migratory- proliferative or osteogenic phenotypes) [2]. Inflammation, oxidative stress, mechanical stretch, angiotensin II, transforming growth factor-β (TGF-β), matrix metalloproteinases (MMPs) are the major stimuli for vascular smooth muscle cells (VSMC) phenotype switching. Synthetic VSMC secrete MMPs allowing the migration of SMC into intima by detaching cells from the basement membrane and extracellular matrix (ECM) [6]. The migrated SMC are involved in proliferation and hyperplasia of intima promoting thickening of the arterial wall. High extracellular levels of calcium and phosphate and the absence of inhibitors of calcification allow VSMC differentiation into osteogenic phenotype, where VSMC have the features attributed to osteoblasts or chondrocytes [7]. Osteogenic phenotype decreases the expression of SMC markers and induces intense calcification of elastic fibers in vascular wall.

The next event that contributes to arterial vascular remodeling is redistribution of ECM proteins in the vascular wall. ECM occupies more than half of the vascular wall mass and contains elastin, collagen, fibronectin, fibrillins, proteoglycans and

leucine-rich glycoproteins [8]. ECM maintains vascular function under normal and pathophysiological conditions. Interaction of vascular wall cells with ECM regulates cell migration, adhesion, proliferation and phenotype. Integrins are the ECM receptors that are engaged by cells to sense the ECM content change that is involved in tissue remodeling.

Elastin is mainly synthesized by SMC, however endothelial cells and adventitial fibroblasts are able to produce tropoelastin. Large resistance arteries contain internal and external elastic membranes and some elastic fibers located between smooth muscle cell fibers. In contrast to large elastic arteries, elastin of small arteries and arterioles is only limited to internal elastic membrane. Large central elastic arteries contain significant amount of elastin (111 mg/g in the rat carotid artery) assisting in mitigation of pressure pulsations. The amount of elastin reduces with a decrease in vessel diameter (15 mg/g in small mesenteric arteries) [3].

Collagen is a protein that is in a high content provides arterial stiffness and limits arterial compliance. Collagen distribution reduces with a decrease of vessel caliber (e.g. from 124 mg/g in carotid artery to 67 mg/g in mesenteric arteries). Collagen vascular wall content reduces towards periphery from 20% to 9% of the wall volume over the mesenteric vasculature [3]. Collagen I and III are expressed in media and highly present in adventitia. The basement membrane is comprised of collagen IV.

The elastic fibers of the large elastic arteries provide an adequate arterial compliance during systole. With aging, elastic laminae fragmentation process occurs, redistributing mechanical load to collagen fibers that are stiffer by nature [9]. Such impairments in vascular wall amplify systolic and pulse pressures that provoke hypertension. Arterial remodeling in hypertension is characterized by increase of media/lumen ratio with or without wall thickening (hypertrophic, eutrophic) due to redistribution of SMCs or ECM proteins [10, 11]. Arterial wall thickening occurs due to elastin degradation and collagen deposition. It reduces arterial compliance and amplifies arterial fibrosis limiting distention of the vascular wall. Therefore, pressure elevation is required to surpass arterial wall stiffness.

Endothelial dysfunction is often, but not always, observed in arteries of hypertensive subjects and plays a significant role in pathogenesis of arterial remodeling [1]. Changes in blood flow and shear stress stimulate nitric oxide (NO) release from endothelial cells (EC) that produce relaxation of VSMC and vasodilation. EC layer damage facilitates impairments in endothelium- dependent vasodilation due to the loss of NO production. In oxidative stress, the presence of reactive oxygen species (ROS) decrease NO bioavailability due to the formation of peroxynitrite that further exacerbates oxidative environment and EC injury [12]. In inflammation, EC produce cytokines [13] and growth factors: monocyte chemotactic protein (MCP-1), TGF-β, C-reactive protein, plasminogen activator inhibitor (PAI-1). They facilitate EC, VSMC, and vascular pericytes proliferation. Altered morphology with disruption of endothelial layer integrity, impaired vasodilation and inadequate vasoconstriction significantly affect vascular tone contributing to pathogenesis of hypertension [14]. Previously, we have described that homocysteine elevation facilitates endothelial cell injury and peripheral vascular remodeling with collagen accumulation in the superior mesenteric artery [15]. Several other reports have con-

firmed endothelial cell injury, vascular endothelial dysfunction and vascular remodeling in hyperhomocysteinemia that contribute to pathogenesis of hypertension [16–18].

21.3 Homocysteine

21.3.1 Homocysteine Metabolism

Homocysteine (Hcy) is a non-protein-coding α-amino acid that is synthesized from dietary protein-derived methionine. Hcy is circulated in plasma in four forms: about 1% is presented as the free thiol, 70–80% of circulated Hcy is bound to plasma proteins (albumin), the rest of 20–30% of Hcy is bound to itself to form Hcy dimers or combined with other thiols including cysteine forming Hcy-cysteine mixed disulfide [19]. The term "total plasma (or serum) Hcy" (tHcy) is determined as all four circulating forms of Hcy combined together [19].

Within the cells methionine converts to S-adenosylmethionine that is a methyl group donor and is essential for various methylation reactions involving DNA, amino acids and proteins [20]. S-adenosylhomocysteine is formed by the methyl group donation of SAM to various substrates [20]. The excess of Hcy in plasma is cleared via two essential pathways: remethylation and transsulfuration pathways (Fig. 21.1). In remethylation pathway Hcy is converted back to methionine with implication of two different enzymes: cobalamin-dependent methionine synthase (MS) that is expressed in all tissues at very low levels and betaine-homocysteine methyltransferase (BHMT) that is specific for kidney and liver and is produced at the very high levels [21]. The first enzyme utilizes cobalamin (B_{12}) as a cofactor and uses 5-methyltetrahydrofolate (5-MTHF) as the methyl donor. 5-MTHF or active form of folate (B_9) is synthesized from 5, 10-methylenetetrahydrofolate by methylenetetrahydrofolate reductase (MTHFR). Betaine-homocysteine methyltransferase (BHMT) that is only expressed in the liver and kidney and is cobalamin-independent enzyme uses betaine as methyl donor to remethylateHcy back to methionine [22]. In transsulfuration that occurs only in the small intestine, liver, pancreas and kidney a cofactor- vitamin B_6 is required to convert Hcy to cystathionine by cystathionine β- synthase (CBS) [23]. Cystathionine is hydrolyzed by vitamin B_6- dependent cystathionine gamma-lyase (CSE) to cysteine that is used as a precursor for synthesis of antioxidant- glutathione or vasodilator-hydrogen sulfide [23]. Nutritional deficiencies in vitamin cofactors (B_{12}, B_9, and B_6) and mutations in MTHFR, CBS and CSE enzymes are the common causes of HHcy.

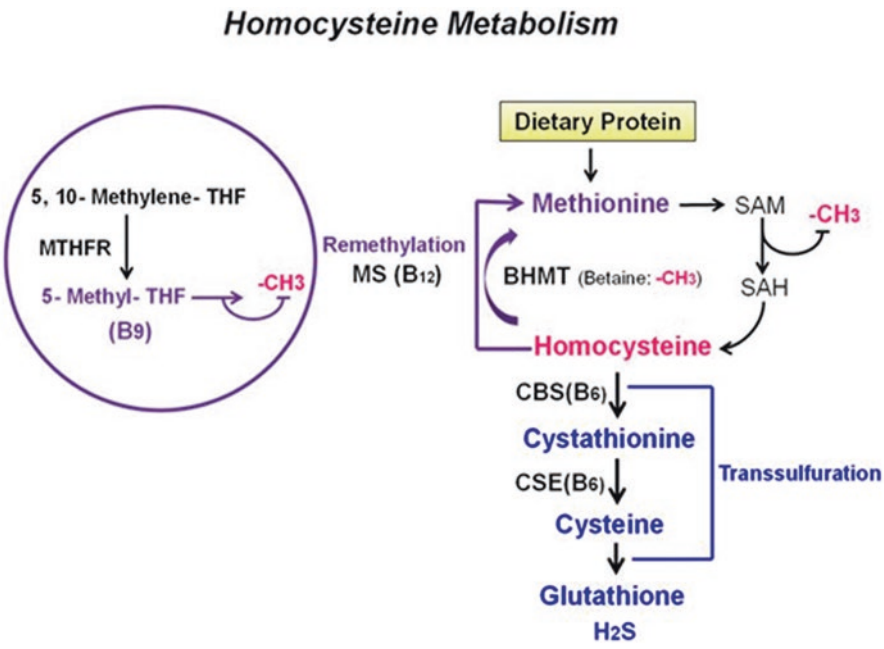

Fig. 21.1 Schematic representation of homocysteine metabolism

21.4 Hyper Homocysteinemia

21.4.1 Epidemiology and Etiology of Hyperhomocysteinemia

The 'normal' range for plasma tHcy is about 5–15 uM/L and hyperhomocysteinemia (HHcy) is defined as an elevation of plasma tHcy level above 15uM/L. HHcy is classified into moderate (16–30 uM/L), intermediate (31–100 uM/L) and severe (more than 100 uM/L) HHcy [19].

Studies have shown that the prevalence of HHcy varies in different countries and is estimated to be 6% in the Costa Rican population, 5% in the United States, 77% in India, 27.5% in China and similar to that estimate in Brazil and Lebanon [24, 25]. Patients with symptomatic atherosclerotic vascular disease have shown 13–47% of HHcy prevalence. Several studies have shown the elevation of tHcy plasma level with age due to age-associated decline in the activity of enzymes that are involved in Hcy clearance and reduction in renal function [26, 27]. The prevalence of HHcy is significantly higher in men than in women that could be explained by the difference in muscle mass, lifestyle, vitamin intake and sex hormones. A clinical study showed that four-month treatment with ethinyl estradiol of male-to-female transsexulas significantly reduced the Hcy plasma levels, however female-to-male transsexuals who received androgen therapy expressed high plasma levels of Hcy [28]. In addition, another clinical study confirmed the beneficial effect of estrogen

replacement therapy in reduction of Hcy plasma levels in postmenopausal women [29]. A significant amount of studies have observed a strong positive correlation between smoking, alcohol and coffee consumption and serum tHcy concentrations [24, 30, 31]. Vermaak et al. have shown that cigarette smokers have significantly lower levels of pyridoxal phosphate that contributes to Hcy metabolism compared to non-smokers [32].

Genetic defects in enzymes that are involved in Hcy metabolism are significant etiological factors that contribute to HHcy. The most common genetic disorder that causes severe HHcy and homocysteinuria is homozygous deficiency of CBS enzyme that is inherited as an autosomal recessive trait and characterized by 40-fold elevation of fasting tHcy [19]. It occurs in 1 in 100,000 live birth and defined by specific phenotype that includes lens disposition, bone impairments, intellectual disability and premature atherosclerosis [33]. There are 60 described CBS enzyme mutations with I278T and G307S are being the most common types [34]. The less common cases of severe HHcy are genetic mutation in MTHFR, MS enzymes and genetic disorders in cobalamin metabolism [35]. The most prevalent type of genetic enzyme disorder that has been associated with moderate increase of plasma tHcy levels is a single point mutation at nucleotide 677 (C-to-T substitution) in MTHFR gene that causes about 50% decline in enzyme activity [36]. There is about 10–13% of prevalence for this specific type of MTHFR mutation (TT genotype) in white population [37]. Nutritional deficiencies in vitamin cofactors such as folate (B_9), cobalamin (B_{12}) and pyridoxal phosphate (B_6) contribute to the development of HHcy [38]. It has been reported that about two-thirds of HHcy is due to low blood concentrations of mentioned vitamin co-factors [39]. Other case that could affect tHcy plasma levels is kidney dysfunction. Clinical studies have shown that patients with chronic renal diseases have elevated tHcy levels due to the impairments in Hcy clearance by renal enzymes [40].

Epidemiological studies have described HHcy as an independent risk factor of atherothrombotic vascular disease complications of which include stroke, myocardial infarction, peripheral vascular disease, miscarriage, pulmonary embolism and coronary heart disease [41, 42]. Several mechanisms have been implicated in pathogenesis of HHcy-induced vascular disease: reactive oxygen species activation, damage of vascular endothelium followed by endothelial dysfunction and promotion of atherosclerosis, impairment in the process of thrombolysis and hypercoagulation [43–45]. Sengwayo et al. have reported that Hcy elevation was significantly associated with increase in both systolic and diastolic blood pressure [42]. It has been implicated that the possible mechanisms that are involved in HHcy- mediated hypertension could be ROS- induced vascular endothelium damage that causes impairment in vasodilation, vascular remodeling with elastin degradation and collagen deposition and VSMC hyper- proliferation [42, 46]. A significant amount of other studies have also shown a positive correlation between Hcy plasma levels and hypertension [47–53]. HHcy has been implicated to be involved in the initiation of vascular inflammation as one of the key mechanisms that contributes to the development of arterial vascular disease predisposing to hypertension [54–57].

21.5 Diabetes

Diabetes is associated with increased risk of renal dysfunction, cardiovascular disease and myopathy due to intense vascular remodeling. The vascular complications observed in diabetes involve altered production of vasoactive substances and superoxide, modification of basement membranes and widespread endothelial dysfunction [58]. Vascular remodeling results in a thickened arterial wall along with reduced lumen which results in increased vascular resistance and hypertension leading to the hypertrophic remodeling in the sub-cutaneous arteries. This further leads to the development of microangiopathy of small vessels.in diabetes the process of arteriogenesis is severely affected that involves growing of collateral arteries to restore blood supply in the ischemic area [59, 60]. The formation of collateral arteries involves recruitment of circulating angiogenic cells (CAC-monocyte-macropahge subsets), that have a strong angiogenic response. The CACs are capable of promoting neovascularization in vivo and are important regulators of wound healing and tissue regeneration [61]. Studies have shown that there is decrease in the number of CACs in type II diabetes and their angiogenic potential is also lowered. In diabetes CACs have reduced proliferation rate, display defective adhesion to endothelium and have less potential to create new vascular structures [62–64]. To find a treatment or therapeutic application to diabetic vascular remodeling, it is important to understand the signaling pathways that promote the proliferation and improvement of CACs.

21.6 Vascular Remodeling: Related Pathways

The revascularization process after ischemia is controlled by HIF1-α (Hypoxia inducible factor-α). In hyperglycemia, there is down regulation of HIF1-α which is accountable for decreased collateral growth triggered by myocardial ischemia in diabetic patients [65].Polymorphism in HIF1-α has been found to be associated with type 2 diabetes mellitus and absence of coronary collaterals in patients with ischemic heart disease [66, 67]. In a study on MI in rats, the infarct size increases in response to hyperglycemia along with reduced production of HIF1-α. Apart from HIF1-α, VEGF (vascular endothelial growth factor) is another factor responsible for vascular remodeling. In patients with diabetes mellitus, both VEGF and its receptor VEGFR are downregulated [68]. In diabetic rats also, the mRNA and protein levels of VEGF-A and its receptor VEGF-R1/R2 are decreased [69]. Lower level of VEGFA in non-obese diabetic mice, are associated with reduction in neovascularization. If no-obese diabetic mice are treated with intramuscular injection of VEGF, normal levels of neovascularization can be achieved.

21.7 Conclusions

Experimental studies have demonstrated that elevated plasma Hcy is a risk factor for the peripheral arterial disease and hypertension. However, the precise mechanism of detrimental Hcy interaction with vascular wall is incompletely understood. In this book chapter we have described different aspects of Hcy action in the development of peripheral vascular remodeling that is a hallmark of hypertension. Diabetes also leads to vascular remodeling due to defective endothelium and abnormal VEGF and HIF-1α.

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Part VI
Pharmacological Therapies

Chapter 22
Mechanisms of Action of Drugs for Treating Endothelial Dysfunction in Diabetes Mellitus

Hina L. Nizami and Sanjay K. Banerjee

Abstract Endothelium is the thin cellular layer lining all the blood vessels, and responsible for regulation of vascular tone, clotting, tissue perfusion, healing and repair. Being in direct contact with blood, it is the primary target of any abnormality, such as hyperglycemia-induced endothelial dysfunction in case of diabetes mellitus. Hyperglycemia and insulin resistance in diabetes cause endothelial injury through diverse mechanisms such as oxidative stress, inflammation and impairment of nitric oxide bioavailability, all of which lead to compromised endothelium-mediated vascular reactivity. Therapeutic management of endothelial dysfunction in diabetes is necessary in order to prevent and treat the resulting microvascular and macrovascular complications such as neuropathy, nephropathy, retinopathy, cardiomyopathy and atherosclerosis. Drug classes used to prevent or treat endothelial dysfunction in diabetes include glucose-lowering drugs, insulin-sensitizers, lipid-lowering drugs, renin angiotensin system-blockers, nitric oxide synthesis modulators, antioxidants, anti-inflammatory drugs and ion channel modulators (discussed in detail). Therapies that show promise as future interventions include vitamin D or its analogues, TRP channel modulators, ivabradine and others. Also, nutritional therapies such as probiotics are being speculated as promising future therapies for diabetes-linked endothelial dysfunction. Though the presence of multiple pathogenic mechanisms in diabetes ensures a large number of drug targets, it also introduces redundancy in signalling pathways and hence the need for poly-pharmacy. Drugs with multiple targets or mechanisms of action hold promise as future therapies of endothelial dysfunction in diabetes.

Keywords Endothelial dysfunction • Diabetes • Inflammation • Insulin resistance • Oxidative stress • Renin-angiotensin system • Nitric oxide • Vascular complication • Metabolic memory

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22.1 Introduction

Human body is a complex machine that involves fine co-ordination of different organ systems to maintain normal physiology. While the organs and tissues differ in structure and function, they rely on the same source for oxygen and nutrient supply, which is blood. For blood to perfuse every tissue, the body consists of a vast and intricate network of blood vessels. Adequate perfusion with blood is essential for any tissue or organ to derive fuel for energy-production and subsequent metabolic function. Inadequacy of perfusion manifests as ischemic-reperfusion injury in various organs such as heart, brain and kidney. Apart from these acute manifestations, slow-progressing impairment in perfusion of a tissue causes organ injury and dysfunction, as in case of chronic diseases such as diabetes [1].

The blood vessels in our body are composed of three layers of tissue i.e. fibrous tissue, smooth muscle and the endothelium. While the fibrous tissue and the smooth muscle maintain the structure and contractibility of the blood vessels respectively, the innermost lining formed by endothelial cells regulates a variety of functions such as blood clotting, blood pressure regulation and leukocyte-endothelium adhesion [2]. Since the endothelium is in immediate contact with blood, any abnormalities caused by diseases, both communicable as well as non-communicable, first affect endothelial layer. These abnormalities stimulate cellular signalling cascades in endothelial cells as well as in adjacent vascular smooth muscle cells and target organs, which form the pathological basis of injury to affected organs [2]. Hence, attenuation of endothelial dysfunction is an attractive premise to tackle target organ dysfunction in chronic diseases.

22.2 Endothelium and Vascular Function

Endothelium is a thin layer of cells lining all the blood and lymphatic vessels in the body, however small or large. Earlier thought to be merely a selective barrier between the blood and the body tissues, endothelium has acquired importance as an important regulator of vascular function, from control of vasomotor tone, to complex biochemical cascades like inflammation and atherogenesis. The adaptability of endothelial cells facilitates tissue healing, repair and remodelling. While the thickness and structure of the connective tissue and muscle layer of blood vessels varies from one type to the other, the endothelial layer is invariably found as a thin layer lining all the blood vessels [3]. A number of regulatory molecules released by the endothelium aid in its autocrine as well as paracrine functions, such as regulation of vascular tone, coagulation cascade, vascular growth and remodelling, angiogenesis etc. The maintenance of these functions relies heavily on the tight equilibrium between these regulatory molecules secreted by the healthy endothelium.

22.3 Endothelial Dysfunction: Triggers and Markers

Any disturbance to the fine co-ordination between the sets of molecules secreted by the endothelium leads to endothelial dysfunction. This could be triggered by communicable as well as non-communicable diseases. In case of communicable diseases, exposure to disease causing-organisms, antigens or toxins triggers immune response marked by secretion of inflammatory cytokines and chemoattractant molecules, promoting leukocyte adhesion [4]. Non-communicable diseases such as hypertension, diabetes and dylipidemia also cause endothelial dysfunction. While hypertension causes shear stress and injury to the endothelium, diabetes and dyslipidemia trigger endothelial damage due to abnormal/excess presence of nutrients/metabolites such as glucose and lipids respectively [4]. Diabetes, with the consistent increase in prevalence, has been recognised as an important target for therapeutic intervention in endothelial dysfunction-mediated organ damage such as neuropathy, nephropathy, cardiomyopathy and retinopathy [5]. While impairment of nitric oxide bioavailability and reduced endothelium-mediated vasorelaxation are hallmarks of endothelial dysfunction, other features include impaired haemodynamics, abnormal coagulation, inflammation, oxidative stress and enhanced permeability of cell layer [6].

22.4 Diabetes and Its Impact on the Endothelium

Diabetes mellitus is a metabolic disorder characterised by high blood glucose levels, either due to insufficient production of insulin or due to resistance of tissues to taking up glucose even in presence of insulin, and is reaching pandemic proportions in prevalence [5]. The global prevalence of diabetes has blown up from 30 million in 1985 to 415 million in 2015. This can be simply explained as every 1 in 10 adults being affected with diabetes currently, with every 1 in 2 adults suffering with undiagnosed diabetes [7]. Apart from worsening of epidemiological indicators such as morbidity and mortality in diabetic patients, the healthcare systems and economy are the indirect sufferers due to healthcare burden. Cardiovascular complications of diabetes form the bigger part of all its complications, followed by damage to almost every organ of the body, including brain, liver and kidney. The pathogenic mechanism common to all these complications is the damage caused by persistent hyperglycemia to the microvasculature, resulting in endothelial dysfunction (Fig. 22.1) [8].

Diabetes and insulin resistance have been linked with endothelial dysfunction, which is not just an epiphenomenon of disease pathology, but may precede the onset of full-blown disease by several years [8]. Because of the extensive crosstalk between nitric oxide signalling and insulin signalling, impairment in nitric oxide pathway is inevitable in diabetes. Diabetes-induced hyperglycemia leads to decreased production of NO, inactivation of NO by oxygen-derived free radicals,

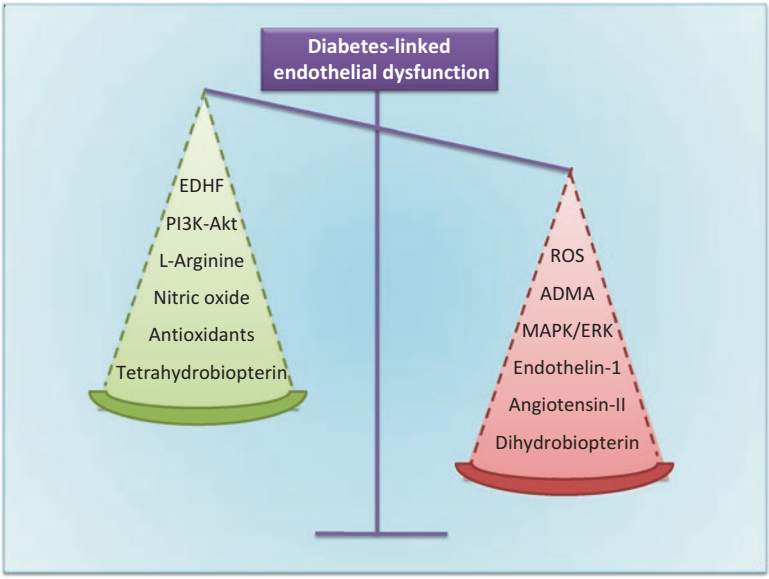

Fig. 22.1 Molecular imbalance in diabetes-linked endothelial dysfunction
Diabetes-linked hyperglycemia and insulin resistance disturb the critical balance between the mediators of endothelial function, favouring the oxidant, pro-inflammatory, prothrombotic and vasoconstrictor species over antioxidant, anti-inflammatory, anti-thrombotic and vasodilator species. (*EDHF* Endothelium-Derived Hyperpolarising factor, *PI3K* Phosphoinositide 3-kinase, *ROS* Reactive Oxygen Species, *ADMA* Asymmetric Dimethyl Arginine, *MAPK* Mitogen-Activated Protein Kinase, *ERK* Extracellular signal-Regulated Kinase)

and/or increased production of endothelium-derived contracting factors, all of which contribute to endothelial dysfunction [9].

Keeping in view the associated macro- and microvascular complications, diabetes is now considered not just a metabolic disorder, but also a disease of the vasculature. Apart from hyperglycemia-induced activation of alternate metabolic pathways such as polyol pathway, advanced glycation end-product formation, PKC activation and hexosamine pathway, diabetes also induces low-grade systemic inflammation through NF-KB, the master-regulator of inflammatory and proatherosclerotic genes [10]. Oxidative stress is believed to unify all these mechanisms, and cause endothelial damage and atherogenic changes. Though glycemic control has been shown to have a beneficial effect on macrovascular complications as well as microvascular complications, search for other promising interventions for diabetes-induced damage in the vasculature attracts significant healthcare spending and efforts [11].

22.5 Diagnosis of Endothelial Dysfunction

Since the hallmark of endothelial dysfunction is impaired NO availability in vascular beds, nitric oxide levels would be an ideal biomarker for assessment of endothelial function. However, since NO is a volatile molecule with a short half-life, it is measured indirectly in the form of its products such as nitrate. In terms of functional assessment, downstream effects viz. the vasodilatation induced by NO production can be studied using various methods. These methods involve assessment of vasodilatation following mechanical (shear stress) or pharmacological (acetyl choline) or mixed induction of NO release [12]. The resulting changes in endothelium can be assessed using quantitative angiography in coronary circulation, intravascular ultrasound in microcirculation, and venous plethysmography in peripheral circulation. However, these methods are limited by their invasive nature, and have been replaced by flow-mediated dilatation. It is a non-invasive method involving ultrasound analysis of brachial artery diameter following 5-min forearm ischemia. Reactive hyperemia-peripheral arterial tonometry (RH-PAT) is another non-invasive, quantitative clinical test used for the evaluation of peripheral endothelial function [13]. RH-PAT, as well as brachial artery flow mediated dilation (FMD), use reactive hyperemia after forearm occlusion as a trigger to detect endothelium-dependent vasodilation. While FMD represents conduit artery vasodilation, RH-PAT represents microvessel vasodilation [13]. However, the use of any of these methods to study endothelial dysfunction in a region warrants caution while interpretation of data, and its correlation with endothelial function in other vascular beds.

22.6 Therapy of Diabetes-Associated Endothelial Dysfunction: Underlying Mechanisms

Endothelial dysfunction is the chief and early culprit behind the macro-vascular and micro-vascular complications of diabetes; hence the need for therapeutic management as early as possible during disease progression is paramount. Glycemic control and enhanced insulin-sensitivity through existing anti-diabetic drugs such as metformin, pioglitazone and others has been shown to improve endothelial function in both animal as well as human studies. Also, drugs used to treat cardiovascular complications such as ACE inhibitors, angiotensin receptor blockers, renin inhibitors, and statins show beneficial response in endothelial dysfunction, sometimes by showing effects different from their primary mechanism of action. These include inhibition of NADPH oxidase (oxidative stress) and activation of GTP cyclohydrolase (*de novo* synthesis of eNOS co-factor BH4) [10]. Apart from these, experimental compounds acting against pathological mechanisms such as AGE formation, PKC activation and protein glycosylation have also shown promise against endothelial dysfunction in experimental studies [10]. These mechanisms and drug targets have been summarised in a schematic diagram (Fig. 22.2). Currently, considerable focus is on

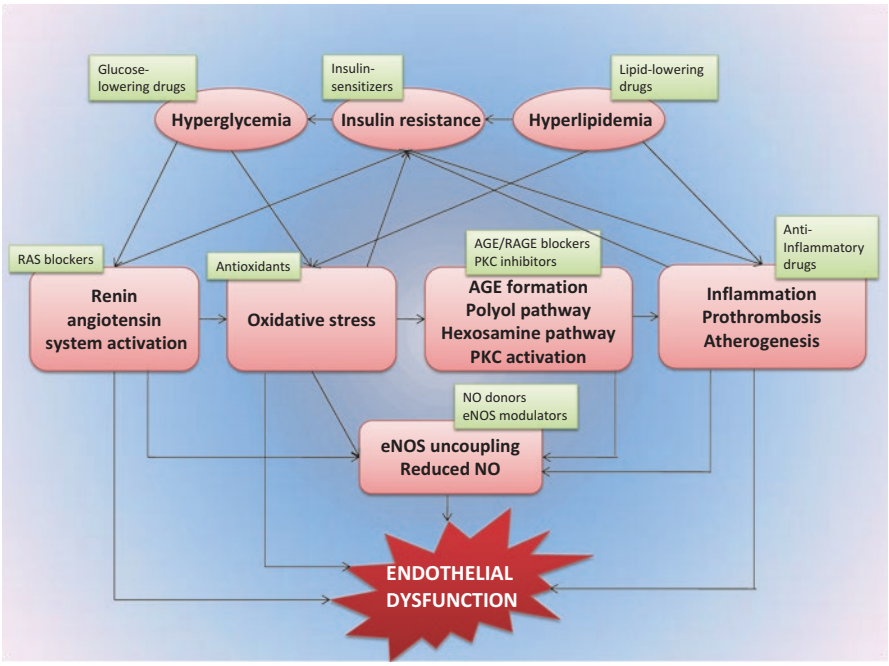

Fig. 22.2 Pathophysiology and drug targets for endothelial dysfunction in diabetes (*AGE* Advanced Glycation End Products, *RAGE* Receptor for Advanced Glycation End Products, *PKC* Protein Kinase C, *NO* Nitric Oxide, *eNOS* Endothelial Nitric Oxide Synthase)

nutritional therapy to modulate endothelial function in diabetes. e.g. Resveratrol, vitamin D, citrus flavonoids, Chinese herbal compounds and probiotics [14].

For the sake of clarity and ease of categorisation, the different existing as well as potential avenues for therapy of endothelial dysfunction in diabetes shall be discussed based on the type of risk factors primarily addressed by them.

22.6.1 Glucose-Lowering Therapies

Glucotoxicity is one of the major risk factors for endothelial damage, as seen in both experimental as well as cross-sectional clinical studies, where direct detrimental role of hyperglycemia to vasculature by impairing endothelium-dependent vasodilatation has been demonstrated. Post-prandial hyperglycemia, especially seen in Asian subjects with carbohydrate-rich diet, has been linked with vascular endothelial dysfunction slowly progressing to other cardiovascular complications [15]. Hence, therapies traditionally used to control these blood glucose spikes, viz. alpha-glucosidase inhibitors, DPP-4 inhibitors and GLP-1 agonists, could also improve vascular function in diabetic patients.

22.6.1.1 Alpha-Glucosidase Inhibitors

Alpha-glucosidase inhibitors are oral anti-diabetic drugs that reduce the breakdown of carbohydrates in the intestine, thereby reducing post-prandial hyperglycemia. Treatment with AGIs, especially acarbose, has also shown to provide beneficial effects on lipid levels, blood pressure, coagulation factors, carotid intima-media thickness and endothelial dysfunction [16]. Voglibose, when given for 12 weeks, has been shown to improve flow-mediated dilatation in Type 2 diabetic patients who had not responded to sulfonyl ureas, metformin or pioglitazone [17]. In a study involving patients with acute coronary syndrome undergoing percutaneous coronary intervention (PCI), one-week treatment with miglitol improved endothelial function as assessed by RH-PAT index [18]. Moreover, this change was found to be correlated with the reduction in post-prandial glucose levels. Miglitol has also been shown to inhibit mitochondrial ROS overproduction and subsequent apoptosis in endothelial cells, through activation of AMPK and phosphorylation of eNOS [19].

22.6.1.2 Dipeptidyl Peptidase-4 (DPP-4) Inhibitors

DPP-4 inhibitors are a class of oral antihyperglycemics that act by inhibiting DPP-4- mediated breakdown of GLP-1. The first drug of this class, sitagliptin, was approved in 2006, followed by other members such as vildagliptin, alogliptin, saxagliptin, teneligliptin and linagliptin [20]. Since these drugs are effective in blunting post-prandial hyperglycemia, they have often been hypothesised to have favourable effects on cardiovascular outcomes as well. Sitagliptin has been shown to inhibit endothelin-1 in aortic endothelium of STZ-induced diabetic rats, by suppressing the NF-KB system through activation of AMPK [21]. In another study, sitagliptin has been shown to stimulate vascular healing by enhanced recruitment of regenerative CXCR4+ endothelial progenitor cells [22]. Sitagliptin exerts protective effect against endothelial dysfunction in rats with diet-induced metabolic syndrome, by regulating nitric oxide-mediated vasodilatation as well as epigenetic signatures such as histone methylation [23]. Other drugs of this class have shown similar effects in experimental models of metabolic syndrome or diabetes. However, some clinical studies negate these claims and hence further clinical evidence is needed to substantiate their possible therapeutic effect in humans [24].

22.6.1.3 Sodium-Linked Glucose Transporter-2 (SGLT2) Inhibitors

A relatively new class of anti-hyperglycemic drugs attracting attention these days is the SGLT2 ingibitors or ‘glifozins’. These are drugs that inhibit reabsorbtion of glucose in the kidneys, mediated by a low-affinity, high capacity transporter-sodium-linked glucose transporter 2 (SGLT2). The members of this class, canaglifozin, dapaglifozin and empaglifozin, are currently approved only for use in Type 2 diabetic patients along with diet and exercise, or other anti-diabetic drugs [25].

However, SGLT2 inhibtion has shown promise in protection against other cardiovascular risk factors such as hypertension also. Empaglifozin has been shown to improve endothelial function in diabetic rats, as well as reduce arterial stiffness and high blood pressure in humans [26]. It has also provided benefit against development and progression of nephropathy in diabetic rats. However, it must be added that it might be early to attribute possible therapeutic effects to this class of drugs in managing endothelial dysfunction in diabetes.

22.6.2 Insulin-Sensitising Therapies

Insulin resistance has been recognised as a common denominator for Type 2 diabetes as well as cardiovascular complications. It fundamentally involves the tilting of insulin receptor downstream signalling from PI3K-Akt towards MAPK/ERK cascade [27]. At the level of endothelium, insulin resistance is linked to impaired NO bioavailability, the key feature of impaired vascular function [28]. Insulin resistance has also been shown to induce certain molecular signatures, which remain unreversed even after dietary switch to normal diet, collectively termed as 'metabolic memory' [29]. Drugs that enhance insulin sensitivity have thus been employed in experimental as well as clinical studies as possible therapeutic measures to inhibit diabetes/insulin resistance-linked endothelial dysfunction.

22.6.2.1 Metformin

Metformin, a favourite for clinicians tending to diabetic patients, acts primarily through improving hepatic insulin-sensitivity and overall systemic glycemic status. In clinical studies, metformin has shown beneficial effect on compromised endothelial function in Type 2 diabetic subjects. However, this benefit might not be proportional to improvement in insulin sensitivity. In experimental models, it has been shown to activate AMPK/eNOS pathway, thereby contributing to improved vascular function in metabolic syndrome [30]. This mechanism has been seen to improve recruitment of endothelial progenitor cells in STZ-induced diabetic mice, promoting angiogenesis and repair of damaged vessels [31]. Activation of AMPK and subsequent phosphorylation of PARP1 by metformin, coupled with increased eNOS activity and Sirt1 expression, showed protective effect in vascular endothelial cells, diabetic and hypertensive mice, and AMPK-α knockout mice [32]. Metformin has also been shown to have protective effects against hyperglycemia-induced endothelial dysfunction in thoracic aortae from db/db mice, through mechanisms involving increased phosphorylation of eNOS and Akt [33].

22.6.2.2 Thiazolidinediones (PPAR-Gamma Agonists)

Thiazolidinediones or 'glitazones' is another class of insulin-sensitising oral hypoglycaemic drugs, prescribed as monotherapy or in combination with bigunaides such as metformin. These act through activation of the gamma subtype of peroxisome proliferator-activated receptor, which is a nuclear receptor otherwise activated by endogenous fatty acids and eicosanoids, causing transcription of genes regulating fatty acid metabolism and improving metabolic profile of type 2 diabetic patients [34]. Though this class of drugs is classically known to act on adipocytes promoting uptake of non-esterified fatty acid from systemic circulation, they show pleiotropic action with tissue-specific actions, endothelium being one of them. Clinically approved members of this class such as pioglitazone, as well as newer experimental compounds, have shown beneficial effects on vasculature on both functional and molecular levels. A recent study demonstrated the effects of three new PPAR gamma agonists [GQ-32 (3-biphenyl-4-ylmethyl-5-(4-nitro-benzylidene)-thiazolidine-2,4-dione), GQ-169 (5-(4-chloro-benzylidene)-3-(2,6-dichloro-benzyl)-thiazolidine-2,4-dione), and LYSO-7 (5-(5-bromo-1H-indol-3-ylmethylene)-3-(4-chlorobenzyl)-thiazolidine-2,4-dione)] on cultured human umbilical vein endothelial cells [35]. The compounds activated PPAR-gamma as shown through reporter assays, and enhanced intracellular NO levels assessed using 4-amino-5-methylamino-2′,7′-difluorofluorescein-FM probe, apart from attenuation of glucose-induced ROS generation. Moreover, enhanced expression of angiogenic molecules such as vascular endothelial growth factor A and interleukin-8 was associated with endothelial cell migration and tube formation. In a randomised controlled study involving patients of metabolic syndrome who received pioglitazone for 12 weeks (15 mg for 6 week, then 30 mg daily for 6 weeks) or matched placebo, pioglitazone treatment improved insulin sensitivity as well as left ventricular diastolic and endothelial function [36]. In another study, pioglitazone used in combination with a calcium channel blocker improved blood brain barrier integrity and endothelial function in a model of pancreatectomy-induced diabetes [37]. Pioglitazone has also been shown to promote angiogenesis and microvessel density in ischemic hind limb of diabetic rats, through regulation of HIF/VEGF hypoxia response pathway [38]. Treatment with 10 uM pioglitazone in-vitro has been shown to improve the viability as well tube-forming capacity of endothelial progenitors cells obtained from individuals with impaired glucose tolerance [39]. These reports suggest beneficial effect of thiazolidinediones in diabetes-associated endothelial dysfunction. However, the benefits need to be weighed against the side-effects such weight gain, fluid retention, atypical fractures and possibly, bladder cancer.

22.6.3 Lipid-Lowering Therapies

Presence of excess lipids in circulation, whether due to dietary habits or metabolic derangements, is a well-established independent risk factor for both endothelial dysfunction and insulin resistance [40]. The strong association between dyslipidemia that exists in type 2 diabetic patients, and cardiovascular abnormalities such as atherosclerosis is thus inevitable. Dyslipidemia includes both sustained increased in circulating lipids as well as post-prandial lipemia, which remains underrated. Elevated levels of LDL-cholesterol, triglycerides and/or non-esterified fatty acids in the circulation collectively cause lipotoxicity i.e. lipid-induced injury or dysfunction at both cellular and organ level. This damage is mediated by diverse mechanisms such as oxidative stress, inflammation, and apoptosis induced by lipid accumulation [40]. Therapies that attenuate dyslipidemia, including use of statins, fibrates, cholesterol binding drugs and lipolysis inhibitors, are thus choices for potential protective effects against endothelial dysfunction.

22.6.3.1 HMG CoA Reductase Inhibitors

Statins, or hydoxy methyl glutaryl CoA (HMG CoA) reductase inhibitors, are widely prescribed for management of hypercholesterolemia. This class includes natural compounds viz. lovastatin, simvastatin and pravastatin, and synthetic compounds such as atorvastatin, fluvastatin and rosuvastatin. They exert their anti-hypercholesterolemic action by inhibiting the conversion of HMG CoA to mevalonate, which is the rate-limiting step in the cholesterol biosynthetic pathway in liver. As intra cellular sterol levels decrease, the hepatocytes enhance LDL-receptor expression in order to enhance uptake of LDL cholesterol from circulation [41]. Statins are known to confer protection against cardiovascular risk factors in metabolic syndrome and diabetes, which includes improved vascular function and reduced systemic inflammation [42]. In an experimental study in type 2 diabetic spontaneously hypertensive rats, treatment with atorvastatin (20 mg/kg/day) for five weeks significantly improved endothelial function by increasing nitric oxide levels, while decreasing peroxynitrite levels as well as mean blood pressure [43]. Rosuvastatin, in combination with a protein kinase C beta inhibitor, improved migration and tube-froming capacity in HUVECs exposed to high-glucose medium [44]. In vivo, this combination improved left ventricular function and microvessel density, and reduced myocardial fibrosis following myocardial infarction in diabetic rats, which was coupled with increased expression of VEGF, phosphorylated Akt and eNOS. In a study involving diet-induced obese mice, pravastatin restored the effect of induced pluripotent stem cell-derived endothelial cells in hindlimb ischemia, mediated through a nitric oxide-dependent mechanism [45].

22.6.3.2 Fibrates (PPAR-Alpha Agonists)

Fibrates, another class of lipid-lowering agents, are generally used in in combination with statins in the pharmacological management of hypertriglyceridemia and mixed hyperlipidemia. Their triglyceride-lowering effect is attributed to activation of PPAR alpha and subsequent transcription of its target genes [46]. In the liver, fibrates reduce triglyceride-VLDL synthesis by enhancing beta-oxidation of fatty acids. In the plasma, they increase triglyceride catabolism by inducing transcription of lipoprotein lipase and inhibiting transcription of apoC-III gene. They also increase high density lipoprotein (HDL)-cholesterol levels by increasing apoA-I and apoA-II gene transcription [46]. With such effects on plasma as well as tissue lipid levels, fibrates are known to provide protection against cardiovascular risk factors that rely on lipotoxicity. In a sub-study of the Fenofibrate Intervention and Event-Lowering in Diabetes (FIELD) study, treatment with fenofibrate caused significant improvement in arterial endothelial function after 4 months [47]. Fenofibrate administration for 12 weeks has been shown to be protective against endothelial dysfunction in kidneys of type 1 diabetic rats through increased mRNA levels of eNOS, AMPK and LKB1, and thus significantly increased renal NO production [48]. Similar effects have also been seen in endothelial dysfunction associated with experimental diabetic neuropathy in db/db mice [49]. A number of other studies report beneficial effects of fibrates in diabetes-associated endothelial function through diverse mechanisms such as inhibition of endothelin-1 expression, improvement of antioxidant status and activation of PI3K/Akt pathway [50–52].

22.6.3.3 Other Lipid-Lowering Therapies

Other lipid lowering therapies include the anti-lipolytic drug niacin, and the cholesterol absorption inhibitor ezetimibe. Niacin/nicotinic acid, which is used to control triglyceridemia owing to its anti-lipolytic action, has been shown to improve endothelial function in experimental models of diabetes as well as in type 2 diabetic patients, along with better compliance and outcomes when combined with statin therapy [53]. Ezetimibe, a cholesterol uptake-inhibiting drug, showed protective effect against peripheral microvascular dysfunction in type 2 diabetic subjects, assessed using reactive hyperaemia index (RHI) [54]. Similar to niacin, addition of ezetimibe to statin therapy improves the lipid parameter outcomes, but whether this relates with endothelial function is yet to be seen [55].

22.6.4 Renin-Angiotensin System-Targeting Therapies

Renin-angiotensin system, the proteolytic cascade with profound effects on vascular structure and function, has been a therapeutic mainstay in patients with hypertension. Its activation has been classically associated with increased salt and fluid

retention, increased vascular tone, impaired vasodilatation and subsequent cardiovascular abnormalities such as hypertension, and cardiac hypertrophy and remodelling [56]. The earlier model of this system, which considered renin, ACE, angiotensin II and AT1 receptors as the effectors of its actions, has been reshaped with the discovery of the functional role of the alternate and physiologically antagonistic axis consisting of ACE2, ang-(1–7), ang-(1–9) and Mas receptor [57]. The activation of RAS in diabetes is one of the primary effectors of kidney dysfunction and cardiovascular abnormalities, though the exact mechanism of activation remains unknown. Apart from its typical actions like impaired vasodilatation, induction of NADPH oxidase-mediated oxidative stress, prothrombotic and proliferative actvity, RAS has also been shown to be linked to insulin signalling in both metabolic and vascular tissues such as the adipose and blood vessels respectively [58]. These effects logically lead to the hypothesis that agents inhibiting this system, which includes angiotensin converting enzyme inhibitors and angiotensin receptor blockers, might afford cardiovascular benefit in diabetic patients.

22.6.4.1 Angiotensin Converting-Enzyme (ACE) Inhibitors

Angiotensin converting-enzyme inhibitors, including captopril, enalapril, lisinopril and ramipril, form the first-line therapy in the management of hypertension, and are also used to reduce morbidity and mortality in congestive heart failure, myocardial infarction, chronic renal insufficiency, diabetes mellitus and atherosclerosis [59]. In a study involving type 2 diabetic patients with stage 1 chronic kidney disease, treatment with ramipril for 12 weeks improved both endothelial as well as kidney function parameters, with a simultaneous decrease in plasma fibroblast growth factor (FGF) 23 [60]. In the experimental model of type 2 diabetes in rats, treatment with ramipril for 8 weeks alleviated insulin resistance, improved endothelium-dependent vasodilatation, and enhanced the levels of antioxidant enzyme haem-oxygenase-1 in the aorta [61]. Upon mechanistic investigation in high glucose-treated rat aortic endothelial cells, ramipril was found to enhance HO-1 expression through the activation of AMPK and Nrf-2. Importantly, the effects of RAS blockade have been shown to persist years after discontinuation of therapy [62].

22.6.4.2 Angiotensin Receptor Blockers

Angiotensin receptor blockade using drugs such as losartan, telmisartan and candesartan is another therapeutic strategy targeting RAS, which is used in the management of hypertension, heart failure and renal dysfunction in diabetes. Owing to their positive effect on nitric oxide-stimulated vasodilatation, and also protective effect against NADPH oxidase-mediated superoxide anion production, these drugs can attenuate endothelial dysfunction in diabetes [63]. In an experimental study involving STZ-induced type 1 diabetic rats, three-month treatment with losartan improved endothelial parameters such as expression of eNOS, bioavailability of nitric oxide,

and acetyl choline-stimulated vasorelaxation [64]. Telmisartan, an angiotensin receptor blocker that also shows PPAR-gamma agonistic effect, has been shown to activate PI3K/Akt/eNOS pathway and improve VEGF-induced angogenic responses from diabetic rats [65]. Azilsartan, an angiotensin receptor blocker with a higher affinity for and slower dissociation from AT1 receptors compared to other ARBs, has been shown to improve vascular endothelium-dependent relaxation in aortic rings obtained from KKAy diabetic mice [66]. This was associated with improved ratio of Ser1177/Thr495 phosphorylation of eNOS, and attenuation of expression of MCP-1, NOX-2 and NOX4. An important characteristic of angiotensin receptor blockade as a therapeutic approach is the molecular crosstalk with PPAR-gamma signalling, thus rendering favourable metabolic effects to many angiotensin receptor blockers, the most prominent being telmisartan [67, 68].

22.6.4.3 Direct Renin Inhibitors

Direct renin inhibition is another therapeutic approach targeting the renin angiotensin system, which came into practice relatively recently. Renin inhibitors block the first rate-limiting step in the renin-angiotensin cascade i.e. the conversion of angiotensinogen to angiotensin I by renin, and hence the production of all subsequent members of this system. This offers a benefit over other RAS-inhibiting drugs, in terms of no compensatory rise in plasma renin activity despite an increase in plasma renin concentration, unlike ACE inhibitors and ARBs whose action stimulates feedback loop that activates renin upon suppression of the RAS [69]. Aliskiren, the only FDA-approved direct renin inhibitor, was licensed in 2007 as an orally active drug for the therapy of hypertension [70]. Owing to its direct effect on the RAS, it has been tested in several studies as a possible therapeutic option for endothelial dysfunction occurring in diabetes. In an experimental study performed in fructose-fed hypertensive rats, four-week treatment with aliskiren (100 mg/kg/day) led to elevated plasma nitrite levels, and reduced systolic hypertension, insulin resistance, dyslipidemia, aortic lipid peroxide levels and aortic wall hypertrophy [71]. In another study where aliskiren (5 or 25 mg/kg/day) was administered via osmotic pump to STZ-induced diabetic rats for two weeks prior to induction of hindlimb ischemia, improved recovery of limb perfusion and capillary density was observed in aliskiren-treated group of animals. This was accompanied by an increase in the number of circulating Sca-1+/Flk-1+ EPC-like cells, elevated levels of the plasma VEGF and stromal cell-derived factor (SDF)-1α in a dose-dependent manner [72]. In clinical studies, treatment with aliskiren has been shown to improve renal haemodynamic and endothelial function in diabetic patients [73]. However, combining aliskiren with an ACE inhibitor or ARB has been contraindicated by the FDA in patients of diabetes, in view of the risk outweighing the benefits of such therapy [74].

22.6.5 Nitric Oxide/Enos Modulating Therapies

Nitric oxide was discovered in 1980 as the key endothelial factor involved in blood vessel relaxation, whether induced by a chemical mediator (acetyl choline, serotonin) or shear-stress. Apart from vasodilatation via cGMP-mediated kinase cascade, nitric oxide is involved in regulation of events such as monocyte and leukocyte adhesion to the endothelium, platelet-vessel wall interaction, angiogenesis, and smooth muscle proliferation [75]. The synthesis of nitric oxide in the endothelium is carried out by endothelial nitric oxide synthase (eNOS), localised in lipid-rich plasmalemmal caveolae as a dimer in its active and stabilized from, through the conversion of L-arginine to L-citrulline. The function of eNOS is regulated by multiple factors such as mRNA expression, L-arginine, influx of Ca^{2+}, and tetrahydrobiopterin (BH4), which is an essential cofactor for eNOS catalysis [76]. Diabetes induces disruption in nitric oxide synthesis by eNOS through a number of mechanisms such as peroxynitrite-mediated disruption of caveolae, impairment of de novo synthesis of BH4 as well as oxidation of BH4 to BH2, high levels of asymmetric dimethyl arginine (ADMA), and other mechanisms [77]. Interestingly, disruption in eNOS functioning is directly correlated with further progression of insulin resistance due to reduced perfusion and glucose uptake, hence fuelling a vicious cycle [78]. Thus, drugs that bear direct significance to nitric oxide synthesis or signalling such as BH4 analogues, ADMA blockers, eNOS modulators, and nitric oxide donors are worthy of discussion in terms of their importance as therapy for diabetes-induced endothelial dysfunction.

22.6.5.1 BH4 Analogues

Tetrahydrobiopterin, the co-factor essential for catalysis by eNOS, acts as an allosteric modulator and converts eNOS to conformationally active form with high affinity for L-arginine. Hyperglycemia in diabetes mellitus leads to reduced levels of BH4 in two major ways: by impairment of its de novo synthesis involving GTP cyclohydrolase, and by conversion of BH4 to BH2 (inactive as eNOS co-factor) due to oxidative stress. Reduced availability of BH_4 alters the function of eNOS, and the enzyme produces superoxide anion (O_2^-) instead of NO, a phenomenon called 'eNOS uncoupling' [77]. Supplementation with BH4 or its physiological precursor sepiapterin has been shown to improve endothelial function in animal models of diabetes, and later in clinical studies [79, 80]. However, its efficacy is compromised by its propensity to enhance BH2 levels causing subsequent uncoupling of eNOS, which is undesirable. Hence, more studies are needed to determine the dose-response relationship, keeping in view the tight stoichiometry between eNOS and BH4 [81].

22.6.5.2 Arginase Inhibitors and ADMA Blockers

L-arginine, the substrate for eNOS normally present in abundance in the body, is also a substrate for arginase that converts it to ornithine and urea. Increased expression of arginase has been seen in diabetes and atherosclerosis, which reduces the availability of arginine for eNOS, and thus reduces NO production [82, 83]. Arginase inhibition seems to be a promising strategy for endothelial dysfunction in diabetes and thus, treatment with the arginase inhibitor Nω-hydroxy-nor-L-arginine has been shown to improve microvascular endothelial function in patients of type 2 diabetes mellitus [84]. L-arginine also faces competition by asymmetric dimethyl arginine (ADMA), an endogenous methylated arginine molecule formed from breakdown of proteins, whose levels are seen to rise in vascular complications associated with diabetes [85].Therapeutic approaches currently used to target diabetic vascular complications are known to only partially act by reducing ADMA. However, search for inhibitors of protein-arginine methyl transferases (ADMA-synthesising enzymes), and modulators of dimethylarginine dimethylaminohydrolases (ADMA-metabolising enzymes) might yield potential therapeutic options for patients of diabetes-related vascular complications [81].

22.6.5.3 eNOS Modulators

Drugs such as metformin, rosuvastatin, miglitol and fenofibrate that activate eNOS through stimulation of the PI3K/Akt pathway have been discussed earlier. Among drugs that directly modulate the function of eNOS, CavNOxin is a cell-permeable, synthetic alanine-substituted peptide analog of caveolin that directly activates eNOS by attenuating the inhibitory effect of endogenous caveolin-1. In a study designed to assess its effect on vascular complications in STZ-induced diabetic ApoE knockout mice, treatment with CavNOxin (2.5 and 5.0 mg/kg every 3 days for 14 weeks) led to lowering of oxidative stress markers, inhibition of the expression of proatherogenic mediators, and blocking of leukocyte-endothelial interactions [86]. Keeping in view the low expression of eNOS in diabetes-related endothelial dysfunction, focussed development of chemical libraries for compounds enhacing eNOS transcription has yielded two molecules, AVE9488 and AVE3085 [81]. Oral administration of AVE3085 (10 mg/kg/day for 7 days) to db/db mice improved vascular function through enhanced NO bioavailability and reduced oxidative stress, thus appearing as a promising approach for diabetic vasculopathy [87].

22.6.5.4 Nitric Oxide Donors

Nitric oxide donors, including organic nitrates and sodium nitroprusside, are the drugs that release nitric oxide in the physiological environment by processes that could be enzymatic as well as non-enzymatic. Organic nitrates are currently in clinical use for management of ischemic heart disease and acute as well as chronic

angina. Their action is mediated by rapid denitration by the mitochondrial enzyme aldehyde dehydrogenase in vascular smooth muscle to release nitric oxide [88]. Glyceryl trinitrate (GTN/nitroglycerin) is the prototype nitrate, other members being isosorbide mononitrate and dinitrate, erithrityl tetranitrate and pentaerithrityl tetranitrate. In an experimental model of STZ-induced diabetes in Wistar rats, treatment with pentaerithrityl tetranitrate (15 mg/kg/day p.o. for 7 weeks) improved endothelial dysfunction by preventing eNOS uncoupling and NADPH oxidase activation [89]. A major limitation, though, in the use of nitrates is the development of tolerance [90]. Another fast-acting nitric oxide donor is sodium nitroprusside, which is clinically used as a drug of choice to lower blood pressure in patients of hypertensive crisis [91]. However, its utility in diabetes-induced endothelial dysfunction is confined to its use as the standard drug for NO-dependent endothelium-independent vasodilatation in clinical studies [92].

22.6.6 Oxidative Stress-Targeting Therapies

In aerobic organisms, the redox energy of mitochondrial electron transport is harnessed in the high-energy phosphate bond of the final product i.e. ATP. The final enzymatic step in this chain is the reduction of molecular oxygen to water, catalysed by cytochrome oxidase C. However, partially reduced oxygen species such as the superoxide anion, hydrogen peroxide and hydroxyl ion may also be formed through these (and other) electron transfer reactions, and are known as 'reactive oxygen species' with the potential to attack lipids, proteins and nucleic acids [93]. Pathological conditions such as diabetes are marked by oxidative stress i.e. imbalance between these pro-oxidant species and the antioxidant defence systems such as superoxide dismutase, catalase and glutathione peroxidase. In the vasculature, ROS cause uncoupling of eNOS, possibly by zinc-depletion of the enzyme and oxidation of its co-factor BH4, leading to production of more superoxide that combines with nitric oxide to yield the toxic pro-oxidant peroxynitrite [77]. Oxidative stress has thus been implicated as the unifying mechanism for diabetes-induced endothelial injury, with superoxide ion overproduction considered as a common and essential precursor for all other pathways such as polyol pathway, advanced glycation end-product formation, PKC activation, hexosamine pathway and NF-KB activation [94]. This makes oxidative stress an attractive target for prevention and therapy of the complications of diabetes, and existing drug treatment of the cardiovascular complications of diabetes is often accompanied by reduced oxidative stress markers.

22.6.6.1 Free-Radical Scavengers

Considering the overproduction of reactive oxygen species in diabetes, the use of free radical scavengers for prevention and therapy of vascular complications was hailed as a potential therapeutic approach [94]. Free radical scavengers, including

natural antioxidants like vitamin A, vitamin C & vitamin E, and alpha-lipoic acid as well as synthetic ones like SOD- and glutathione-mimetics, have been tested for their potential protective effect against vascular complications in diabetes. In a study involving type diabetic patients, treatment with alpha-lipoic acid (infused intravenously at a dose of 600 mg over 30 min) was shown to improve endothelium-dependent vasodilatation; however, translation of this effect to possible vasculoprotective effect was left to future investigation [95]. A similar study using infusion of the antioxidant vitamin C in diabetic patients showed similar improvement in endothelium-dependent vasodilatation measured using forearm blood flow [96]. However, a number of studies have come up and refuted the claims regarding the positive effects of antioxidant vitamins for therapy of vascular complications in diabetic patients. In a recent study by Cazeau et al., there were no positive effects of vitamin C & vitamin E on vascular parameters in diabetic patients [97]. Notably, high dose vitamin E (1800 IU daily) supplementation for 12 weeks has been shown to paradoxically worsen some vascular reactivity parameters in patients of type 1 and type 2 diabetes mellitus [98]. While the translational failure of antioxidants in the therapy of vascular complications does not negate the importance of oxidative stress as a worthy therapeutic target, it shows the need for better study designs and an understanding of the effect of antioxidant interventions on specific ROS moieties. Another free-radical scavenging drug is the SOD-mimetic Tempol, a synthetic analogue of the endogenous antioxidant enzyme superoxide dismutase that catalyses the conversion of superoxide to H_2O_2, further converted to water by catalase. Tempol has been shown to restore the vasodilator response to acetyl choline in the afferent renal arteriole dissected out from alloxan-induced diabetic rats [99]. Similarly, deferoxamine is a scavenger of hydroxyl ions that has been shown to protect against diabetic endothelial dysfunction [100, 101].

Apart from antioxidant vitamins and enzyme-mimetics, natural antioxidant compounds such as bioflavonoids, carotenoids and hydroxycinnamates are important candidates for the treatment of oxidative stress-induced diabetic complications [102]. Literature evidence regarding the protective effect of these antioxidants, along with others like such as curcumin, allicin and piperine, has been listed in the following table (Table 22.1). However, similar to synthetic antioxidants, the claims regarding protective effect of natural antioxidants also remain to be substantiated in clinical setting.

22.6.6.2 NADPH Oxidase Inhibitors

The disappointing results from the use of free-radical scavengers prompted investigations into inhibition of the sources of ROS. NADPH oxidase is a membrane-localised multi-subunit enzyme, which catalyzes the formation of superoxide anion and/or hydrogen peroxide by electron transfer from NADPH to molecular oxygen through the heme groups in its transmembrane domains, utilizing FAD as a cofactor [110]. In diabetes and its vascular complications, excess production of ROS has been attributed partly to enhanced activity of NADPH oxidases, in close

Table 22.1 Natural compounds/antioxidants with protective effect against endothelial dysfunction in diabetes

Compound(s)	Study type	Parameters/results	References
Apigenin & Naringenin	In vivo: Type 2 diabetic rats	Reduced blood glucose, serum lipid, malondialdehyde, ICAM-1, insulin resistance index	[103]
	In vitro: Palmitate-treated endothelial cells	Improved SOD activity and acetylcholine or insulin-induced aortic relaxation	
		Inhibition of NF-κB activation and ICAM-1 mRNA expression and improved nitric oxide production in the presence of insulin (in vitro)	
Daidzein	High glucose-treated human umbilical vein endothelial cells (HUVECs)	Enhanced viability and reduced lipid peroxidation, intracellular reactive oxygen species (ROS) generation	[104]
		Reduced inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and NF-κB protein expression	
Catechin hydrate	STZ-induced diabetes in rats	Reduced serum glucose and vascular oxidative stress	[105]
		Enhanced PI3K-induced eNOS phosphorylation and acetyl choline-induced vasodilatation	
Astaxanthin	STZ-induced diabetes in Wistar rats	Reduced serum oxLDL, aortic MDA levels and LOX expression	[106]
		Improved endothelium-dependent vasodilator responses to ACh and upregulated eNOS expression	
Lycopene	STZ-induced diabetes in rats	Reduced serum glucose, ox-LDL levels, aortic MDA levels and iNOS -activity	[107]
		Enhanced aortic SOD activity, NO levels, and eNOS activity	
Curcumin	STZ-induced diabetes in rats	Decreased superoxide radical production	[108]
		Improved ACh-mediated vasodilatation	
Diallyl disulphide	Obese diabetic rats & High glucose-injured HUVECs	Reduced malondialdehyde (MDA) and ROS	[109]
		Elevated activities of SOD and glutathione peroxidase (GSH-Px) in mitochondrium	

conjunction with the activation of renin-angiotensin system. Apart from existing therapies targeting the renin-angiotensin system, direct NOX inhibitors have attracted significant drug discovery efforts in the quest for therapies targeting oxidative stress. GKT137831 is a highly potent NOX2 and NOX4 inhibitor of the pyrazolopyridine class, and has been shown to prevent hyperglycemia-induced oxidative stress in human aortic endothelial cells [111]. However, since the different isoforms of NADPH oxidase enzymes differ in their subunit composition, activation, physiological and pathophysiological functions, designing drugs with isoform specificity remains a formidable challenge [110].

22.6.7 Inflammation-, Thrombosis- and Atherogenesis-Targeting Therapies

An important physiological role of the endothelium-derived nitric oxide, apart from vasodilatation, is to prevent leucocyte adhesion and maintain the endothelium in an anti-inflammatory state. Also, the endothelium secretes both prothrombotic molecules such plasminogen activator inhibitor-1 (PAI-1), thromboxane, tissue factor, and von Willebrand's factor (vWF), as well as antithrombotic molecules such as NO, heparans, prostacyclin, tissue plasminogen activator, and thrombomodulin, ensuring a fine balance between blood's fluidity and thrombosis [10]. Abnormal conditions that stimulate a pro-inflammatory and prothrombotic phenotype cause impairment of endothelium-dependent vasodilation and promote progression towards atherosclerosis [112]. It is well documented that diabetes induces chronic systemic low-grade inflammation, with enhanced levels of markers such as tumour necrosis factor-alpha (TNF-a), interleukin-6 (IL-6), intercellular adhesion molecule and C-reactive peptide [113]. Diabetes is also known to enhance activation of NF-KB, the master regulator of inflammatory genes and cytokines, which is linked with endothelial dysfunction. Drugs that reduce inflammation and thrombosis, therefore, should be able to attenuate endothelial dysfunction and subsequent complications.

The relevance of anti-inflammatory therapy for diabetes-linked endothelial dysfunction is substantiated by clinical evidence such as reduced NFκB activation in freshly isolated endothelial cells from obese human subjects treated with salsalate (anti-inflammatory drug), along with improved insulin sensitivity and endothelium-dependent vasodilation [114]. AMPK, the major energy-sensor molecule, has also been suggested to suppress endothelial inflammation by blunting TNF-induced NF-κB activation and resulting expression of adhesion molecules in endothelial cells [115]. Thus, activation of AMPK by metformin has been shown to inhibit cytokine-induced NF-κB activation in vascular endothelial cells [116]. Pharmacological inhibition of the prothrombotic vasoconstrictor thromboxane A_2 using S18886 reduces inflammation and slows down atherogenesis caused by diabetes mellitus, possibly by counteracting the effects on diabetes on endothelial function and adhesion molecule expression [117].

A new concept regarding the role of inflammation in diabetes-linked endothelial dysfunction is 'inflammaging', which denotes an amalgamation of accelerated aging and chronic low-grade inflammation [118]. Cellular aging or senescence is associated with the development of a pro-inflammatory phenotype known as the senescence-associated secretory phenotype (SASP), mainly regulated by NF- B, the mechanistic target of rapamycin (mTOR), and the interleukin-1/NLR family pyrin domain containing 3 (IL- 1/NLRP3) inflammasome pathways. Hyperglycemia-induced oxidative stress and other imbalances associated with T2DM (such as altered lipid metabolism, epigenetic alterations, and low-grade inflammation) have the potential to foster premature senescence in the endothelium and promote diabetic complications. Inflammaging has thus

become an area of intense research for therapies of vascular aging in diabetes, with players such as statins, sirtuin family activators, mTOR inhibitors, and ER stress modulators [118, 119].

22.6.8 Ion Channel-Modulating Therapies

Ion channels act as the transmembrane gateways that regulate the flux of ionic species such as Ca^{2+}, Na^{+}, K^{+} and Cl^{-} across the lipid bilayer of plasma membrane otherwise impermeable to them [120]. They help establish an electrochemical gradient in both excitable and non-excitable cells, which is critical for impulse transmission in heart, blood vessels, nerves and skeletal muscles. Apart from these electrophysiological phenomena, ion channels also modulate several processes coupled with ion transport, such as insulin secretion, gastric acid secretion, and regulation of excretion in the renal tubules. Vascular function is also dependent on the concerted functioning of different types of ion channels such calcium channels, potassium channels, and transient receptor potential (TRP) channels that are now gaining increased attention [121]. Ion channels are involved in a number of endothelial cell functions dependent on intracellular Ca(2+) signals, such as the production and release of nitric oxide and PGI(2). Also, ion channels may be involved in the regulation of the traffic of macromolecules by endocytosis, transcytosis, the biosynthetic-secretory pathway, and exocytosis, e.g., Willebrand factor, and tissue plasminogen activator [122]. Ion channels are also involved in controlling intercellular permeability, EC proliferation, and angiogenesis. Considering the extremely sensitive nature of ionic homeostasis, anomalies in ion channels are bound to cause major pathological changes. Diabetes has been shown to induce changes in expression as well as sensitivity of these channels, and this contributes as an etiological change involved in vascular dysfunction [120, 123]. Thus, drugs that modulate the activity of these ion channels have often been explored as therapeutic option for diabetes-linked vascular dysfunction.

22.6.8.1 Calcium Channel Blockers

Entry of calcium through L-type calcium channels in smooth muscle cells of the vasculature is the primary trigger for the contraction of blood vessels. In vascular beds, depolarisation-induced calcium influx triggers the release of calcium from the intracellular stores in sarcoplasmic reticulum (mediated by ryanodine receptor-linked calcium channels) [124]. This increase in cytosolic calcium levels enhances calcium-calmodulin binding and myosin light chain kinase phosphorylation, which promotes actin-myosin interaction and thus, vascular smooth muscle contraction. The use of calcium channel blockers to block voltage-sensitive calcium channels leads to relaxation of blood vessels, especially in arterial beds [125]. Calcium

channel blockers such as amlodipine are already favoured drugs for the management of hypertension, and their role in attenuating impaired vasodilatation in diabetes also has been often explored. Amlodipine is a dihydropyridine-class calcium channel blocker that has been shown to attenuate hypertension in diabetic rats with concomitant reversal of eNOS uncoupling and vascular oxidative stress [126]. However, since the known effects of calcium channel blockers are limited to vascular properties alone and do not extend to metabolic improvement, experimental studies explore their effect on diabetic vasculopathy often in combination with other classes of vasoprotective drugs such as statins, RAS inhibitors and oral antidiabetics. For example, 6-month therapy with a combination of amlodipine and lisinopril improved endothelial as well as carbohydrate metabolism in type 2 diabetic patients with concurrent hypertension, safely and effectively [127].

22.6.8.2 Potassium Channels Openers

Potassium channels in smooth muscle, in their open state, are responsible for potassium efflux-induced hyperpolarization and resulting inhibition of calcium entry, hence promoting vasodilatation. The different kinds of potassium channels in vascular smooth muscle include K_{Ca} (calcium-activated), K_V (voltage-dependent/delayed rectifier), K_{ATP} (ATP-sensitive) and K_{IR} (inward rectifying) [128]. Although the hypotensive action of potassium channel openers like minoxidil was discovered close to sixty years ago, the discovery of K_{ATP} channels in most of the cell types and in mitochondria has further fuelled the possibility of use of potassium channel openers for therapy of cardiovascular diseases. Inactivation and/or reduced sensitivity of these channels in vascular beds have been implicated as one of the possible anomalies underlying impaired endothelial function in diabetes [129, 130]. Nicorandil, a hybrid K_{ATP} potassium channel opener as well as nitrate, has been shown to inhibit intimal hyperplasia after catheter-induced balloon injury in diabetic rats [131]. In diabetic patients undergoing percutaneous coronary intervention, treatment with nicorandil (20 mg once daily for 1 week before and 6 months after PCI) was shown to prevent PCI-related myocardial injury, and improve left ventricular ejection fraction [132]. Diazoxide, another K_{ATP} channel opener, has been shown to improve wound-healing in diabetic mice by improving EPC function [133]. However, an attention-worthy point regarding the use of potassium channel openers in diabetic patients is that their action might be compromised by hypercholesterolemia, and concurrent anti-diabetic treatment with sulphonyl ureas [134].

22.6.8.3 Ivabradine

Ivabradine is a novel first-in-class selective sino-atrial node I_f (f stands for funny current) channel inhibitor. I_f channel is a mixed sodium and potassium channel that belongs to the HCN (hyperpolarization-activated cyclic nucleotide-gated)

channel family and, mediates the inward flow of cations that initiates the spontaneous diastolic depolarization phase, modulating heart rate [135]. Ivabradine selectively inhibits the I_f current without affecting other ionic currents in the heart, and is used to control heart rate in patients with angina. The reduction in heart rate by ivabradine has been shown to improve endothelial function in different conditions such as heart failure and dyslipidemia. It has been shown to reduce vascular oxidative stress, improve endothelial function, and prevent the development & progression of atherosclerosis in two different models of dyslipidemia in mice, the unifying precursor being the reduction in heart rate [136, 137]. In diabetic mice, ivabradine shows cardioprotective effect through reduced apoptosis, and inhibition of expression and activity of MMP-2 [138]. In patients with chronic stable angina with diabetes mellitus, ivabradine has been shown to be safe and effective option for prevention of angina, without any adverse effects on glucose metabolism[139].

22.7 Future Targets/Therapies for Endothelial Dysfunction in Diabetes

Apart from the established pathological mechanisms underlying endothelial dysfunction in diabetes and the drugs used to target them, several new mechanisms and targets are being uncovered in the quest for new and more effective lines of therapy. One of these is the role of vitamin D deficiency with diabetic vascular complications such as coronary artery disease, an association which has attracted significant research efforts [140]. Another approach is the modulation of transient receptor potential (TRP) channels, group of non-selective cation channels located mostly in the cell membrane, being increasingly recognised for newer roles such as in cardiovascular and metabolic regulation [141]. Vascular complications of diabetes are also being linked to endoplasmic reticulum stress, due to protein misfolding and abnormal expression of chaperones such as heat shock proteins [142]. Lower expression of heat shock proteins in insulin-resistant state has been linked with the alternate ACE2/Mas receptor axis of the renin angiotensin system, and modulation of these targets has shown therapeutic potential [143]. Insulin resistance and hyperglycemia have also been shown to induce epigenetic signatures such as DNA methylation and altered expression of micro RNAs, which result in long-lasting changes in endothelial function, collectively known as ‘metabolic memory’ [144]. The common thread through all these mechanisms/targets is the need for translation of experimental evidence (presented in Table 22.2) into safe and effective therapeutic modulators.

Table 22.2 Promising targets/mechanisms for future therapy of endothelial dysfunction in diabetes

Mechanism/target	Modulators (reported)	Link with endothelial dysfunction	References
Vitamin D signalling	Calcitriol, Paricalcitol, Maxacalcitol, Doxecalciferol	Vitamin D treatment rescues endothelial colony forming cells from glucotoxic effects of gestational diabetes mellitus	[145, 146]
		Oxacalcitol prevents endothelial dysfunction in type 2 diabetic rats through reduced p22(phox) expression and improved eNOS uncoupling	
TRP channel modulation	Capsaicin	Capsaicin-induced activation of TRPV1 reduces diet-induced triglyceridemia; also improves endothelium-mediated vasodilatation in genetically hypertensive rats	[147, 148]
Endoplasmic reticulum stress	TUDCA, Argirein	TUDCA administration to diabetic mice normalizes myogenic response and endothelium-dependent relaxation	[149, 150]
		Argirein administration to diabetic rats ameliorates corpus cavernosum dysfunction by enhanced expression of eNOS and ER chaperone BiP	
Heat shock proteins	Heat shock therapy, Geranylgeranyl acetone	Heat shock-induced HSP72 expression in insulin resistant rats is linked with epigenetic regulation of eNOS and improved endothelial function	[143, 151]
		GGA induces activation of Hsp90/AMPK and increases NO-mediated vasodilation in healthy subjects, as well as in smokers	
ACE2-Mas receptor	Ang (1–7)	Ang (1–7) administration to diabetic mice increases nitric oxide bio-availability and inhibits oxidative stress	[152, 153]
		Ang (1–7) treatment-induced ACE2/Ang-(1–7)/Mas pathway activation corrects existing diabetes-induced CD34(+) endothelial progenitor cell dysfunction through improved NO bioavailability	
Metabolic memory (Epigenetic signatures)	Tenegliptin, Metformin	Tenegliptin alleviates chronic high glucose-induced oxidative stress, ER stress and apoptosis in endothelial cells, and overcomes metabolic memory	[154, 155]
		Metformin protects against hyperglycaemic metabolic memory in endothelial cells via activation of LKB1/AMPK/ROS pathway	

(continued)

Table 22.2 (continued)

Mechanism/target	Modulators (reported)	Link with endothelial dysfunction	References
MicroRNAs	miR-181b, AMO-221 (antisense miR-221 oligonucleotide)	miR-181b enhanced Akt phosphorylation and reduced endothelial dysfunction epididymal white adipose tissue	[156, 157]
		AMO-221 abolished the inhibitory effect of high glucose exposure on HUVECs transmigration	
Mitochondrial biogenesis	Epicatechin, Resveratrol	Epicatechin improved endothelial nitric oxide synthase function, mitochondrial activity and markers of mitochondrial biogenesis in high glucose-treated human coronary artery endothelial cells	[158, 159]
		Resveratrol induced mitochondrial biogenesis in human coronary artery endothelial cells and in aorta of db/db mice via Sirt1/eNOS axis	
Soluble epoxide hydrolase (SHE)	AR9281, AR9286, trans-4-[4-(3-adamantan-1-ylureido)-cyclohexyloxy]-benzoic acid (t-AUCB)	Treatment with AR9281 and AR9286 elevated epoxy/diol lipid ratio, and improved endothelial function in models of diabetes, obesity and hypertension	[160, 161]
		Treatment with t-AUCB alleviated signs of metabolic syndrome and reversed cardiac structural and functional abnormalities in high fat-fed rats	
Nutritional modulation (probiotics)	Kefir	Improved NO bioavailability and endothelial progenitor cell recruitment in Kefir-treated spontaneously hypertensive rats	[162]

22.8 Conclusion

Diabetes-induced endothelial dysfunction remains the unifying cause for development of all further vascular as well as metabolic complications, and chronic organ-damage. As is said in possibly every set of guidelines regarding the management of diabetes, the risk of cardiovascular complications in diabetes is significant and necessitates early therapeutic management to prevent complications such as hypertension, atherosclerosis and ischemia. The multifactorial pathology of diabetes and its comorbidites is interesting in many ways; the first being that it ensures that there is no dearth of targets for drug therapy. However, due to the intricate and extensive crosstalk between the pathogenic mechanisms/targets, the drugs used for treating diabetic complications cannot actually be confined to one mechanistic class alone. A prime example is metformin, the anti diabetic drug of choice, whose newer

mechanisms of its action keep showing up. Apart from its classic insulin-sensitising action, it has now been recognised as a potential modulator of 'metabolic memory' and also as a nitric oxide donor. The complexity in signalling networks is responsible for redundancy in pathways, such that the effect of modulating a certain target might be compensated for by some other endogenous mechanism. This has fuelled drug discovery efforts towards designing drugs with multiple targets/mechanism of action with tissue-organ-specific effects. For example, efforts are on to design drugs that possess both insulin secretagogue and nitric oxide-donor properties. Such approach increases the chances for better management of both the causes and effects of endothelial dysfunction in diabetes. Newer non-redundant targets and promiscuous drugs thus seem to define the future of drug therapy for diabetes.

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Chapter 23
Beneficial Effects of Inorganic Nitrate/Nitrite on Vascular Function and Blood Pressure in Diabetes

Zahra Bahadoran, Parvin Mirmiran, and Asghar Ghasemi

Abstract The potential property of inorganic nitrate (NO_3) and nitrite (NO_2) to convert to nitric oxide (NO), a key regulator of vascular homeostasis and a natural vasodilator, has highlighted these anions as therapeutic options in vascular abnormalities and hypertension states. Pre-clinical studies confirmed the protective effects of NO_3 and NO_2 against ischemia-reperfusion injury, arterial stiffness, oxidative stress, and inflammation. Clinical studies also revealed that short-term supplementation with NO_3 and NO_2 could improve endothelial function, decrease arterial stiffness, pro-inflammatory cytokines, and effectively decrease systolic and diastolic blood pressure. In summary, supplementation with inorganic NO_3 and NO_2 represents a promising therapy for treatment of vascular dysfunction and hypertension in humans. To confirm cardioprotective effects of inorganic NO_3/NO_2 in diabetic patients, clinical studies with a dose-response design and a longer-duration, are highly recommended.

Keywords Nitrate • Nitrite • Vascular function • Hypertension • Type 2 diabetes

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23.1 Introduction

23.1.1 Vascular Dysfunction in Type 2 Diabetes

It is well known that impaired vascular function, subsequent elevated blood pressure and vascular disease are important undesirable complications of developing insulin resistance and type 2 diabetes; the therapeutic approach focusing on vascular dysfunction, is a priority in management of type 2 diabetic patients [1–4].

Endothelial dysfunction in diabetes mellitus is characterized by changes in proliferation, barrier function, adhesion of other circulating cells, and sensitivity to apoptosis [5]. Several vascular physiological components, including the endothelium, vascular smooth muscle and platelet are disrupted in diabetes [1]. The angiogenic and synthetic properties of endothelial cells is also mainly affected by hyperglycemic condition [6].

23.1.2 Nitric Oxide Pathway, Vascular Function and Type 2 Diabetes

23.1.2.1 NO Production

Currently, NO is known as a biologically active hormone, with several functional properties, including regulation of vascular homeostasis and blood pressure, inhibition of platelet activation, regulation of energy and lipid metabolism as well as mitochondrial biogenesis, and modification of various physiological pathways [7, 8].

Both enzymatic and non-enzymatic pathways have been identified for NO production. Conversion of L-arginine to NO via three isoforms of NO synthase (NOS), including neuronal NO synthase (nNOS), inducible NO synthase (iNOS), and endothelial NO synthase (eNOS), is the classical recognized NO production pathway. All three NOS isoforms progressively oxidize and reduce L-arginine to L-citrulline to product NO [9].

In 1994 it has been shown that inorganic NO_3/NO_2could act as a substrate or backup system for non-enzymatic endogenous generation of NO [10]; so these onions are now considered as important alternative sources of NO instead of the classical L-arginine-NO-synthase pathway, particularly in the hypoxic states [11, 12]. It has been shown that under certain conditions, different enzymes including hemoglobin, myoglobin, xanthine oxidoreductase, mitochondrial cytochrome oxidase, aldehyde dehydrogenase 2, cytochrome P450 reductase (CPR) and cytochrome P450 (CP), can reduceinorganic NO_3/NO_2 to NO[2]; CPR catalyzes NO_3 reduction, producing NO_2, whereas CP can mediate further NO_2 reduction to NO [13].

The impaired eNOS-dependent L-arginine-NO pathway along with an increased degradation of NO through scavenging by reactive oxygen species (ROS), lead to a substantial decreased NO bioavailability, during some pathophysiological condi-

tions [14]. The impaired NO metabolism, has been recognized as a risk factor for development of cardiometabolic disorders, especially vascular dysfunction, cardiovascular disease, chronic kidney disease, endocrine disorders, insulin resistance, metabolic syndrome, and type 2 diabetes [15–18].

23.1.2.2 The Role of NO Pathway in Cardiovascular Function

Nitric Oxide, an endothelium-derived factor, is a powerful vasodilator and contributes to normal vascular physiology; NO can also inhibit platelet activation, leukocyte adhesion, cell proliferation, and pro-inflammatory transcription factors such as nuclear factor-kappa B [1]. The first identified mediator of NO function wascyclic guanosine monophosphate (cGMP), generated bysoluble guanylyl cyclase (sGC), however further studies revealed that NO exerts a ubiquitous influence in a cGMP-independent manner [2]. The main underlying mechanisms of cGMP-mediated protective effects of NO on vascular function are summarized in Fig. 23.1. NO-dependent relaxation of vascular smooth muscular cell (VSMC) is mediated through decreasing intracellular Ca^{2+} concentrations by two pathways: (1) NO activates soluble guanylyl cyclase (sGC), promotes the conversion of guanosine triphosphate (GTP) to guanosine 3′5′ cyclic monophosphate (cGMP) and increases the intracellular levels of cGMP which results in a decreased intracellular Ca^{2+} levels, by blocking voltage-gated Ca^{2+} channels in the cytoplasm membrane; (2) NO-dependent increased cGMP levels also promotes phosphorylation of phospholamban (the sarcoplasmic reticulum Ca^{2+}-ATPase regulatory protein) through activation of cGMP-dependent protein kinase (PKG) that leads to sequestration and decreased intracellular concentration of Ca^{2+}[1]; decreased intracellular Ca^{2+} levels prevents the activation of myosin light chains kinase (MLCK) by complex calcium-calmodulin (Ca^{2+}-CaM), and consequently, inhibits the myosin light chains phosphorylation and consequently promotes the vascular smooth muscles relaxation [1, 19].

Moreover, NO-dependent increased cGMP levels could also activate cAMP dependent protein kinase (PKA) through decreasing degradation of cAMP by interacting with phosphodiesterase III (PDE III); an increased cAMP in vascular smooth muscle causes reduction of intracellular Ca^{2+} concentration and reduces contraction [19]. In the VSMC, PKA also inhibits the mitogen-activated protein kinase (MAPK), reduces DNA synthesis, and consequently, cell proliferation [20].

Nitric oxide can also regulates endothelial cell functions by inhibition of pro-inflammatory transcription factors such as nuclear factor-kappa B (NF-κB); in this pathway, NO increases the expression of I-κBα, which inhibits NF-κB, and consequently, inhibits expression of adhesion molecules and chemokines, such as vascular cell adhesion molecule (VCAM) and monocyte chemotacticprotein-1 (MCP-1), prevents leukocyte migration and the beginning of the atherosclerotic morphologic alterations [2].

Currently it is clarified that several effects of NO are mediated by S-nitrosylation of some functional proteins involved in vascular [21]; protein S-nitrosylation, a reversible modification occurs on cysteine residues [22, 23], activate or inhibit

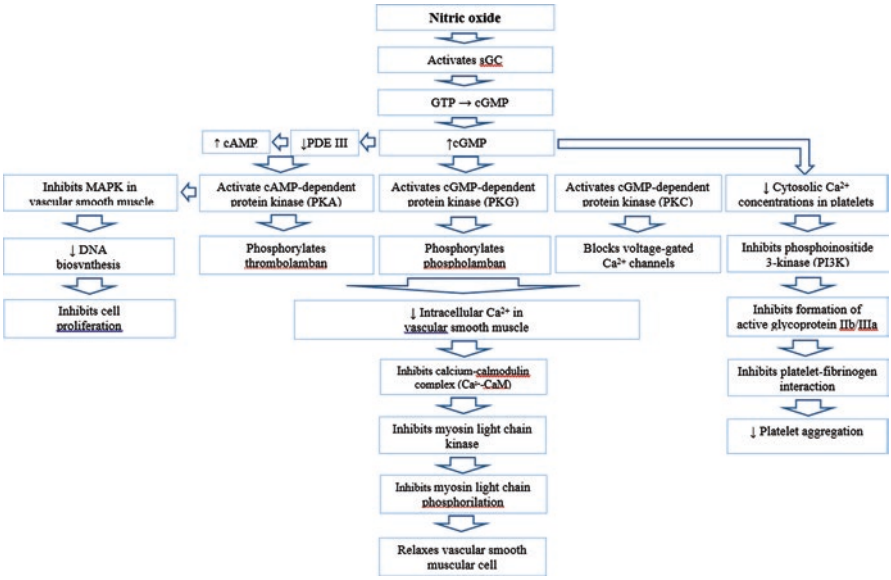

Fig. 23.1 The underlying mechanisms of cGMP-mediated protective effects of NO on vascular function

Nitric oxide increase cGMP production, consequently activates cGMP-dependent protein kinase (PKG), reduces intracellular Ca^{2+} concentration, inhibits the activation of myosin light chains kinase and consequently, inhibits the myosin light chains phosphorylation and the contraction. Nitric oxide also increases the levels of cAMP, activates cAMP-dependent protein kinase (PKA); in the vascular smooth muscle cells, PKA inhibits the mitogen-activated protein kinase (MAPK), reduces DNA synthesis, and consequently, cellproliferation. In platelets, NO reduces cytosolic Ca^{2+} concentration, inhibits activation of glycoprotein IIb/IIIa, and decrease platelet-fibrinogen interaction. activate cAMP dependent protein kinase (PKA) through decreasing degradation of cAMP by interacting with phosphodiesterase III (PDE III); an increased cAMP in vascular smooth muscle causes reduction of intracellular Ca^{2+} concentration and reduces contraction [1, 2]

protein function [24] and be beneficial or detrimental [23]. NO directly nitrosylates transition metals while NO_2, N_2O_3, and transition-metal NO adducts nitrosylate cysteine residues [25].

S-nitrosylation is specific and only some proteins are nitrosylated despite presence of cysteine residues on almost all proteins and production of NO by most cells [25]. S-nitrosylated-dependent effects of NO on vascular tone can be exerted by four pathways: (1) NO increasesthe activity of sarco/endoplasmic reticulum calcium ATPase (SERCA) via S-nitrosylation which accelerates calcium depletion and induces relaxation; (2) NO can also S-nitrosylates G protein-coupled receptors (GPCR) which leads to inhibition of the binding of ligands for the receptor or G-protein coupling; (3) NO-dependent S-nitrosylation of G protein-coupled receptor kinase 2 (GRK2) prevents the desensitization and internalization of β-adrenoceptors; (4) S-nitrosylation of β-arrestin 2 increases receptor internalization [2].

23.1.2.3 Impaired NO_3-NO_2-NO Pathway and Type 2 Diabetes

Findings from *in vitro* and animal investigations have raised the hypothesis that the NO_3-NO_2-NO pathway may play a critical role in glucose homeostasis, insulin secretion, and insulin signaling [26, 27]. Current data indicates there is a significant association between endothelial NO synthase (eNOS) gene polymorphisms and type 2 diabetes; moreover,decreased eNOS expression in skeletal muscle, and reduced NO production and bioavailability in insulin resistance states, clearly underlines the role of NO and its metabolites in the development of hyperinsulinemia, insulin resistance, and type 2 diabetes [28–31].

Undesirable changes in the metabolism of NO_3/NO_2 as well as impaired NO pathway in type 2 diabetes has been linked to consequent metabolic disorders and vascular complications [32]. Decreased NO bioavailability activates the proinflammatory transcription factor NF-κB, and consequently increases the expression of leukocyte adhesion molecules and production of chemokines and cytokines; these actions promote monocyte and vascular smooth muscle cell migration into the intima and formation of macrophage foam cells, characterizing the initial morphological changes of atherosclerosis.

Some well-known mechanisms could describe the impaired NO pathway in type 2 diabetes; first, chronic hyperglycemia, increased oxidative stress and NF-κB activation, as well as accumulation of advanced glycation end products (AGEs) in diabetic condition are the substantial disruptive factors in the NO_3-NO_2-NO pathway [33]; second, increased plasma levels of asymmetric dimethylarginine (ADMA), an endogenous competitive inhibitor of NO synthase, in diabetic condition is also known as hyperglycemia-induced impairment factor of NO pathway [34]. Third, decreased NOS activity and production of NO from L-arginine as well as increased arginase activity, which leads to decreased L-arginine bioavailability and NO synthesis via NOS, are other disrupted pathways in NO homeostasis in diabetic conditions [35]. Finally, since activation of NOS is regulated by insulin and the Akt signaling pathway, impaired insulin secretion and insulin resistance due to diabetes could affect L-arginine-NO pathway and NO biosynthesis [36]. Mechanistically, insulin can increase L-arginine transport *via* increased expression of cationic amino acid transporter-1 (CAT-1), the predominant L-arginine transporter expressed in endothelial cells [37]. Recent evidence suggests that insulin resistance may contribute substantially to the onset and development of cardiovascular disease in type 2 diabetic patients via abnormal insulin-mediated regulation of L-arginine transport [38].

Considering the disrupted L-arginine-NO pathway in diabetic states, and NO-like bioactivity of NO_3/NO_2, these onions are now considered as supplementary treatment in management of type 2 Diabetes [26, 39].

23.1.3 Inorganic NO_2 and NO_3

Inorganic NO_3 and NO_2 are both naturally occurring as well as food additive compounds in the human diet. Vegetables and other plant based foods are the most common sources of dietary intake of NO_3 and contribute up to 85% total dietary NO_3 intake; green leafy vegetables including lettuce and spinach, cabbage, rocket lettuce, red beetroot, and radish have higher concentrations of NO_3 [40, 41]. Drinking water could also provide considerable amounts of NO_3; main sources of dietary NO_2 are processed meat and animal food products [41].

Some acute toxicities such as methemoglobinemia, potential endogenous conversion to N-nitroso compounds (NOC), mutagenic, teratogenic, and carcinogenic effects, as well as development of thyroid disorders, thyroid cancer and type 1 diabetes have been main health concerns for a long time regarding the NO_3 and NO_2 exposure [42–44].

In recent years, following the discovery of potential ability of inorganic NO_3 and NO_2 as an important back-up system for impaired NOS-derived NO generation, the historical conception of the scientific community focused on the potential hazards of NO_3 and NO_2 exposures [43, 44] shifted towards therapeutic properties of these compounds in cardiometabolic disorders [26, 45–54]. Theoretically, reductions of NO_3 and NO_2 to NO could restore NO homeostasis, maintain the steady-state NO levels, and are considered as stable storage pools for NO-like bioactivity [55]. So, considering the role of NO as the key regulator of vascular homeostasis and natural vasodilator, supplementation with inorganic NO_3 and NO_2 have been investigated as potential therapeutic options in metabolic disease, including hypertension, cardiovascular disease and in states renal dysfunction [56–59].

Currently, a large body of evidence supports a crucial role of NO_3 and NO_2 in the regulation and modulation of blood flow, endothelial function and blood pressure [12, 58, 60]. Pre-clinical studies also confirm protective effects of NO_3 and NO_2 against ischemia-reperfusion injury, arterial stiffness, oxidative stress, and inflammation [58], however, the nutritional aspects of the vasculoprotective effects of these anions are not clear and their long-term effects are still unknown [61]. Some evidence also indicates that NO_3/NO_2-rich foods such as green leafy vegetables play a protective role against type 2 diabetes and cardiovascular disease [62]. It has also been suggested that high-NO_3 diet as well as inorganic NO_2/NO_3 supplementation may have a beneficial role in management of type 2 diabetes, and related disorders especially endothelial dysfunction and vascular complications [63].

23.2 Cardioprotective Effects of Inorganic NO_3 and NO_2

23.2.1 The Proposed Underlying Mechanisms

The capacity of inorganic NO_3 and NO_2 to produce NO, a key regulator of vascular homeostasis and a natural vasodilator, has highlighted these anions as therapeutic options in vascular abnormalities and hypertension states [59]. In addition, inorganic NO_3 and NO_2 have different cardiovascular protective effects including anti-platelet activity, lipid-lowering effects, anti-inflammatory effects, and improvement of blood flow in hypoxic and ischemic tissue [59, 61, 64]. Pre-clinical assessment of inorganic NO_3/NO_2 also revealed its properties to protect against ischaemia-reperfusion injury and reduce arterial stiffness, and inflammation [61].

Although hypotensive effect of inorganic NO_3/NO_2 has been confirmed in both normotensive and hypertensive subjects, the specific location where NO_2-derived NO exerts its effects is largely undetermined, and its underlying mechanisms for the cardioprotective properties in human subjects are still not fully understood [65]. It has been proposed that NO_2 could be a stable endocrine carrier and transducer of NO-like bioactivity within the circulation; acute hypotensive effects and systemic vasodilation following consumption of dietary NO_3 and NO_2 are mainly mediated through the NO-cGMP pathway [12, 61].

The novel mechanisms recently investigated in a model of natural aging-related cardiovascular and metabolic abnormalities, suggest that inorganic NO_3 mediates its therapeutic effects through restored cGMP signaling and increased NO bioavailability, decreased angiotensin II (ANG II) type 1 receptor gene expression, improved endothelial function, increased insulin release and reduced NADPH oxidase activity and superoxide generation [57]; these favorable effects were associated with reduced plasma creatinine, improved endothelium-dependent acetylcholine-induced relaxation, and attenuated contractility to ANG II in resistance arteries [57].

Another underlying hypotensive mechanism of NO_2 is attributed to its potential to generate NO°, nitrous anhydride, and other nitrosating species at low pH, which promote S-nitrosothiol formation when NO_2 is in the stomach [66].

It has been shown that gastric S-nitrosothiol formation mediates the antihypertensive effects of oral NO_2/NO_3; increased S-nitrosothiol levels following consumption of NO_2, may increase S-nitrosylation of ANG II type 1 receptor, decrease its affinity for angiotensin II, and subsequently inhibit constriction of vascular smooth muscle cells. More interestingly, treatment with omeprazole could attenuate plasma S-nitrosothiol concentrations and inhibit the antihypertensive effects of NO_2 [66].

S-nitrosylation of proteins have different outcomes on cardiovascular physiology and several essential roles of S-nitrosothiol have been previously found in vascular homeostasis, cardiac function, and protection against myocardial injury [67, 68]. S-nitrosylation of essential regulators of β-adrenergic receptor signaling and calcium cycling improve cardiac function; moreover, in the vasculatures, S-nitrosylation of some proteins decreases inflammation and apoptosis, regulates blood flow and oxygen delivery, induces neovascularization and transducing hypoxic signals [67, 69, 70].

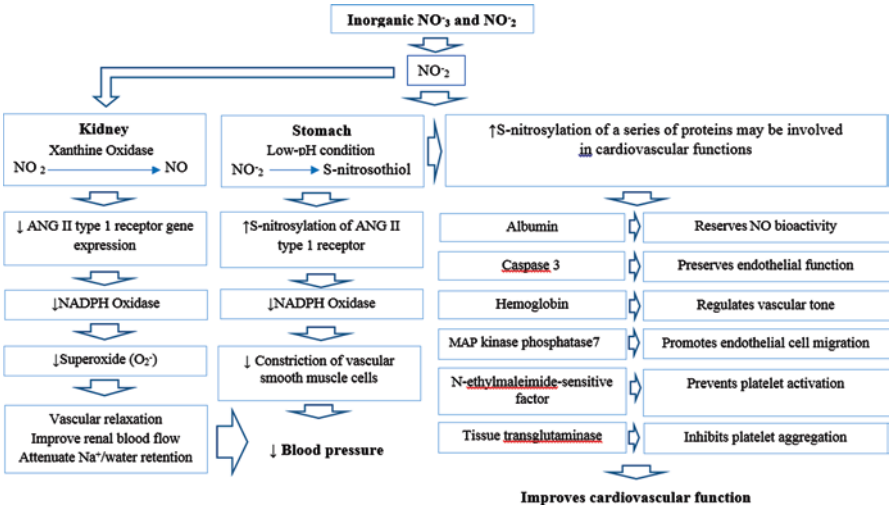

Fig. 23.2 The possible underlying mechanisms of hypotensive properties of inorganic NO_2/NO_3 Inorganic NO_2 after reabsorption by the gastrointestinal tract is converted to NO by xanthine oxidase in preglomerular afferent arterioles; NO inhibits angiotensin II type I receptor, inhibit NADPH oxidase activity and reduces superoxide generation. These actions lead to vascular relaxation, improve renal blood flow, attenuate sodium/water retention, and subsequently decrease blood pressure [57, 65]. NO_2 can also promote S-nitrosothiol formation in the stomach, and S-nitrosylation of ANG II type 1 receptor, decreasesits affinity for angiotensin II, and inhibit of the constriction of vascular smooth muscle cells. S-nitrosylation of a series of proteins involved in cardiovascular functions may mediate cardioprotective effects of inorganic NO_2/NO_3 [21, 66, 69]. NADPH: Nicotinamide adenine dinucleotide phosphate; ANG II: Angiotensin II

Figure 23.2 illustrates the proposed underlying mechanisms of hypotensive properties of inorganic NO_2/NO_3as well as its metabolite, S-nitrosothiol, on cardiovascular function

23.2.2 Animal Models

In animal models, administration of sodium NO_3 enhanced revascularization, increased migration of bone marrow-derived cells to the ischemic-site, and attenuated apoptosis of regenerative myoblasts in ischemic tissue [71]. Supplementation with sodium NO_3 in eNOS-deficient mice could reduce blood pressure [72]; a similar effect was also observed following treatment with inorganic NO_3 in hypertensive and eNOS deficient rat, against hypertension and myocardial ischemia-reperfusion injury [11, 73]. These findings indicate the compensatory effects of exogenous NO_3 for a disrupted NO pathway and related disorders in pathological conditions. Administration of sodium nitrite in high-fat induced hypercholesterolemic rat models had anti-inflammatory effects and inhibits the leukocyte adhesion and

emigration and prevented the vascular dysfunction [74]. Low (0.1 mmol/kg/day) and moderate (1 mmol/kg/day) doses of NO_3 significantly improved endothelial function and atherosclerotic plaque composition and stability in ApoE−/− mice fed a high-fat diet [75]. Administration of dietary NO_2 (50 mg/L in drinking water) was effective in the treatment of vascular aging in mice, which was related to a substantial decreased aortic pulse wave velocity, normalization of NO-mediated endothelium-dependent dilation and improved vascular dysfunction; the observed effects were mediated by attenuation of oxidative stress and inflammation, and increased mitochondrial biogenesis and health [76].

23.2.3 Human Studies

Several clinical trials are being performed to determine the broad therapeutic potential of increasing NO_2 bioavailability on human health and disease. Most recent findings from clinical human investigations imply on beneficial cardioprotective outcomes following short-term administration of inorganic NO_3 [26, 77]; improvement of endothelial dysfunction and a blood pressure lowering effect of NO_3/NO_2 are the most observed end points in these studies.

It has been shown that after consumption of dietary NO_3, systemically absorbed $NO_3$was concentrated 10-fold in the salivary glands and enteredin an entero-salivary circulation and consequently reduced to NO_2 by bacterial NO_3 reductases, and swallowed into the stomach providing a source of systemically available NO_2/NO; afterward, NO_2 was transported in the arterial circulation to resistance vessels, where lower O_2 tension favors the reduction of NO_2 to NO, causing vasodilatation, with consequent lowering of blood pressure [78]. Administration of 500 ml of a commercially available NO_3-rich beetroot juice beverage (containing ~9.4 mmol of NO_3), in two age-groups healthy adults including young (mean age=25 years) and old (mean age=64 years), resulted in a significant decreased peripheral and aortic blood pressure in both young and older adults, however aortic wave reflection assessed by aortic augmentation index, decreased only in young adults (range of change from baseline over time: aortic augmentation index@75bpm, −4.3 to −8.8%)[79]; these findings suggest that effects of dietary NO_3 supplementation on vascular function may be affected by some physiological status such as aging.

Effects of NO3/NO2 supplementation on blood pressure, vascular function and cardiovascular health are summarized in Table 23.1.

Administration of an acute high-dose of NO_3 (8 mg/kg of body weight)in healthy subjects demonstrated that inorganic NO_3 decreased systemic blood pressure and increased vascular conductance, with the potential to increase peripheral tissue oxygenation; these effects occur without altering cardiac electrical activity suggesting that inorganic NO_3 may be a safe complementary treatment in heart failure [87].

Four-weeks administration of inorganic NO_3 (150 μmol/kg body weight $NaNO_3$ ~ 300 g spinach) in elderly volunteers with mild hypertension and increased cardiovascular risk, improved their endothelial function, reduced vascular stiffness and

Table 23.1 Effects of NO_3/NO_2 supplementation on blood pressure, vascular function and cardiovascular health

Author	Study design and population	Intervention	Effects of nitrate
Kapil et al. 2015 [49]	Randomized double-blind, placebo-controlled clinical trial on patients with hypertension	Dietary supplementation for 4 weeks with either dietary NO_3 (250 ml daily, as beetroot juice) or placebo	Improves endothelial function ↓Arterial stiffness and blood pressure
Shepherd et al. 2015 [80]	Randomized clinical trial on patients with mild-moderate chronic obstructive pulmonary disease	70 ml of beetroot juice(6.77 mmol NO_3) or NO_3-depleted beetroot juice twice a day for 2.5 days	No significant change in systolic and diastolic blood pressures
Berry et al. 2014 [81]	Randomized, single-blind, crossover study on patients with chronic obstructive pulmonary disease	140 ml commercial beetroot juice(7.58 mmol NO_3) or placebo (<0.01 mmol NO_3)	Reduces resting systolic and diastolic blood pressures
Bondonno et al. 2014 [46]	Randomized controlled crossover trial on individuals with high-normal blood pressure	A 7-day high- NO_3diet (at least 300 mg/day NO_3 from green leafy vegetables) or a 7-day low- NO_3 diet	No significant change on arterial stiffness and blood pressure
Liu et al. 2014 [50]	Randomized controlled cross-over trial on healthy adult men and women	A meal with high NO_3 (220mg of NO_3 derived from spinach) or low NO_3	Increases large artery elasticity index, reduces pulse and systolic blood pressures
Rammos et al., 2014 [52, 53]	Randomized, placebo-controlled double-blind trial on older adults	4 weeks supplementation with 150 μmol/kg sodium NO_3or 150 μmol/kg sodium chloride	Improvement of endothelial function and vascular stiffness ↓Pro-inflammatory cytokines ↓Systolic blood pressure
Ingram et al. 2014 [48]	Two clinical settings: 1- Double blind, cross-over study on patients with inducible myocardial ischemia 2- Double blind study on healthy subjects	1-Administration of low-dose sodium NO_2 (1.5 μmol/min for 20 min) or saline during dobutamine stress echocardiography 2- Intravenous infusion of low-dose sodium NO_2 (1.5 μmol/min for 20 min) or saline into the contralateral arm before and after ipsilateral forearm ischemia	↓Effects of ischemia and inducible myocardial ischemia protects against ischemia injury-mediated vascular injury

(continued)

Table 23.1 (continued)

Author	Study design and population	Intervention	Effects of nitrate
Hobbs et al. 2013 [82]	Randomized, controlled crossover trial in healthy subjects	Dietary NO_3 via 200 g beetroot bread containing 100 g beetroot (1.1 mmol nitrate) or 200 g white bread (<0.01 mmol NO_3)	↑ Endothelium-independent vasodilation ↓Diastolic blood pressure
Hobbs et al. 2012 [83]	Randomized, controlled, single-blind crossover trial in healthy subjects	Beetroot juice in dose of 0, 100, 250 and 500 g, or three bread products, control bread (0 g beetroot), red beetroot- and white beetroot-enriched breads	Beetroot juice decreased both systolic and diastolic blood pressure, in a dose-dependent manner Both red- and white-beetroot breads decreased systolic and diastolic blood pressures, compared to control
Webb et al. 2008 [78]	An open-label crossover design clinical trial on healthy subjects	500 mL beetroot juice ~ 1395 mg NO_3	↓Systolic and diastolic blood pressure over 24 h with peak reductions of 10.4 and 8 mm Hg, respectively
Sobko et al. 2010 [84]	A 10-days randomized, cross-over trial in 25 healthy volunteers	Japanese traditional diet (~18.8 mg/kg/bw/day NO_3) compared to control diet	↓ Diastolic blood pressure on average 4.5 mmHg No changes in systolic blood pressure
Ashworth et al. 2015 [85]	A 7-days randomized, crossover trial in healthy subjects	High-NO_3 vegetables diet (salad, such as lettuce, rocket, celery, leeks, fennel and mixed salad leaves) compared to control diet	↓Systolic blood pressureon average 4 mm Hg
Bailey et al., 2016 [86]	A6-days cross-over experiment in healthy smokers and healthy non-smoking controls	140 mL/day NO_3-rich (8.4 mmol NO_3) beetroot juice compared with NO_3-depleted (0.08 mmol NO_3) beetroot juice as control	↓Systolic blood pressure in non-smokers
Hughes et al. 2016 [79]	A clinical trialin healthy adults in two age-groups: young (mean age=25 years) and old (mean age=64 years)	500 ml of a commercially available NO_3-rich beetroot juice beverage (9.4 mmol of NO_3)	↓ Peripheral and aortic blood pressure in both young and older adults ↓ Aortic wave reflection only in young adults (range of change from baseline over time: aortic augmentation index@75bpm, −4.3 to −8.8%)

(continued)

Table 23.1 (continued)

Author	Study design and population	Intervention	Effects of nitrate
Alsop et al. 2016 [87]	A clinical study in young adults	One dose of oral NO_3 consumption (8 mg/kg)	↓ Diastolic blood pressure No effect on pulse pressure or rate-pressure product ↓ Vascular compliance prior to isometric grip exercise and ↑ vascular compliance following exercise
Jonvik et al. 2016 [88]	A semi-randomized crossover study in healthy adults	Four different beverages, each containing 800 mg (~12.9 mmol) NO_3: sodium nitrate (NaNO3), concentrated beetroot juice, a rocket salad beverage, and a spinach beverage	↑ NO_3 and NO_2concentrations after ingestion of all four beverages ↓ Systolic blood pressure 150 min after ingestion of beetroot juice (5 mm Hg) and rocket salad beverage (6 mm Hg) and 300 min after ingestion of spinach beverage (7 mm Hg), but did not change with $NaNO_3$ ↓ Diastolic blood pressure 150 min after ingestion of all beverages and remained lower at 300 min after ingestion of rocket salad and spinach beverages
Vanhatalo et al. 2010 [89]	A clinical study in balanced crossover design in healthy subjects	Supplementation with beetroot juice 500 ml/day ~ 5.2 mmol NO_3) or placebo (500 ml/day low-calorie juice cordial)	↑ Plasma NO_2 concentration ↓ Systolic and diastolic blood pressure ↓ Steady-state Vo2 during moderate exercise
Jajja et al. 2014 [90]	A randomized clinical study in older, overweight subjects	Beetroot juice concentrate or blackcurrant juice	↓ Daily home-measured systolic blood pressure but not resting clinic or 24-h blood pressuremeasuremnet
Gilchrist et al. 2013 [91]	A double-blind, randomized, placebo-controlled crossover trial in type 2 diabetes	250 ml beetroot juice daily (active) or 250 ml NO_3-depleted beetroot juice (placebo)	No significant changes in blood pressures, macro-vascular or micro-vascular endothelial function

significantly reduced systolic blood pressure, whereas diastolic blood pressure, heart rate and carotid intima-media thickness remained unchanged [53]. Short-term consumption of NO_3-rich spinach decreased systolic blood pressure, by 2.7 mmHg, and also increased brachial artery flow mediated dilatation, by 0.5%, in healthy volunteers [46]. After a 3-day supplementation with sodium nitrate (0.1 mmol/kg body weight/d ~ 150–250 g of a nitrate-rich vegetable such as spinach, beetroot, or lettuce), a lower diastolic blood pressure and arterial pressure was observed compared to placebo group [92].

A randomized crossover study demonstrated a dose-dependent decrease in blood pressure and vasoprotection after inorganic NO_3 ingestion in the form of either supplementation with potassium NO_3 or high-NO_3 beetroot juice [93]. Administration of beetroot juice also induced acute blood pressure lowering effect, vasoprotective, and anti platelet properties [78]. A single ingestion of a dietary NO3 (500 mL beetroot juice ~ 1395 mg NO_3) reduced systolic and diastolic blood pressure over 24 h with peak reductions of 10.4 and 8 mm Hg, respectively [78]. Anti-hypertensive effect of NO_3/NO_2 has been proposed to be mediated by an improvement of endothelial function.

A recent meta-analysis of 15 clinical trials showed that supplementation with inorganic NO_3 and high-NO_3 beetroot juice decreased bothsystolic blood pressure(−4.4 mm Hg; 95% CI= −5.9, −2.8;*P*< 0.001) and diastolic blood pressure(−1.1 mm Hg; 95% CI= −2.2, 0.1; *P* = 0.06) [94]. Meta-analysis of clinical studies confirmed a dose-response beneficial effect on vascular function following supplementation with inorganic NO_3 and beetroot juice [95].

Some evidence from epidemiological studies also showed an inverse association between high-NO_3/NO_2 diet and development of hypertension. In a prospective study, conducted on 1546 non-hypertensive adults aged 20–70 years, a significant inverse association was observed between NO3-contaninig vegetables and the development of hypertension in the highest tertile category (relative risk = 0.63, 95% confidence interval= 0.41–0.98, *P* for trend = 0.05) [96]. In another 6-years follow-up study, the highest compared to the lowest intake of dietary NO_2 (median intake ≥ 12.7 *vs.*< 6.04 mg/d) was accompanied with a significant reduced risk of HTN, in the fully adjusted model (OR=0.68, 95% CI=0.47–0.98; *P* for trend=0.054) [97].

In a cross-over randomized clinical trial, administration of 140 mL/day NO_3-rich (8.4 mmol NO_3) beetroot juice compared with NO_3-depleted (0.08 mmol NO_3) beetroot juice as control, decreased systolic blood pressure only in non-smoker healthy but not smoker subjects [86]. The authors suggested that inorganic NO_3 metabolism was compromised in smokers leading to an attenuated blood pressure reduction compared to non-smokers following NO_3 supplementation; they also indicated that their observations may provide novel insights into the cardiovascular risks associated with cigarette smoking and suggested that this population may be less likely to benefit from cardioprotective effects of NO_3 [86].

Cardioprotective effects of NO_3/NO_2 have not yet been confirmed in diabetic patients [91]. In a double-blind, randomized, placebo-controlled crossover trial, 2-weeks supplementation with 250 ml/d beetroot juice (7.5 mmol NO_3/day) or 250 ml/d nitrate-depleted beetroot juice (0.002 mmol NO_3/day), increased plasma

concentration of nitrite and nitrate, but no significant changes were observed in systolic and diastolic blood pressures, or in macro- or micro-vascular endothelial function [94]. Data shows that impaired metabolic pathways in diabetes could lead to diminished NO production and bioavailability, inactivated NO and impaired eNOS activity and endothelium-dependent vasodilatation [33, 98], indicating the NO-like bioactivity and blood pressure lowering effects of dietary NO_3 supplementation observed in healthy subjects, would be mild in diabetic patients.

23.3 Summary

In 1994, NO_3-NO_2–NO pathway discovered as a complementary to the classical L-arginine-NOS pathway. Accordingly, the historical conception of the scientific community focused on the potential hazards of NO_3 and NO_2 exposures shifted towards therapeutic properties of these compounds in cardiometabolic disorders. Currently, accumulating evidence suggests that inorganic NO_3/NO_2 is an opportunity for novel NO-based therapeutics. Animal and human clinical studies of both inorganic NO_3 and NO_2 supplementation have implied on the beneficial effects in blood pressure, platelet function, vascular function and exercise capacity. To confirm cardioprotective effects of inorganic NO_3/NO_2 in diabetic patients, clinical studies with a dose-response design and a longer-duration, are highly recommended.

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Chapter 24
Myeloperoxidase (MPO): Do We Need Inhibitors?

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Abstract Neutrophils, monocytes and selected tissue macrophages are the predominant sources of myeloperoxidase (MPO). Under physiological chloride concentration, the MPO hemo protein can catalyze the reaction of formation of hypochlorous acid in presence of another oxidant, hydrogen peroxide. MPO-mediated oxidants play a significant part in the inflammatory response, though, the MPO is traditionally viewed as an unspecific microbicidal enzyme and some intermediates are important for immune defense system against invading pathogens. Inflammation plays a lead role in the manifestation and the amelioration of atherosclerosis and other cardiovascular diseases. Hence, there is continuing interest on MPO as a target in the diagnosis and development of potent therapeutic aid against this oxidative enzyme. The necessity of developing a drug to inhibit MPO is getting steady momentum and through this review, we assess the current status of the literature on the source of MPO, its primary physiological role, currently available inhibitors and application of *in silico* screening, harmful effects on the increased levels of MPO and its implication in multiple disease progression. We critically analyze current efforts toward the development of suitable inhibitors using small organic molecules, unexplored organometallic scaffolds, utilization of aptamers as

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myeloperoxidase inhibitor and future perspective on the scope of therapeutic intervention for this attractive target.

Keywords Myeloperoxidase • Inflammation • Oxidative stress • Atherosclerosis • Drug target

Abbreviations

TMB	3,3′,5,5′ Tetramethylbenzidine
SCN	Thiocyanide
SOD	Superoxide dismutase
NADH	Reduced nicotinamide adenine dinucleotide
SP1	Specific Protein 1
ARDS	Acute Respiratory Distress Syndrome
COPD	Chronic Obstructive Pulmonary Disease
WHHL	Watanabe heritable hyperlipedemic
MMP	Matrix metalloprotease
VEGF	Vascular endothelial growth factor
MPOI	Myeloperoxidase inhibitor

24.1 Introduction

24.1.1 MPO and Its Role in Oxidative Stress and Inflammation

Inflammation has been implicated in most, if not all, chronic diseases. Traditionally aspirin and its analogs and corticosteroids have been used as anti-inflammatory agents [1–5]. Oxidative stress and inflammation have been observed to go hand-in-hand [6, 7] and there have been numerous reports of antioxidants suppressing the expression and induction of inflammatory mediators [8]. Myeloperoxidase (MPO) is an enzyme that is associated with immune response and inflammatory cells and has been the topic of interest for many investigators across the globe [9]. MPO is secreted into both the extracellular milieu and the phagolysosomal portions following phagocyte activation by various agonists [10]. Highly oxidizing species like chloramine, HOCl, tyrosyl radical and nitrogen dioxide are generated by MPO [11–13]. It is notable that the milieu of the phagolysosome is specifically orchestrated to provide ideal oxidative and proteolytic environment for killing of overrunning pathogens [14, 15]. Primary function of MPO under normal physiological environment is to provide anti-bacterial defense against invading pathogens.

However implication of its role in pathophysiology by innumerable research work has made to investigate on its mechanism of action in the manifestation of disease condition. Its pathogenic role in atherosclerosis has been suggested for over two decades, and attempts have been made to develop inhibitors that could be used pharmacologically [16–18]. In this review, we provide a molecular and theoretical basis for potential inhibitors of MPO both from small molecules and metal-based structures, its function and suggest that MPO action could be controlled nutritionally.

Structurally, MPO is a basic lysosomal heme protein, produced at the time of myeloid variation process that has a major component of neutrophil primary granules known as azurophilic granules [19–22]. It is also expressed at much lower levels in monocytes [23] and tissue macrophages [24, 25]. Neutrophils are the predominant source of MPO, whereas monocytes hold about 30% of the MPO present in neutrophils [26–28]. Pro-myelocytes and promyelomonocytes vigorously synthesize MPO during granulocyte segregation in the bone marrow [29, 30].

Neutrophil azurophilic granules are also considered as primary lysosomes [31, 32]. Different types of lysosomal granules in neutrophils have been represented in Fig. 24.1. In addition to MPO, neutrophil primary (azurophilic) and secondary granules contain a variety of other enzymes including alkaline phosphatase, azurocidin, β-galactosidase, β-glucuronidase, cathepsins, DNases, defensins, elastase, lipases, lysozyme, lysosome hydrolases, phospholipase, protease, trypsinase, and permeability increasing factors [33–36]. Whether these enzymes work alone or in unison, has not been explored. Considering that all these enzymes are catabolic in nature, it is likely that these enzymes have an elaborate mechanism for anti-bacterial actions. In general, the process of degranulation of azurotrope can be achieved by elastase, a specific marker enzyme [37]. MPO plays a major role in oxygen dependent and independent killing of microorganisms in association with other enzymes *via* various reactive oxygen species (ROS) [38, 39]. Evidence suggests that MPO is the major contributor in terminating the respiratory burst by regulating the activity of NADH oxidase [10, 40], as pictorially represented in Fig. 24.2. The role of oxidative stress in the actions of other enzymes has not been fully understood and it requires systematic investigations to establish the link.

24.1.2 MPO as an Oxidant and Enzymology

Due to the imbalance in the oxidant generation and its defensive role, the concept of oxidative stress evolves. In an elevated oxidative stress condition, endogenous antioxidant capacity is down regulated, leading to the ensuing event of increase in levels of ROS and reactive nitrogen species (RNS) [41].

Several lines of evidence in the literature delineate that free radicals play a substantial role in chemical signaling and functional utility of the cells [42, 43]. However, production of free radicals beyond to certain optimum levels will complicate the physiological functions. Particularly, structural modification of biomolecules like, alteration in lipids motif, DNA and proteins which would affect the

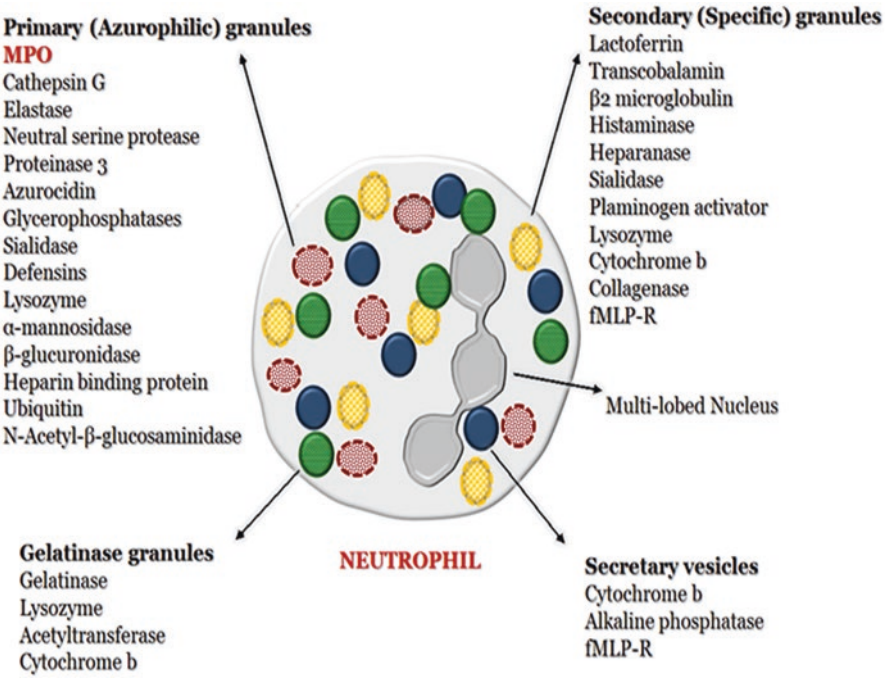


Fig. 24.1 Various types of lysosomal granules in neutrophils

normal cellular and physiological functions. Available data clearly delineate that there is a correlation between increase in the incidence of multiple disease including cardiovascular disease, cancer and neurodegenerative disease with the increase in free radicals mediated oxidative stress [44].

24.2 Reactive Oxygen and Reactive Nitrogen Species

Free radicals are chemically reactive species comprising unpaired electrons in their atomic or molecular orbitals. Oxygen metabolism during normal cellular respiration generates reactive oxygen species (ROS) as by-products. The free radical mediated reaction is terminated when two highly reactive chemical species are within the vicinity of chemical reactions and the resultant product is relatively less reactive chemical entities. Propagation of radical mediated reaction initiates when neutral species react with a labile radical component resulting in the production of new radical intermediate. Free radical quenching under physiological condition is primarily governed by Nrf2 anti-oxidant mechanistic pathways [45]. Nrf2 is a group of endogenous anti-oxidant enzymes, heme oxygenase (HO-1), superoxide dismutase (SOD3), glucuronosyltransferase-1a6 (UGT-1a6) responsible for scavenging of

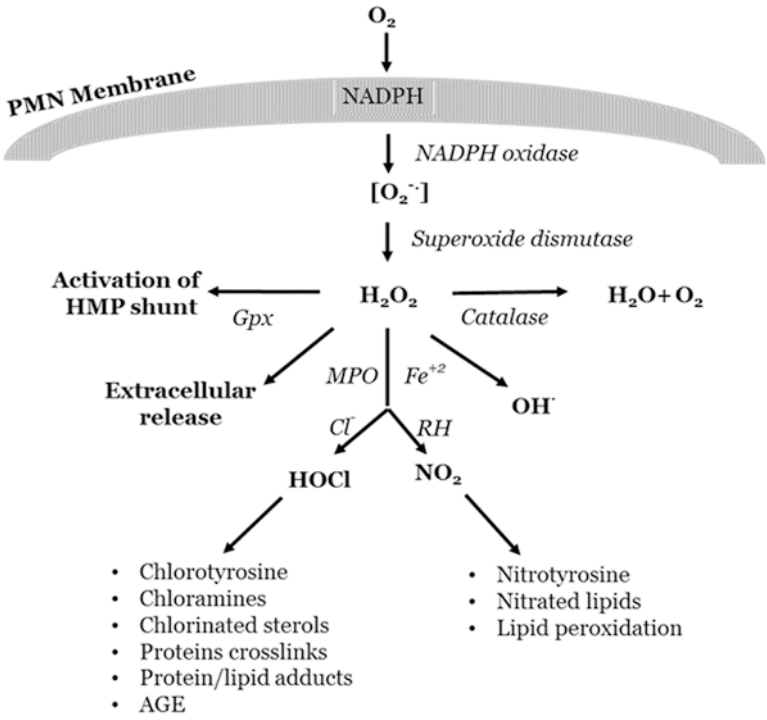


Fig. 24.2 Generation of superoxide anion catalyzed by NADPH and MPO-mediated generation of hypochlorous acid and regulating the activity of NADH oxidase

toxic radicals [46]. These endogenous anti-oxidant enzymes are cytoprotective and protect biological targets from deleterious effects of free radicals before they cause irreversible damage. Several natural as well as synthetic chemical entities act as non-enzymatic anti-oxidant mediators on the quenching of free radicals by way of terminating free radical chain reactions [47–49]. Excessive production of ROS, exceeds the capacity of antioxidants for free radical neutralization, causes severe damage to cells and tissues by reacting it with biomacromolecules. This can contribute to pathological conditions of various disease manifestations. In order to prevent the free radical chain reaction in biological systems, one way of looking at a solution, is to convert superoxide anion into molecular oxygen by oxidative elimination of an electron. This is possible because molecular oxygen carries a specific electronic configuration. Generation of secondary radical species, ROS or RNS, is facilitated by primary ROS during a chemical reaction with other molecules in a physiological environment. This process can happen with the help of either in the presence of any catalyst or any oxidative enzyme or sometime with metal-catalyzed chemical reaction. Such reaction, sometimes, can happen without the aid of any chemical mediators. Generation of multiple highly oxidative reactive-species such as hydrogen peroxide, hydroxyl radical and peroxynitrite is primarily produced by

superoxide anion (O_2^-). The formation of RNS is due to a reaction between superoxide anion and nitric oxide (NO), a small molecule vasodilator. Due to this chemical conversion, the effective concentration of nitric oxide bioavailability decreases, leading to rigid blood vessels thereby obstructing a smooth blood circulation. This may lead to onset of complications in vasculature.

24.3 Chemical Substrates

A variety of MPO substrates such as 3,3′,5,5′ tetramethylbenzedine (TMB) [50], O-dianisidine, guaiacol [51], urate [52], estradiol [53], tyrosine [54], serotonin [55], norepinephrine [56], ascorbate [40], phenols [57], aniline [58], halides [59], thiocyanate [60] and nitrite [61] have been reported in the literature. However, halides and pseudohalide (SCN, a chemical species resembling with halogen in reactivity) are considered as major substrates (Fig. 24.3).

Under normal physiological conditions, chloride ion concentration in human plasma is between 100-400 μM which is higher than bromide or pseudo-halide [62]. For halogenation activity, chloride ion is a primary substrate and hypochlourus acid is a primary product in the halogenation cycle of MPO mediated conversion. The next level of halogenation cycle is bromide and its concentration lies between 20-100 μM. Unlike chlorination mediated by hypochrlous acid, due to presence of very low concentration of bromide ion in physiological condition, oxidative bromination is not observed very prominently. The pseudo-halide, thio cyanate (SCN^-) is also an important substrate for MPO activity and its available plasma concentration also in the range of 20–100 μM. However, MPO can utilize a pseudo-halide, SCN^-, as a competitive substrate when its concentration reaches over normal plasma level concentration. This reactivity is attributed due to its higher rate constant compare to chloride or bromide ions. The reactivity of pseudo-halide with MPO is similar to that of reaction of chloride and MPO. Due to presence of more reactive sulfur functionality in pseudo-halides, in presence of MPO, it catalyzed the reaction in the formation of thiocyanates, carbamates and thiocarbamates.

24.4 Requirement of H_2O_2

MPO is able to utilize diverse co-substrates, eg. hydrogen peroxide, to produce reactive oxidants as intermediates [12]. Several stable final products engendered by these reactive intermediates were characterized [63]. MPO enhances the oxidizing capabilities ofhydrogen peroxide by employing it as a primary substrate to produce a multiple reactive oxidants and penetrable radical species via a classic peroxidase cycle [64]. During the process of immune defense against microorganism, the presence of hydrogen peroxide is essential. In the presence of halide ion, MPO in its

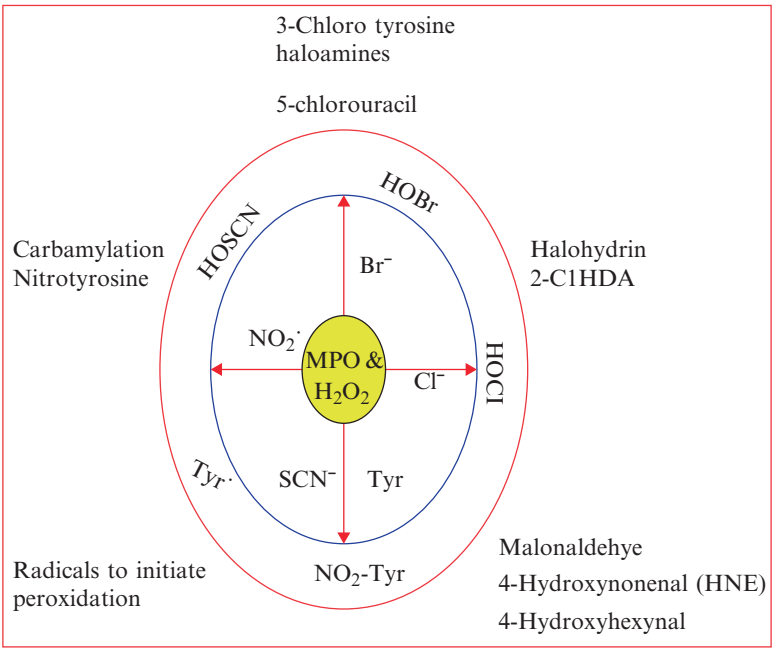


Fig. 24.3 Biomarkers of MPO-catalyzed oxidation in the physiological outcome

native form converts hydrogen peroxide into more powerful oxidant, hydrochlorous acid. The availability of hydrogen peroxide comes from a multiple sources. Superoxide anion is generated due to respiratory burst essentially mediated by NADPH oxidase [10, 65, 66] and this superoxide anion will act as a precursor for the generation of hydrogen peroxide. The reactivity of MPO intrinsically depends on the intermediates, Compound I and Compound II, through which redox mechanism operates. The redox reaction between compound I and compound II decides the mode of MPO reaction in presence of enzyme substrate, and superoxide anion is generated MPO which would enable the formation of highly oxidizable chemical entity [41, 67–69].

24.5 Catalytic Reaction and Mode of MPO Reaction

The connectivity between MPO as an element of immune response to entering foreign microorganisms that can cause havoc to physiological functions has been thoroughly studied way back in 70s and 80s. Invading pathogens are killed, through innate defense mechanism, by the production of highly reactive hypohalous chemical entities, which are an important process in fighting against microorganism. Essential role of immune cells and leukocytes in the generation of ROS shows

visible correlation between these entities. Multiple protein subunits of NADPH oxidase are present in astrocytes responsible for generating precursors for oxidizable species. Some of these subunits are gp91phox, p22phox, p40phox, p47phox and p67phox [70, 71]. Indiscriminate oxidation of biomolecules by powerful oxidants during bactericidal activity leads to impaired functions of many organs possibly due to inflammatory actions of enzyme. Existing evidence indicates the role of MPO in the manifestation of oxidative stress leading to inflammation. During neutrophilic burst, circulating monocytes and tissue macrophages in presence of MPO from intracellular storage granules along with additional protein units and other materials, plays a vital role in the inflammatory environment. The NADPH present in intracellular space catalyzes the reaction in the formation of superoxide radicals (O_2•) from oxygen molecule which occurs at the phagolysosomal membrane or in the plasma, known as oxidative burst. The quenching of superoxide anion radical, chemically inert radical and weak redox agents ensues by way of dismutation mediated by superoxide dismutases, SOD to yield hydrogen peroxide and oxygen molecule. Mechanistically, hydrogen peroxide in presence of MPO with Fe(III) porphyrin native state undergoes rapid reaction with a kinetic rate constant of $k \sim 1.4107$ M yields an intermediate, a Fe(IV)-oxoporphyrin radical-cation species (Fe(IV) = O $Por^{\cdot+}$**,** also referred as compound I via a 2-electron oxidation reaction. The formation of hypohalous acids from halides and hypothiocyanide from pseudohalide is catalyzed by electron deficient species indicative of formation of other reactive intermediates. Oxidative carbonylation of cytosolic proteins during phagocytic neutrophils was observed when reactive aldehyde, hydroxynonenal (HNE), was added [72]. This pathway is further corroborated to NADPH oxidase linked to MPO activity and was successfully deactivated by anti-oxidants such as butylated hydroxytoluene and Trolox, showing the involvement of MPO-dependent lipid peroxidation.The peroxidation cycle is attributed to generation of aldehydes from polyunsaturated fatty acids. MPO-mediated lipid peroxidation generates reactive aldehydes *viz.* malonaldehyde from poly unsaturated fatty acid, 4-hydroxynonenal from ω-6 polyunsaturated fatty acyl chains and 4-hydroxyhexinal from ω-3 polyunsaturated chains. On the other hand, the process of halogenation cycle is explained when Fe (II) in native state of enzyme undergoes a 2-electron reduction with a complete reaction cycle involving (native enzyme ------ > Compound I ------ > native enzyme). The process of formation of compound II from compound I is well explained when it likely undergoes single electron reaction with hydroxylated aromatic compounds (polyphenols or simple of substituted phenols) or various exogenous anti-oxidants (Fig. 24.4). Structurally, the compound is a Fe(IV)-oxo species. Subsequently, second electron transfer from substrate would generate a radical ion and MPO in its native form with the Fe (III) state. The process of transformation of enzyme to its native form by second electron transfer is also known as peroxidase cycle. Many organic and inorganic substrate molecules easily transfer electrons to compound I and compound II intermediates due to their tendency to accept electrons.

Investigations on the mechanism of myeloperoxidase-catalyzed reactions have shown a complex interrelationship between the substrates H_2O_2 and the halide

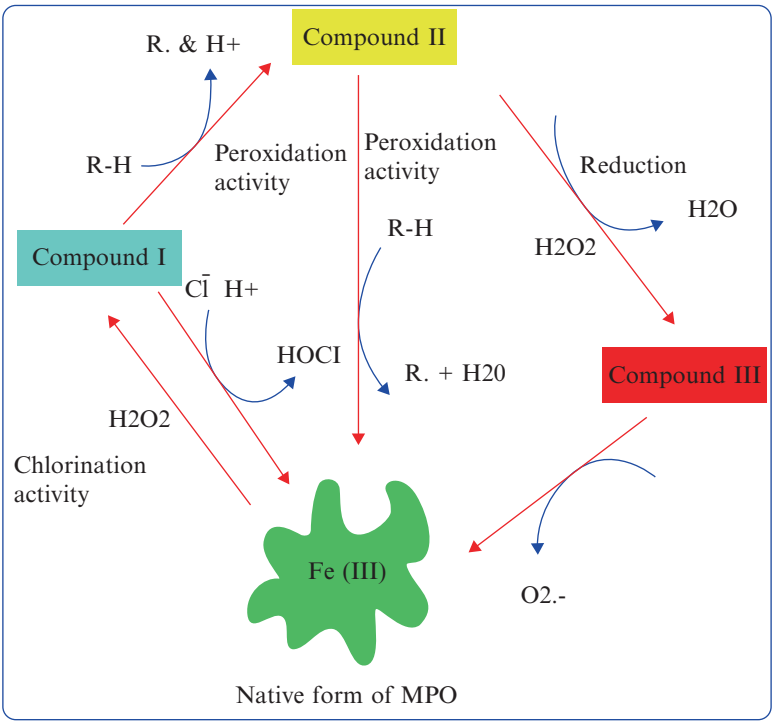


Fig. 24.4 Mechanistic pathways depicting chlorination activity, peroxidation activity, compound I and compound II generation

[73, 74]. The channelling of MPO activity depends on the rate of superoxide anion production, the presence of H_2O_2 and other co-substrates. By way of preventing MPO to reach H_2O_2 or its subsequent reduction products, the inhibitors fully inactivate the native enzyme. The underlying mechanism, in both inhibition cycles, is the reaction of Fe (III) form of MPO with H_2O_2 to form compound I - an oxoiron (IV) species with a porphyrin π-cation radical. Due to its powerful oxidant nature, the compound I catalyzes both one and two electron oxidation. The operative mechanism of MPO inhibition occurs depending upon the kinetics of the electron transfer reaction and concentration of donor species [39, 75]. The generation of Fe (III) form of MPO from compound I in the halogenation cycle releases a corresponding hypohalous acid. In case of peroxidation type mechanism, many organic small molecules tend to donate two successive elections to compound I and compound II. Compound I is reduced *via* compound II by one-electron reduction happening successively in two times (Fig. 24.5).

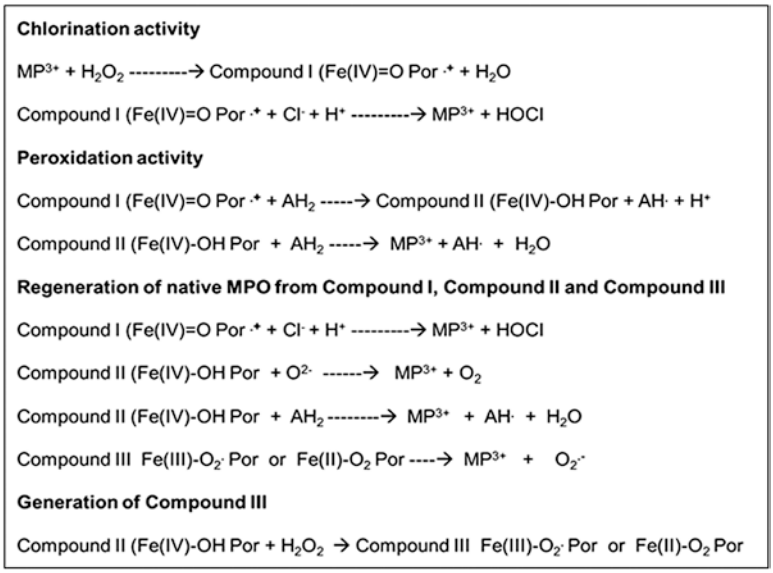


Fig. 24.5 Redox modification of heme in multiple oxidation states with subsequent generation of reactive intermediates in the catalytic cycle of MPO reaction

24.6 Immune Response System

MPO is an essential microbicidal component of innate immunity [76]. Reactive oxidants generated by phagocytes are of important application in host defenses [77], tumor surveillance, and inflammation [78] (Fig. 24.6). The combination of trio, namely, myeloperoxidase–hydrogen peroxide–chloride system provides critical pathways to generate powerful halogenating agents [79]. Mechanistically, the formation of redox intermediate compound I [80], with a ferryl oxygen, an Fe(IV) ion, and a porphyrin ð-cation radical [81] are possible due to donation of two electron by ferric state peroxidase to convert H_2O_2 to water molecule. Through compound I, MPO substrate is oxidized by way of taking one electron and one hydrogen atom and changed it into a reactive radical species. Due to this, peroxidase is getting attached to compound II, with a hydrogen atom bound to the intermediate via either to ferryl oxygen or apoprotein and porphyrin ð-cation radical abolished by the donated electron [82]. The peroxidase can get another electron and another proton from a substrate to get ferric state from compound II which subsequently changes it into a radical. MPO can additionally regain the ferric state directly from compound I by peroxidation of halide ions to toxic hypohalous acids in a two-electron transfer reduction [83]. Contribution of MPO as an immune defense system against foreign microorganism is chiefly operated through this mechanism [79, 84].

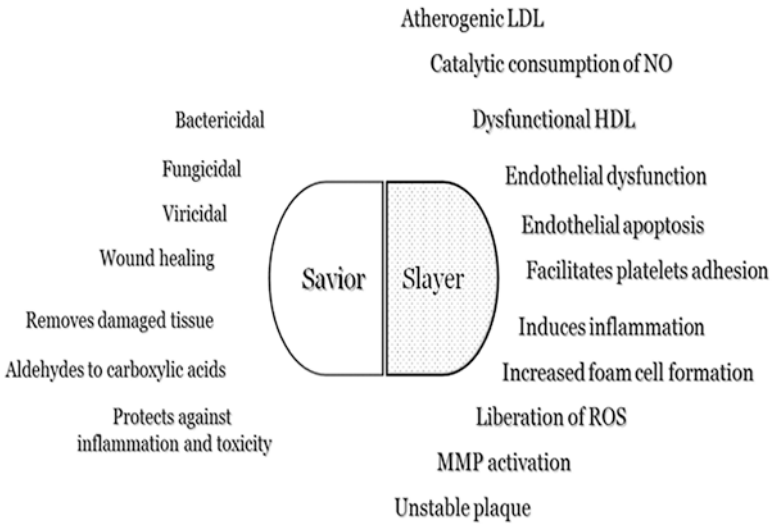


Fig. 24.6 Implication of MPO in favorable entity as a savior. On the other hand, substantial evidence to show the pathogenesis of MPO mediated diseases

24.7 MPO Gene and Variation Amongst Species

Chromosomal location as well as number of exons of MPO differs in different species as shown in the Table 24.1.The ClustalW2 analysis demonstrated 83% similarity of protein sequence between human and mouse.

To date, a single functional polymorphic site within the MPO gene is known. This variation is at position 463 due to single replacement of G to A base in consensus sequence of SP1 transcription factor. A related study demonstrated that this variation might also lead to high and low expressions of genotypes G/G and A/A, A/G respectively in in vitro gene expression assays as well as promotor activity. G) [85]. Later this observation allowed researchers to assume that carriers of the low-expressing A allele may have fewer enzymes available for the activation of MPO-mediated reaction pathways.This polymorphism has been previously identified in multiple disease conditions, such as acute promyelocyticleukemia, Alzheimer's disease [86], lung cancer [87] and multiple sclerosis [87] and atherosclerosis, however, the association of MPO genetic variation with atherosclerosis is not yet known. A related study showed that A allele is less frequent in subjects with angiographically documented coronary artery disease (CAD) [88]. In contrast, an allele of healthy population which is allied with increased lipid levels suggests its role with the risk of CAD [89].Several MPO deficiency causing genetic mutations have been identified, still several have to be undiscovered. Recent studies imply that in acquired cases, the MPO deficiency is temporary with a fraction of the polymorphonuclear

Table 24.1 Number of exons of MPO and chromosomal location of different species

Common name	Species	Chromosome location	Exons
Human	Homo sapiens	17q23.1	14
Mouse	Mus musculus	11.52.22	16
Rat	Rattusnovergicus	10q26	15
Cattle	Bas taurus	19	15
Dog	Canis lupus familiaris	9	14
Pig	Susscrofa	12	13
Horse	Equscaballus	11	13
Rhesus monkey	Macacamulatta	16	12

leukocytes (PMNs) and resolves with improval of provocative conditions. Several disease conditions, including but not limited to, diabetes, severe infection which would lead to assimilated MPO deficiency [90].

24.8 Structure, Active Site Pocket and in Silico Modelling of MPO

MPO is a dimeric and a strong cationic glycosylated protein located on human chromosome 17 in segment q12–24 with 146 kDa molecular weight [91, 92]. Two dimers of this oxidative enzyme are connected with a single disulphide bridge between symmetry-related halves (73 kDa) [91]. Each dimer consists of a light subunit of 14.5 kDa molecular weights and heavy subunit of 58.5 kDa. The heavy subunit contains a protoporphyrinIX group with a central iron atom and both heme groups are functionally identical.

To achieve an efficient approach for MPO inhibition, a complete detailed knowledge of MPO protein structure is essential to rationally design therapeutic agents. Several docking methods have been reported in the literature while aiming at creating potential inhibitors for this enzyme [93–95]. Many reversible and irreversible MPO inhibitors have been reported in the literature. Most of the reported inhibitors were tested under in vitro conditions [96, 97]. Computer-aided inhibitor design comes handy in creating rationale structures. Computational docking methods provide valuable tools to create a compound library. Feasibility of docking procedure can be validated and assessed by comparing it with experimental parameters. Crystal structures of MPO in the literature are fully known whereby Fe is in the form of ferric state [98, 99]. The active site pocket of MPO consists of two areas one distal heme cavity and funnel-shapped channel. This channel is oriented in such a way that it is connecting distal cavity to the outer surface of the MPO enzyme. There are water molecules get accumulated in the distal cavity and are hydrogen bonded with three amino acid residues namely, Gln91, His95, and Arg239 and there form hydrogen bonding between these residues within the vicinity of the distal cavity.

24.9 In Silico MPO Inhibitor Design Using SYBYL Suite

Although docking experiments could only provide interaction of inhibitors in MPO active site with non-covalent interactions among various amino acid residues and picturing tight binding mode between enzyme and inhibitor, the results provided key information on designing a new type of MPO inhibitor using ferulic acid building block. Scientifically proven technique, computational docking can be used in an efficient way; however docking results need to be analyzed in a perspective model of inhibitor design. Docking model and results need to be used as a guiding principle and carefully analyzed results can provide valuable information for target identification and validation.

In our efforts toward the creation of a new class of compounds for inhibiting MPO enzyme, several ferulic acid analogues have been designed and analyzed in a docking model using SYBYL suite platform. Several key parameters are used to maximize our designed search. Compare to other peroxidase enzymes, Compound I and Compound II reduction rates in MPO are higher and this is largely attributed due to aromatic π stacking in MPO available in heme D ring. Difference in structural features between ferric, compound I and compound II can contribute significantly to docking mode in MPO inhibition. This has greater impact than electrostatic changes or ferry oxygen addition in other peroxidase. By visualizing MPO compound I and compound II and its chemical and electrochemical illustrations, no characteristic difference in docking mode was noted. Observed redox potentials are in agreement with small differences, however, the requirement for structural features for the differences in redox potentials remains unexplored. To study the reaction rates of MPO, compound I and compound II charge distribution need to be studied in detail in addition to realize the importance of the structural features of the enzyme.

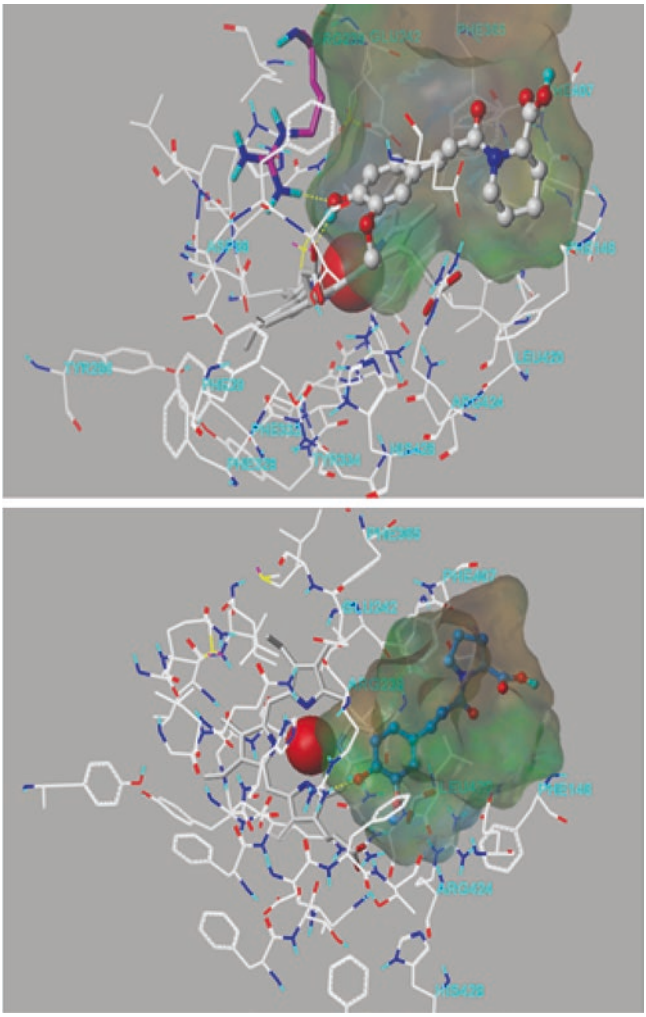
With our own extensive research work on this protein and effort toward the development of pharmacological inhibitor, we laid out a quality stride in successful drug development program on this interesting protein. Beginning with, we have extracted MPO protein structure with the code 3F9P having a resolution of 2.7 Å and it is employed as a representative example for docking methodology. SYBYL docking module has been extensively used in medicinal chemistry platform in lead identification and lead optimization for various drug discovery programs. Application of SYBYL docking in MPO inhibitor design based on ferulic acid building block in the cardiovascular therapeutic development program is unknown in the literature.A recent effort from our laboratory on the development of MPO inhibitor using ferulic acid derivatives has provided valuable information on the rational designing of MPO inhibitor(80). Ferulic acid is a known anti-oxidant possessing various medicinal values [100]. Ferrulic acid is a hydroxylated aromatic phytochemical, related to trans-cinnamic acid present in the cell wall of the plants that has been shown to exert potent anti-oxidant functions including MPO catalyzed reactions. Ferulic acid has been previously shown to reduce oxidative stress reactions [101, 102].Ferulic acid or its chemically modified derivatives could be used as

inhibitors of MPO. The molecular docking of ferulic acid derivatives at the active site of MPO provided valuable information on MPO inhibitor design.The docking methodology includes the energy computations for the interaction between molecular probe-target connections for active site and pathway to the active site. The docked mode of MPO inhibitors in the active site pocket is closer to heme edge wherein accessibility of heme edge easy for effective inhibition. For MPO, the dimensions are 20 Å X 35 Å X 30 Å. The docking probe can access through distal cavity of hemo protein and ligand position and notably the ferric ion was seen through all the investigated probes. The molecular surface of probe contact channel is 2505 Å3 which is equivalent to the volume enclosed by the solvent accessible molecular surface.

There are four distinct chains namely A, B, C and D present in the enzyme structure. Only the A and C chains are considered for modeling, while the crystal symmetry-related B and D chains are omitted. In pre-docking preparation, protons are added to the heme structure and the NH_3^+ and COO^- charged ions are kept at the terminal residues. All the coordinate bonds present in the heme moiety of the complex are manually removed and the iron atom of the heme moiety was assigned atom type corresponding to Fe^{++} with a charge of +2. In addition, it was ensured that the heme atom types were correctly assigned with two of the pyrrole nitrogen atoms assuming formal charge of −1 each. Thus, the entire heme moiety was maintained in a neutral form. Furthermore, the coordinate bonds between the Ca^{++} and the neighboring oxygen atoms were removed manually. The main reason for removal of the coordinate bonds is the inability of the force field to deal with such entities during energy optimizations. Sidechains carrying all the amide bonds of asparagine and glutamine are placed in such a way to keep their interactions with neibhoring residues and group of atoms to get maximum optimized interactions. The process of staged minimization of in the enzyme consists of the following protocols: (a) The positions of hydrogen atoms are minimized (b) sidechain atoms are minimized (c) Calcium atoms are omitted with minimization of complete protein, (d) heme structure is minimized (e) all the atoms are minimized. By utilizing Tripos Force field, energy minimization with conjugate gradient is carried out with 100 cycles of steepest descent in each step of staged minimization. For the purpose of eliminating unwanted elements attached to original X-ray structure, the process of staged minimization is used to get refined structure of the protein. This is specifically performed after the addition of hydrogen atoms and excluding coordinate bonds. The purpose of aforementioned protocol is to have an intact docking structure of 3F9P by the value of 0.2 Å between the minimized and X-ray protein atoms.The myeloperoxidase-heme complex was used as input in Sybyl-X to generate a protomol using the "automatic" preference. This was done since the complex in the X-ray complex has no ligand bound to it. The protomol is generated in the large cavity on the correct side of the heme moiety and comprising of a series of clustered probes that characterize hydrophobic (methane) and hydrophilic (NH and C = O) links with the protein atoms. Therefore, the enzyme active site pocket is a specific arrangement of sequence of amino acid residueswhich otherwise called, protomol, signifies active site of the entire protein structure. The 3-dimensional structures of the ferulic acid

derivatives used in the Surflex-docking studies were obtained by subjecting their 2-D structures to CONCORD5 within the ligand preparation module of Sybyl-X (version 1.3). In case of compounds containing the proline moiety, only the S configuration (corresponding to the natural amino acids) was considered. On the other hand, both R and S configurations of the stereochemical center in the piperidine moiety were considered. For the purpose of docking simulated studies, structures generated from CONCORD5 will have usual bond length and bond angles so that it does not alter during simulation experiment. However, the initial three dimensional structures come with required dihedral angle which would very well vary during the course of docking of ligands. The ligand structures thus prepared were docked into the 3F9P using the Surflex-dock technology described in the literature. The most accurate docking mode GEOMX was employed with default parameters. Protein flexibility was not employed while ligand ring flexibility was explored. Depending on docking score, nearly 20 structures (poses) were saved for each of the ligands. The special tool, Results Browser within Surflex-dock GUI in Sybyl-X 1.3, will analyze high-scored poses for all the top-scored ligands.Top-ranked docked poses of ferulic acid derivative (shown in ball and stick rendering) in the active site of myeloperoxidase structure in 3F9P is shown in the Figs. 24.7 and 24.8. Also shown is the surface (colored by lipophilic potential) that envelops the protomol used in the docking studies. The porphyrin ring of the heme group is shown in capped stick representation while the iron atom is represented by the red sphere. Hydrogen bonding interactions between the hydroxyl groups on the aromatic rings of 1 and 2 on one hand and the guanidine moiety of Arg239 are shown by yellow dotted lines connecting the oxygen atom on the ligand to one of the hydrogen atoms on the N atom. As can also be deemed from these illustrations, the ligand is docked in the active site of myeloperoxidase structure through a range of non-specific hydrophobic interactions in the large binding site. For example, the piperidine moiety is hydrophobically (lipophilically) enclosed by the sidechains of Phe146, Leu420, Phe407 and Phe365. However, the piperidine moiety does not optimally fill the binding cavity, suggestive of potential substitutions that can be explored to potentially increase the affinity of the ligands leading to greater inhibition of myeloperoxidase.

Analysis using the program MUSE to explore R-group substitutions on the core of lead compound provided some insight on the structure of the enzyme. Scoring function that includes theSurflex-dock scoring function and a set of penalties for deviations from drug-like properties of the newly invented moleculesare employed for R-group expansion. The R-groups used for substitutions were chosen from a database of nearly 1000 fragments commonly employed in medicinal chemistry, using a genetic algorithm that included pre-defined rules and guidelines for mutations of various kinds.



Figs. 24.7 and 24.8 Docking module with ferulic acid analogue in MPO active pocket. The pictures depict the tight binding of MPO inhibitor surrounded by critical amino acid residues in the active site region. Red ball represents heme ion at the middle of porphyrin structure

24.10 Implication of MPO in Disease Targets

In last two decades, MPO has gained much attention and a number of pioneering studies demonstrated that, except as a crucial component of the phagocytosis process, MPO had a number of unique pleotropic effects [103]. A wide range of MPO actions have been recognized in human health and diseases [24, 104]. MPO-mediated oxidative products are clinically identified and characterized in human atherosclerotic areas [105, 106] rich in macrophages and consistent with its role on

oxidative damage of biomolecules including lipids and proteins by high-sensitive mass spectrometric analysis [107]· [108, 109].Macrophages / monocytes are primarily involved in the formation of lesion generated by hypochlorous acid via the hydrogen peroxide-chloride system [105, 110, 111]. This is possibly due to co-localization of MPO and CD68-positive cells in type V and type VI lesions [112, 113]. Enzymatically active MPO exhibited enhanced secretion of cytokines from macrophages, including TNFα, IL-1, and IFa/b, and enhances the capacity to phagocytosis and also intracellular killing of microorganisms [114]. The role of mature inflammatory macrophages is to engulf fresh neutrophils. This specific functional utility cannot be performed by freshly isolated monocytes or resident lung macrophages [115, 116]. This specific observation is related to the resolution of inflammation due to manifestation of MPO-mediated oxidants [117, 118]. Critical analysis of manifestation of disease biomarkers revealed that MPO is invariably aiding at either early onset or progression of multiple diseases. MPO derived oxidants with numerous cell functions play a major role in tissue injury [22]. Elevated levels of MPO have been associated with several inflammatory diseases such as Alzheimer's [87, 119], anti-neutrophil cytoplasmic autoantibody (ANCA) mediated glomerulonephritis, Acute Respiratory Distress Syndrome (ARDS), Bechet's disease, bone marrow transplantation-induced injury, cancer, asbestos-induced injury, Chronic Obstructive Pulmonary Disease (COPD), cystic fibrosis, cirrhosis, fibrosis, dermatitis herpetiformis, diabetes, inflammatory bowel disease, ischemia-reperfusion injury, multiple sclerosis, Parkinson's disease, periodontal disease, rheumatoid arthritis, systemic inflammatory response syndrome and vasculitis (Fig. 24.9). In cardiovascular arena,

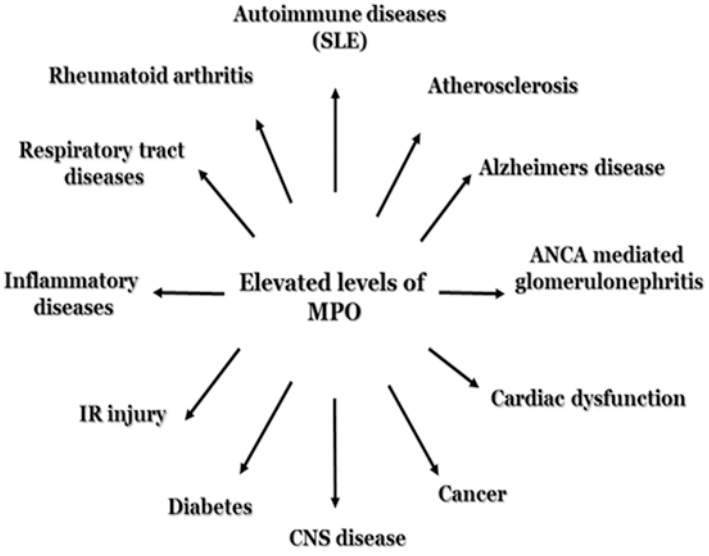


Fig. 24.9 Implication of MPO in various disease state

MPO has the most important, prominent effect in pathophysiology of cardiovascular diseases (CVD) [120–123]. Discussion on the role of MPO in all cardiovascular diseases is beyond the scope of this review. However, in view of our continued interest in the investigation on the mechanism of pathogenesis of atherosclerosis and to identify a suitable therapeutic remedy, in this review, we limit our discussion pertaining to the prospective role of MPO in atherosclerosis only.

24.11 Role of MPO in Atherosclerosis

While MPO is suggested to be implicated in killing bacteria and other pathogens, it may also be involved in the pathogenesis of many other diseases (Figs. 24.1 and 24.2). Depending on the type of infection, MPO levels will be elevated from 1.8 to100 ng/ml [124]. MPO's potential involvement in atherosclerosis has gained tremendous importance in recent years. It has been shown that macrophages in human atherosclerosis contain MPO, suggesting that MPO might play a special role in pathogenesis of atherosclerosis [125]. MPO has been localized in the atherosclerotic lesion although the presence of MPO transcript in such locations has not been demonstrated [126]. Recent pioneering studies [127] show that MPO-derived products are elevated in subjects with CVD [128, 129]. Patients with coronary heart disease specifically in acute coronary syndromes (ACS) have significantly higher leukocyte intracellular MPO due to the degranulation of circulating phagocytes [130, 131]. Evidence suggests that in normal controls MPO levels are 15-18 ng/ml whereas in atherosclerosis and coronary artery disease subjects it varies from 20 ng/ml to 80 ng/ml depending on disease severity [132]. Using state-of-the-art methodologies, MPO-derived biomarkers have been identified to be present in increasing concentrations in subjects with coronary artery diseases. These products include nitrated, chlorinated or cross-linked tyrosine residues, as well as products of lipid peroxidation and changes in the amino groups of amino acids [133–136]. While tyrosine cross-links or nitration could be accounted for by other pathways, chlorination of tyrosine has been suggested to be specific for MPO action. MPO levels of leukocytes and free blood MPO, as detected by immune assays, mainly associated with the manifestation of coronary artery disease. Studies have also shown that MPO contributes considerably to plaque vulnerability. Individuals who possess MPO levels in the fourth quartile among sequential subjects undergoing diagnostic cardiac catheterization were 15- to 20-fold more likely to demonstrate abnormal coronary angiograms compared with subjects in the lowest quartile. This relationship remained significant after statistical adjustments for Framingham risk score and for C-reactive protein. Recent studies seem to indicate that MPO is an important marker in subjects with heart failure, even when corrected for brain natriuretic peptide (BNP) [137, 138].

Several clinical studies have implicated MPO in CVD [139] specifically probed characteristic marker for coronary artery disease. The same study also showed that increased blood-MPO level was significantly greater in subjects with established

CAD compared to healthy controls. Two independent studies in 2003 revealed that blood levels of MPO could powerfully predict the incidence of major cardiac events following the initial visit of patients presenting with ACS or chest pain [129]. Interestingly, these studies also revealed that MPO predicted adverse outcome even when troponin T did not predict, a marker of myocardial necrosis commonly used to predict risk of CVD. The results of these studies shed the light on the importance of the predictive value MPO in the absence of circulating troponins, as this suggests that MPO release actually precedes myocardial infarction and identifies patients with unstable plaques before complete microvascular obstruction.

In contrast to human results, data from animal studies of atherosclerosis have failed to demonstrate such relationships. It has been identified that the irradiation of LDLR knockout mice followed by subsequent infusion of MPO knockout mice's bone marrow significantly increased the atherosclerotic lesions [19]. Similarly, MPO-knockout in apolipoprotein E knockout mouse model also did not show any impact on atherosclerosis development. These data may pointout that MPO might act as an atheroprotective agent in atherosclerotic mouse models. However, in the absence of specific MPO biomarkers, chlorotyrosine, in atherosclerotic lesions in both LDLR KO and APOE KO mouse models may indicate other mechanistic pathway operating in the system. Furthermore, murine leukocytes were identified to carry a less amount of MPO per cell as compared with corresponding human leukocyte types. There is also evidence that specific MPO gene regulatory elements might be missing in mouse MPO.In contrast, studies showed that when human MPO was over expressed in mice, there was a marginal but significant increase in atherosclerosis [140]. While the MPO concentration and genetics might play a role in the atherosclerotic susceptibility of mice, it also needs to be kept in mind that rodent atherosclerosis development is a very high cholesterol-fed, accelerated atherosclerosis. MPO's involvement might be at very early stages of atherosclerosis when oxidation is initiated. Non-specific propagation of oxidation might overwhelm the disease process under such conditions, particularly when other pathways of oxidation have been noted to be important. A recent report has shown that the Watanabe heritable hyperlipidemic(WHHL) and high cholesterol-fed rabbit model to test the involvement of MPO and showed MPO expression in macrophage-rich lesions and correlates well with atherosclerosis development [110]. In view of conflicting results on the effect of MPO in human and animals, care should be taken to draw a definitive conclusion whether to aggressively approach for anti-MPO therapy in the atherosclerosis treatment. Perhaps, a comprehensive approach in consultation with medical practitioners and drug developers has to be worked out to address the need for affected patient population. A reasonable solution can be achieved on MPO therapy taking the advantage of wide range of MPO therapeutic window. By adopting this approach immune response to bacterial infection and deleterious effects of MPO on their functional utilities can be addressed in a balanced way.

24.12 Early and Late Lesions, M1 Versus and M2 Macrophages

MPO plays a role in early and late stage lesions due to Its localization in mature human atherosclerotic plaques. It also plays a major role in plaque rupture. Macrophages can be grouped in two main categories: classically activated or M1 and alternatively activated or M2. Different subsets of macrophages have been reported. Macrophages polarize to an M1 phenotype by stimulation with bacterial component lipopolysaccharide (LPS) or interferon (IFN-γ) and M2 macrophages are induced by IL4, IL13 or IL10 [141]. Over the years, this classification has been proven to be more general as several other sub-populations have been described [142].

24.13 Activators and Inhibitors

While the implication of MPO in several disease targets has been undoubtedly well established, the remedial measures to control the effects of this enzyme are either mostly in early preclinical stage development or yet-to-start stage. This is largely due to structural variability, complex redox reactivity and undefined pathways with which chemical entities react with the protein. Under normal physiological condition, the reactivity of MPO in innate immune defense process is influenced by presence of specific chemical entities that can trigger the activity of heme enzyme. MPO undergoes a series of redox reactions in biological host medium *via* Compound I and Compound II generation. The specific reactivity of MPO on surrounding molecular environment is characterized either by peroxidation pathway or halogenation cycle. While these activators stimulate the reactivity of MPO, its oxidants indiscriminately oxidize the biological molecules, including lipids, proteins and DNA and impair them altogether. In the absence of complete deficiency of this oxidative enzyme, the role of other MPO-independent bactericidal protocols will automatically be initiated. In view of this compensatory scenario, a link between normal functioning of neutrophils and its direct interaction with MPO need thorough investigations. During the last two decades, more research papers on deleterious effect of MPO in disease manifestation are reported in the literature. However, no single MPO modulator as a drug is available to treat MPO-specific disease. There is no specific inhibitor is available for this esoteric enzyme, possibly though, it is difficult to ascertain the reactivity of this protein. There are several non-specific heme inhibitors are available for this hemo protein. Specifically, sodium azide has been widely studied as a non-specific heme inhibitor [143], needless to mention, this azide entity can also inhibit other proteins such catalase, involved in neutrophil oxidative metabolism, quenches O_2 and –OH. In view of its high toxicity profile, this structure may not be suitable for cytotoxicity studies. Although monospecific anti-(myeloperoxidase) serum can inhibit the extracellular activity of this enzyme,

the role of myeloperoxidase in normal neutrophil function can only be critically assessed when a more specific neutrophil-permeable myeloperoxidase inhibitor is available. Some reported inhibitors like, salicylhydroxamic acid [144] and amino benzoic acid hydrazide [145], inhibitors of the alternative, cyanide-insensitive [146], oxidase of plant mitochondria and some other redox enzymes, seem to act as specific MPO inhibitors.

24.14 Perspective in Inhibitor Design: Small Organic Molecules as MPO Inhibitors

Several lines of evidence suggest that MPO system damage normal tissue leading to multiple disease progression [68]. In view of its potential threat to normal functioning of health systems, it is paramount important to develop a suitable therapy to overcome its deleterious effects. Development of efficient MPO inhibitor can be possible taking into consideration of wide therapeutic range of MPO activity. This could potentially address the role of MPO inhibitors to inhibit MPO activity within therapeutic range at the same time still within a functional range for immune response. The issue of bacterial infection due to MPO deficiency can be easily addressed in view of its large therapeutic range of MPO activity [147]. Additionally, apart from MPO, there are other mechanistic pathways, like leukocyte MPO, are established to involve in the defense mechanism against invading pathogens. Many small organic molecules were reported to inhibit MPO at submicromolar or high nanomolar concentration of the inhibitor [97, 148, 149]. However, the MPO inhibitors that have been reported in the literature are only in laboratory level investigation [95, 116, 150]. Development of salicylhydroxamic is the benchmark development in the area of MPO inhibitor development. Inhibition of MPO in luminol based assay on human neutrophils stimulated by phorbol 12-myristate 3-acetate using salicylhydroxamic acid was believed to open up the window for MPO inhibitor development [98, 144, 151, 152].There are a number of experimental inhibitors of MPO are published in the literature although none have reached the shelf (Table 24.2). These include resveratrol, curcumin, betamin, triazoles, benzoxalone, 4-amino benzoic acid, 5-flurouracil, tryptophan, xanthinethione, salicylhydroxamates, penicillium extract, methylated caffeolyquinine derivatives, and methyl tridecanoic acid. In addition, common drugs such as phenylbutazone, piroxicam, salicylates, olsalazine, sulfasalazine, sulfindac, ibuprofen, aspirin, naproxen, and fulfenamic acid have been reported to inhibit MPO [153–156]. Recently it has been noted that *Ocimum sanctum* (holy basil) can also act as a MPO inhibitor *in vitro* and *in vivo* [157, 158]. Several known blood pressure reducing drugs, the inhibition of angiotensin converting enzyme (ACE), are known to inhibit MPO. For example, captopril, enalapril maleate, lisinopril, ramipril, and sodium fosinopril or of their active counter-parts are known deactivate the hemo protein [159]. Despite their

Table 24.2 Small molecular MPO inhibitors, used for enzyme inhibition and type of inhibitors

Name of the small molecule	Inhibitor type
Salicylhydroxamic acid	Reversible and competitive inhibitor
Hydrazine sulphate	Suicide substrate
Benzoic acid hydrazide	Irreversible inhibitor
4-Aminobenzoic acid hydrazide (ABAH)	Irreversible inhibitor
Indole derivative: Tryptamine, 5-Fluorotryptamine, 5-Chloro -tryptamine, melatonin, serotonin, Indazole, Indazalone	Competitive reversible inhibitor
Anti-inflammatory drugs, Dapsone, Mefenamic acid Sulphapyridine, Quinacrine, Primaquine, Aminopyrine	Reversible, competitive inhibition
Phenylbutazone, Piroxicam, methyl salicylate, Olslazine benzocaine, sulfasalazine, Diclofenac, acetaminophen Deferoxamine, Niflumic acid, Tenoxicam, indomethacin Flufenamic acid	Reversibe, competitive inhibition
2(3H)-benzoxazolone derivatives	Reversible competitive inhibition
Methimazole	Irreversible competitive inhibition
Isonicotinic acid hydrazide (isoniazid)	Suicide substrate
Propylthiouracil	Irreversible competitive inhibition
Mercaptomethylimidazole	Reversible competitive inhibition
Dimethylthiourea	Mechanism of inhibition is not known
Ambroxol, Dithiocarbamate, vitamin C, Chlorogenic acid D-penicillamine, Tiopronin, sodium aurothiomalate Thiacetazone, Aurothioglucose	Reversible inhibition of selected molecular entities
Ferulic acid derivatives	Reversible, competitive inhibitor

anti-inflammatory nature, most of these inhibitors have not been tested for their ability to inhibit the atherosclerotic or oxidative processes.

24.15 Organometallic Scaffolds as MPO Inhibitors

Considering unsustained progress in MPO inhibitor development using small molecules, there is a need to rethink the strategy whether the regular organic molecular scaffolds currently used in medicinal chemistry research encompass adequate structural diversity and structural preorganization to accomplish the desired selectivity. Hence, new strategies are needed for the design of specific molecular framework to design biological regulators to direct required biological processes. In recent times, increase in the interest for metal-organic scaffolds in drug development and biomolecular chemistry is evident from the number of published articles appearing in the

literature [160–163]. The rationale behind this change in trend is largely due to beneficial properties that it can offer to target disease. Due to its structural uniqueness, favorable kinetics, balanced redox reactions, existence of radioisotopes,and characteristic spectroscopic features, MPO is considered as a suitable platform for the modulation and target validation in drug development. In recent times, organometallic therapeutics are gaining considerable attention by different levels of researchers. One area the organometallic compounds showing prominence is cancer. The advantages of organometallic compounds over small organic molecules in drug development area are innumerable, particularly they are relatively simple structure with potent activity, specific interactions are strong at the targeted site, metal-part of the molecule provides synergetic effect. Furthermore, they are exclusively target selective due to existence of sufficient structural complexity and structural preorganization to achieve the desired protein binding selectivity Moreover, many of these metal-based structures are bio-compatible and non-hazardous. Nevertheless, direct usage of organometallic compounds to deactivate the disease causing enzymes has not been explored till date. It is, therefore, unique approach that organometallic compounds would effectively inhibit this oxidative enzyme without causing much discomfort in terms of manifesting infections for complete absence of this enzyme. In this line of investigation, we have created a series of organometallic compounds embedded with different metal ions. Our preliminary *in silico* studies with the synthesized compounds in molecular docking validated the proof of concept idea and further investigation is underway on this development (Rajagopal Desikan, FebinPrabudass, – unpublished results – 2017). Figure 24.10

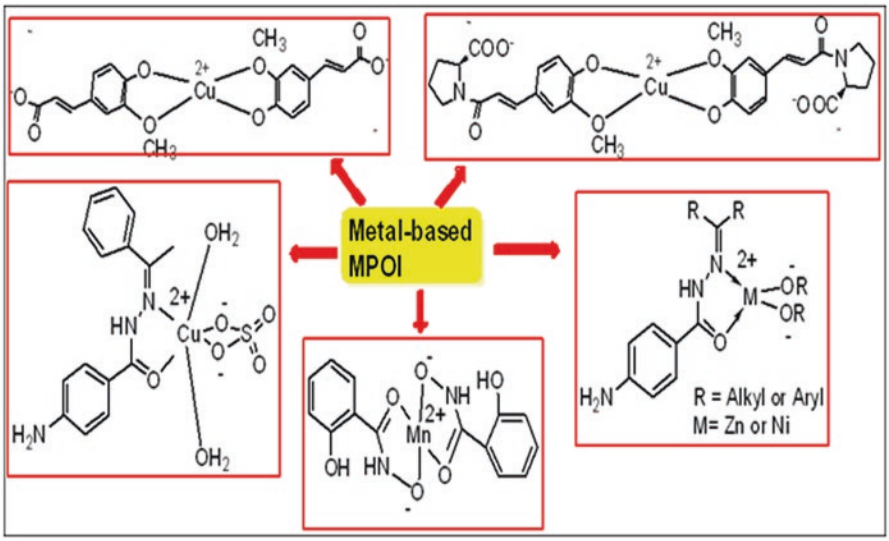


Fig. 24.10 Representative structures based on organometallic molecular scaffolds for MPO inhibition

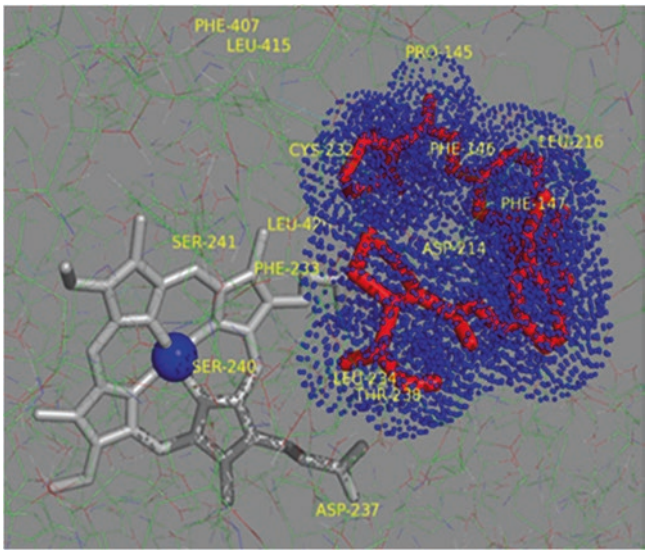


Fig. 24.11 Docking picture of ferulic acid-Cu complex in MPO protein binding

shows representative structures based on organometallic molecular scaffolds specific to MPO inhibition. Figure 24.11 represents the docking picture of ferulic acid metal complex in MPO protein binding.

24.16 Role of Nutrients in MPO Inhibition

A well-balanced diet protects against chronic oxidative stress-mediated diseases [164–167]. Fruits and vegetables contain many polyphenolic compounds that can act as antioxidants under certain physiological conditions [168–170]. The phenolic anti-oxidant mechanism has been well established in the literature [171, 172]. Advantage of polyphenols in oxidative stress management is, it can act even at low concentrations and can provide protection against harmful radical species such as superoxide radical and peroxide radical derived from reactive oxygen species and peroxynitrile derived from reactive nitrogen species. These biological agents have been reported to have antioxidant properties and scavengers of radicals [173]. Prominent molecules in these categories are anthocyanin, berries and berry products, catechins, flavonoids, green tea, phenolic acids, resveratrol, stilbenoids, and tannins are potentially excellent antioxidant sources. Some of these bioactive molecules have been tested for MPO inhibition and due to free radical scavenging ability of polyphenols these bioactive structures are prominent MPO inhibitors (155, 156, 165–167). The documentation of biomarkers of exposure, and effectwill be critical in finding those who will benefit most from bioactive molecules

available in food items carrying polyphenols are expected to interfere with MPO-mediated oxidative pathways, thereby inhibiting the formation of ROS including H_2O_2 and HOCl responsible for manifestation of disease progression. Although scant reports are available on the role of food in MPO inhibition, development of food based MPO inhibitor would certainly open up new avenues for identification of new molecular scaffold against MPO and to address many risk factors associated with MPO.

24.17 MPO, Unexplored Territory in Drug Discovery

The role of MPO in multiple disease targets has been studied only during the past decade although myeloperoxidase is known for over 25 years. Multiple approaches for MPO inactivation have been developed as likely targets as therapeutic intervention while developing specific inhibitors for this enzyme [174].

Available MPO inhibitors in the literature have diverse structural features. Most of these inhibitors can specifically interact with peroxidase. Additionally, these structures have ability to act as generic antioxidants [175]. Several lines of evidence indicate that inhibition of MPO is much more beneficial to patient population having cardiovascular complications; however, complete MPO inactivation can also lead to severe unwanted adverse effects. Important reaction to complete MPO deficiency is immune dysfunction. Mechanistically, an alternate immune response is also proposed in the literature. Leukocyte myeloperoxidase (L-MPO), like MPO from eutrophilic burst, can kill invading pathogens. This effective bactericidal system can perform its function by reacting with halide ions in the presence of hydrogen peroxide. It is well documented that leukocytes from a subject with complete MPO deficiency and from some subjects with partial deficiency have impaired bactericidal activity against certain bacterial stain [21, 176]. In MPO deficient leukocytes system, there is an increased superoxide generation and decreased chemiluminescence. Several lines of evidence suggest that clinical implications will be minimal in leukocyte MPO deficiency due to inherited defect, offering the protocol for an alternate pathway on leukocyte bacterial killing systems.

Development of new class of MPO inhibitors should aim at specific targets cells and its mechanistic pathways should focus at inhibiting MPO *via* pathological approach. The specificity of MPO inhibitors can be achieved by elaborately working out a model which would be interacting with redox intermediates (Compound I and Compound II). Several new approaches are available in the literature and more precisely a few remarkable methods can be utilized for developing more potent MPO inhibitors. Aptamers and small molecule methodologies are more viable approaches for developing efficient MPO inhibiting molecules [177]. Apart from these two, mAB approach can be utilized which would attach to specific target resulting in either activation or inactivation of enzyme structures. Application of specific antibodies for inactivation of protein targets is novel way to identify therapeutic agents. For targets like p22phox, Nox2, and Nox4, antibodies are known in

the literature which can specifically bind to targets. Nonetheless, no therapeutic effects are observed with the developed antibodies. Inactivation of MPO that is expressed in plasma membrane using specific antibody may not provide fruitful outcome due to inability of antibody to cross cell membranes. However, interference of antibody in controlling oxidative stress resulting in inhibiting ROS formation within this plasma membrane may yield a viable pathway for therapeutic development.Synthetic organic small molecular entities having 0.8kD molecular can change the activities of several bio-molecules including specific enzymes and DNA molecules. With the advent of high throughput screening capabilities, identification of lead MPO inhibitor for large data base like ZINC library may provide easy access to locate specific MPO inhibitors. Apart from MPO, using this high through put method, multiple inhibitors for other targets have been identified. The uniqueness of MPO inhibitor development strategy should involve targeting specific disease component and at the same time maintaining a normal physiological activity. It will be very interesting to note that whether partial inhibition of MPO activity has any protective role against specific disease. The role of small molecules in inhibiting MPO under *in vitro* condition and its capabilities in reducing CVD *in vivo* needs to be assessed carefully for successful therapeutic development.

Recently, nucleotide based therapeutic development, namely aptamers techniques, is gaining considerable attention for efficient approach to targeted drug discovery. Incidently, the nucleotide based aptamer technology can provide opportunity for the development of specific MPO inhibitors. Aptamers are classified as small between 25–50 nucleotides and they are single-stranded DNA or RNA macromolecules. These aptamers can form specific connections through intermolecular interactions. Aptamers get specific 3D structures which can efficiently bind to a specific target [178]. Experimentally, a large number of nucleotide sequences can be synthesized and this provides wide diversity of 3D structures of aptamers to have ability to attach itself to nearly any target. By employing exponential enrichment protocol, careful evolution of ligands by exponential enrichment methodology, SELEX, aptamers are made to undergo continuous rounds of *in vitro* selection in order to select for aptamers that have interactions with specific targets. The advantages of aptamer methodology are: (a) Nucleotides can identify targets with high specificities due to affinity, (b) To mimic native counterparts, aptamers can be chemically synthesized and it will increase the resistance against nuclease, (c) Protein isoforms can be easily targeted using aptamers, targeting particular Nox homolog over another can be easily achieved, (d) Recognition of particular form sequence which is applied for increased selection for activated proteins, (e) Therapeutic application of aptamer technology has reached FDA approval particularly multiple vascular proteins of vascular endothelial growth factor (VEGF) and factor IXa, (f) Delivery of aptamer to a specific cell type can be easily achieved by taking aptamers which can enter one cell or tissue type and make use of that pool to counter-select against aptamers that are capable of entering other cell types, (g) By integrating small molecules with aptamer to target specific cell type, expected therapeutic outcome can easily be achieved by generating chemically modified conjugates with aptamers. Therefore, aptamers would function as MPO inhibitors, and

delivery vectors for other class of MPO inhibitors. Aptamer methodology is inearly stage development, however, it has the greatest opportunity to change the approach of rendering treatment to several diseases.

24.18 Future Prospects

While our understanding of MPO-mediated events has increased and our ability to intervene in this setting has broadened dramatically, it is still very important to appropriately allocate interventions and resources, for the purposes of optimizing bringing out a molecule as a drug with minimal risk benefits ratio. It would make sense that future efforts determine a highly sensitive and specific algorithm for the determination of risk, so that clinicians caring for patients can be appropriately treated. Future discovery of this pathway will help to guide large-scale health care policy decisions.

24.19 Conclusion

It is important to consider multiple intricate factors that have been involved to deeply influence the outcome of the development of MPO inhibitors as an important therapeutics. This is not only for cardiovascular disease, but other diseases of which MPO has been implicated. The MPO inhibitors are anticipated to exert their effect on disease target, but at the same time exert little effect on immune suppression addressing the concern on the development of infections.

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