Endothelial dysfunction — A major mediator of diabetic vascular disease

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Abstract

The vascular endothelium is a multifunctional organ and is critically involved in modulating vascular tone and structure. Endothelial cells produce a wide range of factors that also regulate cellular adhesion, thromboresistance, smooth muscle cell proliferation, and vessel wall inflammation. Thus, endothelial function is important for the homeostasis of the body and its dysfunction is associated with several pathophysiological conditions, including atherosclerosis, hypertension and diabetes. Patients with diabetes invariably show an impairment of endothelial-dependent vasodilation. Therefore, understanding and treating endothelial dysfunction is a major focus in the prevention of vascular complications associated with all forms of diabetes mellitus. The mechanisms of endothelial dysfunction in diabetes may point to new management strategies for the prevention of cardiovascular disease in diabetes. This review will focus on the mechanisms and therapeutics that specifically target endothelial dysfunction in the context of a diabetic setting. Mechanisms including altered glucose metabolism, impaired insulin signaling, low-grade inflammatory state, and increased reactive oxygen species generation will be discussed. The importance of developing new pharmacological approaches that upregulate endothelium-derived nitric oxide synthesis and target key vascular ROS-producing enzymes will be highlighted and new strategies that might prove clinically relevant in preventing the development and/or retarding the progression of diabetes associated vascular complications.

1. Introduction

1.1. Vascular function and endothelium

The endothelium is a monolayer of cells covering the vascular lumen. For many years this cell layer was thought to be relatively inert, a mere physical barrier between circulating blood and the underlying tissues. It is now recognized, however, that endothelial cells are metabolically active with important paracrine, endocrine and autocrine functions, indispensable for the maintenance of vascular homeostasis under physiological conditions [1,2]. The multiple functions of vascular endothelium are summarized in Fig. 1 and include regulation of vessel integrity, vascular growth and remodeling, tissue growth and metabolism, immune responses, cell adhesion, angiogenesis, hemostasis and vascular permeability. The endothelium plays a pivotal role in the regulation of vascular tone, controlling tissue blood flow and inflammatory responses and maintaining blood fluidity [3–5].

Endothelium-derived factors with vasodilatory and antiproliferative effects include endothelium-derived hyperpolarization factor (EDHF) [1], nitric oxide (NO) [8,9] and prostacyclin (PGI2) [10], while endothelin-1 (ET-1) [11], angiotensin II and reactive oxygen species (ROS) are among the mediators that exert vasoconstrictor effects [12,13]. Endothelial cells also produce antithrombotic (NO and PGI2 both inhibit platelet aggregation) and prothrombotic molecules [von Willebrand factor, which promotes platelet aggregation, and plasminogen activator inhibitor-1 (PAI-1), which inhibits fibrinolysis] [5].

As a major regulator of vascular homeostasis, the endothelium maintains the balance between vasodilation and vasoconstriction, inhibition and promotion of the migration and proliferation of smooth muscle cells, fibrinolysis and thrombogenesis as well as prevention and stimulation of the adhesion and aggregation of platelets (Fig. 2) [5]. Disturbing this tightly regulated equilibrium leads to endothelial dysfunction.

1.1.1. Potential therapeutic target

Oxidative stress
apoptosis and inflammation having an overall antiatherogenic effect (Fig. 3) [18].

The half-life of NO is very short (less than 4 s). It is rapidly metabolized to nitrite and then to nitrate before being excreted in the urine [4]. Alternatively, NO can also be an endocrine vasoregulator, modulating blood flow in the microcirculation [19]. Importantly, reduced eNOS expression and/or NO bioavailability is associated with endothelial dysfunction [20,21].

1.2.1. Decreased formation of NO

eNOS is a dimeric enzyme depending on multiple cofactors for its physiological activity and optimal function. eNOS resides in the caveolae and is bound to the caveolar protein, caveolin-1 that inhibits its activity. Elevations in cytoplasmic Ca²⁺ promote binding of calmodulin to eNOS that subsequently displaces caveolin and activates eNOS [22,23].

eNOS utilizes L-arginine as the substrate, and molecular oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as co-substrates. Flavin adenine dinucleotide, flavin mononucleotide, tetrahydrobiopterin (BH₄) and calmodulin are the cofactors [4]. A reduced expression and/or activity of eNOS could be responsible for a decrease in NO production. Oxidative stress leads to eNOS uncoupling, a process where eNOS is converted from an NO-producing enzyme to an enzyme that generates superoxide anion (O₂⁻). Mechanisms implicated in eNOS uncoupling include oxidation of BH₄ [24], depletion of the enzyme substrate L-arginine, and accumulation of endogenous methylarginines [25]. More recently, S-glutathionylation of eNOS has also been proposed as a mechanism that leads to eNOS uncoupling and decreased NO bioavailability [26]. Additionally, increased expression of caveolin-1 in the endothelium (as described in diabetes and obesity [190]) leads to impaired activation of eNOS.

eNOS activity within the endothelial cell is also modulated by circulating factors like insulin. Insulin is an essential hormone in metabolic homeostasis with a vasodilator action exerted through the phosphatidylinositol-3 kinase (PI-3K)/AKT pathway-dependent eNOS activation [27]. Insulin can modulate eNOS activity by increasing BH₄ synthesis [28]. Insulin-stimulated endothelial dependent vasodilatation is impaired in insulin resistance [29,18]. Conversely, eNOS plays a major role in the regulation of insulin sensitivity due to the functions of NO in peripheral tissues [30]. Previous studies have shown that mice lacking eNOS are more likely to develop insulin resistance [227]. Apparently, modulation of eNOS phosphorylation in mice is sufficient to affect systemic insulin sensitivity indicating that eNOS phosphorylation may be a novel target for the treatment of insulin resistance [228].
eNOS may be inhibited by endogenous products of arginine metabolism such as asymmetric dimethyl-l-arginine (ADMA) [1]. Following oxidative stress or angiotensin II administration, the observed elevation in ADMA levels reduces NO formation and leads to endothelial dysfunction. Indeed, in several prospective studies, ADMA has been noted to be an independent predictor of cardiovascular events [33–35].

Another factor that regulates eNOS activity in the setting of metabolic disease is adropin, which was recently recognized to be an important regulator of energy homeostasis and insulin sensitivity. Lovren and colleagues [36] demonstrated that adropin is expressed in endothelial cells and improves angiogenesis-related responses via activation of Akt, eNOS, and extracellular signal regulated kinase 1/2. Like adiponectin and leptin, adropin may be an endocrine factor that influences both insulin resistance and endothelial functions such as vasodilation and angiogenesis [36].

1.2.2. Accelerated breakdown of NO

Accelerated degradation of NO by ROS is probably the major mechanism impairing NO bioavailability in states of cardiovascular disease [37,38]. In a diabetic milieu, an increment in O$_2^•^-$ levels is observed in the vasculature (Fig. 4). O$_2^•^-$ readily reacts with NO to form peroxynitrite (ONOO$^-$), reducing NO bioavailability and contributing to impaired vasorelaxation [39]. Fig. 5 shows an increase in nitrotyrosine staining in the aorta of a type 2 diabetic animal model, indicative of peroxynitrite formation. Additionally, lipid peroxyl radicals react with NO at almost diffusion-limited rates and may be a source of NO inactivation [40]. Also, oxidized low-density lipoprotein (LDL) cholesterol may react with endothelial NO before it reaches the vascular smooth muscle cell and therefore reduce total NO-mediated vasodilation [41].

1.3. Prostacyclin

PGI$_2$ is the major metabolite of arachidonic acid produced by cyclooxygenase in the endothelium. PGI$_2$ activates adenylate cyclase, leading to increased production of cyclic AMP and VSMC vasodilation. Additionally, PGI$_2$ is a potent antiproliferative agent in vascular smooth muscle cells, and it reduces oxidative stress and prevents cellular adhesion to the vascular wall [42]. PGI$_2$ also inhibits platelet aggregation. Clinical and experimental models of diabetes are associated with decreased secretion of PGI$_2$ [3,27].
1.4. Endothelium derived hyperpolarizing factor

There are smaller arteries in which endothelium-mediated vasodilation is predominately affected by endothelium-dependent hyperpolarization of vascular smooth muscle cells. The mechanism partially responsible for the endothelium-dependent vasodilation of these arteries, which persists in the presence of inhibitors of eNOS and prostacyclin, was first attributed to a non-characterized endothelial factor termed EDHF [43–45]. The relative importance of the EDHF mediated mechanisms to NO mediated mechanisms alters with vessel size [46]. NO is an important endothelium-dependent mediator of vascular tone in relatively large arteries and larger arterioles. At the level of the aorta, reduced NO bioavailability is proposed to be the main marker for endothelial dysfunction. In resistance arteries, NO, prostacyclins and EDHFs are thought to be involved in mediating endothelial function [47]. Alterations in EDHF-mediated responses have been reported in diabetes [48]. Interestingly, EDHF synthase/cytochrome P450 epoxygenase is also a source of superoxide anion [52].

2. Endothelial dysfunction

In the earliest stages, the principal endothelial alteration is merely functional. Functional impairment of the vascular endothelium is found in all forms of cardiovascular disease [3,12] and also in people with insulin resistance, obesity and type 2 diabetes [18]. The hallmark of endothelial dysfunction is the impaired NO bioavailability. Additionally, endothelial dysfunction is characterized by one or more of the following features: reduced endothelium-mediated vasorelaxation, hemodynamic deregulation, impaired fibrinolytic ability, enhanced turnover, overproduction of growth factors, increased expression of adhesion molecules and inflammatory genes, excessive generation of ROS, increased oxidative stress, and enhanced permeability of the cell layer [53–57].

Numerous risk factors directly contribute to endothelial dysfunction. Some of the more important are: elevated LDL cholesterol and oxidized LDL; low high-density lipoprotein (HDL) cholesterol; elevated triglycerides; hypertension; elevated C-reactive protein (CRP) and circulating lipoprotein-associated phospholipase A2 (Lp-PLA 2 — a specific marker of vascular inflammation); hyperglycemia; elevated omega-6:omega-3 ratio [58]; hyperinsulinemia; elevated homocysteine levels; increased fibrinogen and PAI-1; smoking; insufficient vitamin D; among others [59–61].

The presence of endothelial dysfunction has been implicated in the pathogenesis of atherosclerosis and thrombosis, both for the loss of its protective capability and for the induction of proatherothrombotic mechanisms. The major features associated with endothelial dysfunction are summarized in Table 1.

2.1. The impact of diabetes on the vasculature

Diabetes is not only a metabolic disease but also considered as a vascular disease because of its effect on macro and microcirculation of many vascular beds. The link between diabetes and an increased incidence of cardiovascular disease is well established [62,63]. Recent evidence shows that etiopathogenesis of endothelial dysfunction differs in types 1 and 2 diabetes [64]; it is present at the earliest stages of metabolic syndrome and insulin resistance, and may precede the clinical diagnosis of type 2 diabetes by several years [65].

The metabolic milieu in diabetes (i.e. hyperglycemia, excess free fatty acid release and insulin resistance) induces a vicious circle of events in the vascular wall, involving increased endothelial dysfunction, oxidative stress, low-grade inflammation and platelet hyperactivity, in the early stages of diabetic disease. Thereby, activation of these systems impairs endothelial function, augments vasoconstriction, increases inflammation, and promotes thrombosis [62,63]. In Fig. 6 multiple mechanisms that promote atherogenesis are summarized.
2.1.1. Hyperglycemia

Prolonged hyperglycemia and also transient, acute hyperglycemia have been proven to impair endothelial function in both macro- and microvascular beds in animal studies and in human subjects [67–69]. Although the effect of intensive glycemic control on the prevention of macrovascular disease is less profound than on the reduction of microvascular complications [70].

Hyperglycemia causes vascular damage in different cells of the vascular wall (Table 2). The mechanisms are diverse and include: 1) increased flux of glucose and other sugars through the polyol pathway; 2) augmented intracellular formation of advanced glycation end products (AGEs); 3) increment in the expression of the receptor for AGEs (RAGE) and its activating ligands; 4) activation of protein kinase protein kinase C (PKC) isoforms; and 5) overactivation of the hexosamine pathway [74]. The common pathway is oxidative stress. ROS decreases the metabolism of glucose through glycolysis, and the flux through the alternative polyol and hexosamine pathways is increased. Hyperglycemia induced oxidative stress [71] leads to DNA damage and activation of nuclear poly(ADP-ribose) polymerase (PARP) that in turn increases production of polymers of ADP-ribose reducing glyceraldehyde 3-phosphate dehydrogenase activity. Ultimately the levels of all upstream glycolytic intermediates increase. The accumulation of glycolytic intermediates activates damaging mechanisms: PKC pathway, hexosamine and polyol pathways and AGE formation. The overall effects of these mechanisms are increased oxidative stress, apoptosis and vascular permeability [74].

Additionally, glucotoxicity induces a low-grade proinflammatory condition, due to the activation of transcription factors such as nuclear...
factor-κB (NF-κB) [74–76]. NF-κB is a key mediator that regulates multiple proinflammatory and proatherosclerotic target genes in endothelial cells, VSMC, and macrophages. Activation of NF-κB leads to an increased production of adhesion molecules, leukocyte-attracting chemokines and cytokines activating inflammatory cells in the vascular wall. A prothrombotic state is generated by the increased production of lesion-based coagulants, such as tissue factor, and the inhibitors of fibrolysis, such as PAI-1 (Table 1).

Vascular tone and remodeling are enhanced through reduced NO and an increased activity and production of vasoconstrictors (ET-1, angiotensin II, and prostanoids) and an increased activity and production of vasoconstrictors (ET-1, angiotensin II, and prostanoids). Insulin signaling is transduced via two major pathways: metabolic and hemodynamic effects are mediated by PI-3K and the Ras–mitogen-activated protein kinase (MAPK) pathway is mainly involved in gene expression regulation, cell growth and differentiation [88]. Normally, insulin stimulates NO production in endothelial cells by activating NO synthase via the PI-3K pathway. In insulin resistance (IR) this pathway is impaired, and the production of NO is diminished [89]. Instead, insulin resistance activates MAPK leading to endothelial dysfunction. Insulin stimulates production of the vasoconstrictor ET-1, and increases PAI-1 and cellular adhesion molecule expression [90]. In addition to the direct effects of IR on the endothelium, it also stimulates VSMC proliferation, migration, and altered reactivity, for example, through retnin–angiotensin activation.

Elevated glucose can foster glycation of proteins, promoting formation of AGEs (Fig. 8), protein cross-linking, and ROS formation. Accumulation of AGEs alters the functional properties of matrix components and mediates sustained cellular changes. Glycation modifies the structure of the molecules and disturbs their function and receptor recognition properties. In turn, binding of AGEs to their RAGE receptor increases intracellular enzymatic superoxide production [79,80] and promotes macrophage-mediated inflammation in the vessel wall [81]. AGEs also decrease NO bioavailability and eNOS expression [82,83] and increase expression of ET-1 in endothelial cells [84]; therefore altering the balance between NO and ET-1 to favor vasoconstriction and endothelial dysfunction.

Thus, accelerated formation of multiple biochemical species under hyperglycemic conditions such as nonenzymatic reactive Amadori products, 3-deoxyglucosone, diaclyglycerol, methylglyoxal [85], AGEs, ROS, and nitrosylated species, greatly contributes to endothelial dysfunction in diabetes. The increased oxidative stress seems to be the common alteration, triggered by a type 2 diabetes milieu, in which hyperglycemia is adjoined by insulin resistance, hyperinsulinemia, and dyslipidemia [86].

### 2.1.2. Insulin resistance

Insulin resistance refers to a decreased ability of insulin to promote glucose uptake in skeletal muscle and adipose tissue and to suppress hepatic glucose output [67]. Insulin signaling is transduced via two major pathways: metabolic and hemodynamic effects are mediated by PI-3K and the Ras–mitogen-activated protein kinase (MAPK) pathway is mainly involved in gene expression regulation, cell growth and differentiation [88]. Normally, insulin stimulates NO production in endothelial cells by activating NO synthase via the PI-3K pathway. In insulin resistance (IR) this pathway is impaired, and the production of NO is diminished [89]. Instead, insulin resistance activates MAPK leading to endothelial dysfunction. Insulin stimulates production of the vasoconstrictor ET-1, and increases PAI-1 and cellular adhesion molecule expression [90]. In addition to the direct effects of IR on the endothelium, it also stimulates VSMC proliferation, migration and in adipose tissue is associated with excessive release of free fatty acids (FFAs), which evokes pathogenic gene expression through PKC activation and increased oxidative stress [93]. IR-induced excess of FFAs is essential also in the development of dyslipidemia, which further promotes the development of a proatherogenic lipid profile.

Ultimately, insulin resistance and type 2 diabetes are associated with low-grade inflammation being reflected in increased serum levels of tumor necrosis factor-α (TNF-α), interleukin-6, PAI-1, ET-1 and high-sensitive C-reactive protein, also related to endothelial dysfunction [98].

#### 2.1.3. FFAs

Excessive release from adipose tissue and diminished uptake by skeletal muscles increase circulating levels of FFAs in diabetes [99,100]. Acute infusion of FFAs reduces endothelium-dependent vasodilation in animal models and in humans in vivo [75,101].

Lipotoxicity by FFAs may impair endothelial function by a number of related mechanisms, including increased production of ROS, increase AGE formation and activate PKC, the hexosamine pathway, and proinflammatory signaling to the same extent as diabetic levels of hyperglycemia. FFAs have been shown to induce ROS production in the vasculature via mitochondrial uncoupling and by increasing the expression and protein content of NADPH oxidases [74,93]. FFA-induced overproduction of superoxide inactivates two important antiatherogenic enzymes: prostacyclin synthase and eNOS. ROS also decreases the concentration of intracellular glutathione and makes vasculature more prone to oxidative damage.
Oxidative stress describes the condition wherein an excessive production of ROS overwhelms endogenous antioxidant defense mechanisms. The resultant elevation in ROS levels has a detrimental effect on cellular function, a consequence of ROS-induced damage to lipid membranes, enzymes and nucleic acids [108].

Risk factors for cardiovascular disease (CVD), including type 2 diabetes, are characterized by excess vascular production of ROS [108,109]. One of the earliest consequences of oxidative stress in human subjects is impaired endothelium-dependent vasodilation [108]. Thus, accessing oxidative stress in the vasculature could evaluate the risk for development of vascular disease (Table 3).

2.2. Oxidative stress

2.2.1. Reactive oxygen species: major sources in the vasculature

All layers of the vascular wall have enzymatic systems capable of producing ROS. ROS include the superoxide anion, the hydroxyl radical, NO, lipid radicals, hydrogen peroxide (H₂O₂), hypochlorous acid and peroxynitrite [108].

The most important sources of ROS generation in the vasculature include the mitochondrial electron transport chain [114–116], NADPH oxidases [117–119] and xanthine oxidase [108,120]. In addition, uncoupled eNOS and enzymes, such as lipoygenase and cyclooxygenase, cytochrome P450s, peroxidases and other hemoproteins [108] are sources of ROS.

2.2.1.1. NADPH oxidases. NADPH oxidases are multicomponent enzymes functional in membranes of various cell types including endothelial cells and smooth muscle cells. NADPH family is the predominant source of O₂•− in the human vasculature [117,119]. Of the seven Nox isoforms discovered, only Nox1, Nox2, Nox4 and Nox5 are expressed in blood vessels, with different cell-specific expressions, mode of activations and functions (for a review see [110,112]).

Activation of NADPH oxidases in the vasculature occurs in response to angiotensin II, other vasoactive hormones (e.g., ET-1), growth factors (e.g., transforming growth factor-β), cytokines, and mechanical stimuli (shear stress and stretch), among others [121,110]. Evidences from the literature clearly point to a role of Nox isoforms in vascular disease although their relative contribution remains unclear [110]. Nox1 and Nox2 have been implicated in the pathogenesis of cardiovascular disease [110].

Table 3

<table>
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<tr>
<th>Approach</th>
<th>Examples</th>
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<tr>
<td>Markers of increased pro-oxidant activity</td>
<td>Increase in oxidant-generating systems (NADPH oxidases, xanthine oxidase, mitochondrial ROS, NOS)</td>
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<tr>
<td>Markers of decrease in antioxidant activity</td>
<td>Direct measurements of ROS/RNS generation (reduction of NBT, oxidant-sensitive dyes, direct radicals measurement by ESR)</td>
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<tr>
<td>Altered cellular redox state</td>
<td>Low-molecular-weight antioxidants (vitamins C and E, GSH)</td>
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<tr>
<td>Oxidative damage parameters</td>
<td>Antioxidant enzymes (SODs, GPx, GR, catalase, thioredoxin system, paraoxonase)</td>
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<td>Total antioxidant capacity</td>
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<td>Resistant to an external oxidant</td>
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ROS, reactive oxygen species; RNS, reactive nitrogen species; NOS, nitric oxide synthase; NADPH oxidases, nicotinamide adenine dinucleotide phosphate oxidases; NBT, nitroblue tetrazolium; ESR, electron spin resonance; SOD, superoxide dismutase; GR, glutathione reductase; GSH/GSSG, reduced glutathione/oxidized glutathione ratio; GPx, glutathione peroxidase; 4-HNE, 4-hydroxynonenal; MDA, malondialdehyde.
2.2.2. Endothelial nitric oxide synthase

Oxygenase also been shown to prevent hyperglycemia-associated production of ROS in blood vessels and seems to have a role in endothelial and VSMC growth [112].

2.2.3. Mitochondria

Enzymes of the inner mitochondrial membrane transfer electrons along the electron transport chain which generates a proton gradient, enabling ATP synthase to generate ATP. Under physiological conditions, this process produces ROS as byproducts [116,131,132]. Several mitochondrial antioxidant systems are in place to protect against ROS-induced damage to mitochondrial proteins, lipids and nucleic acids. However, under conditions of oxidative stress, these antioxidant systems are overwhelmed, allowing ROS to exert their deleterious effects and ultimately change mitochondrial function [116,131,134].

2.3. Mechanisms of defense against oxidative stress

The vasculature is endowed with protective antioxidant defense mechanisms, both enzymatic and nonenzymatic, to counteract the detrimental effects of ROS. Non-enzymatic antioxidant molecules include ascorbic acid (vitamin C), α-tocopherol (vitamin E) and glutathione, while superoxide dismutases (SODs), catalase, glutathione peroxidases (GPx) and thioredoxins represent important antioxidant enzymes which act to directly scavenge ROS, converting them to less reactive species [138–140].

2.3.1. Superoxide dismutases

The SODs represent the first and most important line of enzymatic antioxidant defense against ROS. A ubiquitous family of enzymes, SODs catalyze the conversion of O$_2^-$ to H$_2$O$_2$ and O$_2$ [138,139,141]. Three distinct isoforms of SOD have been identified in vascular tissue: Cu/Zn SOD (encoded by SOD1 gene) is located in the cytoplasm, MnSOD (encoded by SOD2 gene) in the mitochondria and extracellular SOD (encoded by SOD3 gene).

The importance of SODs as an antioxidant defense mechanism has been highlighted by gene transfer studies wherein SOD overexpression improved endothelial function [142,143]. Overexpression of SOD2 has also been shown to prevent hyperglycemia-associated production of O$_2^-$, activation of PKC and AGE formation [144], supporting a role for mitochondrial ROS production in diabetic macrovascular disease.

2.3.2. Catalase

Catalase is a highly catalytically efficient enzyme, primarily located in peroxisomes but also functions in the cytosol and catalyzes the conversion of H$_2$O$_2$ to water following dismutation of O$_2^-$ by SOD [138,139,145]. Inherited catalase deficiency has been linked to elevated cardiovascular risk and increased incidence of diabetes mellitus [139]. However, experimental investigation has provided evidence that catalase provides only moderate protection against oxidative stress [146].

2.3.3. Glutathione peroxidases

GPx are a family of enzymes with an important role in antioxidant defense. Like catalase, GPx reduce H$_2$O$_2$ to water and lipid hydroperoxides to their corresponding alcohols. Detoxification of secondary oxidation products is vital and GPx play an important role, reducing lipid peroxides [138,139,147].

There are several isozymes, GPx1 is the most abundant form in mammalian tissues. Mice with a disrupted GPx1 gene exhibit increased susceptibility to oxidative stress-inducing agents [148], while induction of this isozyme has been shown to provide protection against oxidative damage in endothelial cells [149]. In apo-deficient mice, the deficiency of GPx1 accelerates and modifies atherosclerotic lesion progression [31,150]. Furthermore, transgenic GPx1 expression was observed to impair endothelial dysfunction [151]. Similarly, deficiency of GPx3 has been associated with decreased NO bioavailability and increased platelet-dependent thrombosis [139]. GPx4 knockout mice are not viable; they die during early embryonic development.

Glutathione is the principal low molecular weight, non-protein thiol in the cell [138]. Mainly found in the reduced state, glutathione has numerous functions in metabolism, signal transduction and gene expression [152]. GSH acts as an electron donor and can directly scavenge ROS but also acts as a cofactor in the conversion of H$_2$O$_2$ to H$_2$O by GPx [138,139].

Additional selenoproteins with similar antioxidant activities to GPxs include the thioredoxins [139], while the glutathione-S-transferases (GSTs) are examples of selenoproteins containing enzymes of significant importance in secondary oxidative stress defense, acting to detoxify reactive electrophiles [139,147].

2.3.4. Thioredoxin

Thioredoxin (Trx) seems to exert most of its ROS-scavenging properties through Trx peroxidase (peroxiredoxins), which uses endogenous SH groups as reducing equivalents. Thioredoxin is present in endothelial- and vascular smooth muscle cells. Trx scavenges ROS and ONOO$^-$ and also reduce disulfides in proteins, peptides, and oxidized glutathione (GSSG) [140,153].

2.3.5. Heme oxygenase

Heme oxygenase (HO) has indirect antioxidant effects through breakdown of free heme and the production of carbon monoxide, as well as biliverdin and bilirubin, which themselves have antioxidant properties [154]. There are two isoforms of this enzyme, a constitutive heme oxygenase, HO2, which is ubiquitously expressed in endothelial cells, and HO1, which is induced in response to oxidative stress, probably as an adaptive response. There is extensive evidence that HO1 can protect against vascular damage and atherosclerosis [14,124,155]. The carbon monoxide has antiproliferative and anti-inflammatory as well as vasodilatory properties [156]. Genetic models of HO1 deficiency or overexpression of HO1 suggest that the actions of HO1 are important in modulating the severity of atherosclerosis [157].

2.3.6. Paraoxonase

The paraoxonase (PON) family of enzymes acts as vascular antioxidant defense and protects against vascular disease [158]. The PON1 and PON3 enzymes are synthesized in the liver and circulate in plasma associated with HDL. The capacity of HDL in decreasing HDL and LDL lipid peroxidation largely depends on its PON1 content [158]. PON1 knockout mice are more prone to atherosclerosis [159] and low PON1 activity predicts acute cardiovascular events in human prospective studies [160]. Deletion of PON1 gene increases oxidative stress in mouse macrophages [161]. PON2 is expressed in many cell types. The enzyme has been shown to reduce ROS in human endothelial cells and vascular smooth muscle cells [162]. PON2-deficient
mice with an apoE \(^{-/-}\) background developed more atherosclerotic lesions, whereas PON2-overexpressing mice were protected against those lesions [163].

Diabetes is characterized by increased oxidative stress and by decreased PON1 activity [164]. The ability of PON1 to protect against oxidative stress involved in major diseases such as atherosclerosis and diabetes underlines the notion that strategies aimed at increasing PON1 activity and/or expression would have several benefits.

3. Potential therapeutic targets

In type 2 diabetes, glucotoxicity, lipotoxicity, insulin resistance and a mutual interaction between these factors occur to foster the development and progression of endothelial dysfunction. Conventional therapies to reduce hyperglycemia, dyslipidemia and insulin resistance represent important clinical options to improve endothelial function and delay the progression of vascular complications [165]. These conventional therapies and their effect on vascular function have been evaluated and reviewed elsewhere [166–169]. Noteworthy, most of these therapies are not completely effective in slowing vascular disease and would benefit from adjunct cardiovascular protective therapies.

3.1. Cardiovascular therapies targeting the endothelium

The endothelium is a highly important target for therapy in cardiovascular disease [170]. It is rapidly and preferentially exposed to systematically administered agents and establishes a link with the underlying tissue, providing the researcher with a useful therapeutic target.

3.1.1. Potential therapeutic options for treating endothelial dysfunction by modulating eNOS

The vascular tone of arteries is primarily controlled by the bioavailability of NO, a key factor in vascular protection by preserving vessel reactivity. Thus, multiple potential therapeutic targets have been identified along the L-arginine–NO pathway that could increase NO bioavailability. In Fig. 9 these sites are identified and include: at the level of the substrate, L-arginine; at the level of the NO-generating enzyme, eNOS; at the level of the soluble guanylyl cyclase, its main target; and at the level of the main effector of NO action, cGMP [5].

Administration of NO donors such as pentaerythritol tetranitrate (PETN) reduces oxidative stress (probably by inducing HO1) and improves endothelial dysfunction [135]. Thus, diabetic patients would benefit greatly from organic nitrate treatment devoid of classical adverse effects, such as nitrate-induced vascular oxidative stress, nitrate tolerance, and endothelial dysfunction (cross-tolerance) [125].

NO availability can be increased augmenting NO production by eNOS. The simplest way to modulate eNOS is administration of its substrate L-arginine [126] or its essential cofactor BH4 or BH4 analogs (Figs. 9, 10). Folic acid and its active form 5-methyltetrahydrofolate can modulate eNOS by improving BH4 bioavailability in the vasculature by preventing its oxidation [218]. Midostaurin, betulinic acid and ursolic acid upregulate eNOS and simultaneously decrease NADPH oxidase expression. Novel small molecules AVE9488 and AVE3085 are two eNOS transcription enhancers that reverse eNOS uncoupling and preserve eNOS functionality and consequently increase NO bioavailability [136,171]. There is evidence that a cell-permeable peptide antagonizes the inhibitory actions of caveolin-1 on eNOS leading to increased NO production [217]. Statins, angiotensin II type 1 receptor blockers, estrogens and erythropoietin enhance BH4 synthesis by stimulating GTP cyclohydrolase 1 (GCH1) expression or activity. In vivo activation of AMP-activated protein kinase (AMPK) normalizes endothelial function due to an inhibition of GCH1 degradation associated with diabetes [137]. Statins, angiotensin II receptor blockers, ACE inhibitors, the aldosterone antagonist eplerenone and the renin inhibitor aliskiren prevent BH4 oxidation by decreasing the expression and/or activity of NADPH oxidase (Figs. 9, 10). Statins can also directly activate eNOS via post-translational mechanisms involving activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway [172].

In addition, cGMP levels may also be increased by inhibiting its metabolism by the phosphodiesterase-5 (PDE5) enzyme. The strategy of increasing the downstream mediator cGMP without affecting NO levels may be preferred due to the mixed outcomes in stroke reported in animal models following alterations in NO levels [173].

sGC stimulators and activators can treat the 2 forms of sGC insufficiency (i.e., diminished NO bioavailability and reduction of the catalytic capacity of sGC). Preliminary studies with both PDE5 inhibitors and sGC-targeted drugs have shown promising results [174–176].

3.1.2. Therapeutic approaches to reduce oxidative stress and/or increase antioxidant defense systems

Given the crucial role of ROS in endothelial function, considerable efforts have been made to discover therapies to reduce ROS in the vasculature. Despite promising initial observations, clinical trials with antioxidant vitamins C and E failed to show an improved cardiovascular outcome. Eventually new antioxidant molecules, targeted to the precise locations where ROS concentrations are elevated may, at an early stage, inhibit the mechanisms leading to diabetic complications [177].

The ability of PON1 to protect against oxidative stress and hydrolyze homocysteine thiolactone, a metabolite of homocysteine that can impair protein function promoting endothelial dysfunction, underlines the notion that strategies aimed at increasing PON1 activity and/or expression can be beneficial. Certain drugs (e.g. hypolipemic and antidiabetic compounds), dietary and lifestyle factors (e.g. antioxidants, polyphenols, moderate wine consumption) appear to increase PON1 activity [180,195]. Promoting an increase in PON1 activity may prove beneficial to prevent diabetes development [229] and slow down its cardiovascular complications [180,181,230].

Substances able to inhibit NADPH oxidases and prevent superoxide production may be useful for treatment of endothelial dysfunction [224]. Several inhibitors of the NADPH oxidase have been developed to specifically target NADPH oxidase with potential benefits [182–184, reviewed in 110 and 112]. Many cardiovascular drugs interfere with NADPH oxidases although most likely by indirect mechanisms. Additionally, flavonoids exhibit an inhibitory effect on NADPH oxidase combined with \( \text{O}_2^- \) scavenging [184]. Nox-signaling pathways in the vasculature are likely to offer novel therapies. Discovering gene therapy targets towards enzymes involved in the homeostasis of vascular redox state is essential. Recently, the design and application of nanocarriers for delivery of antioxidants to the endothelium was performed with favorable outcomes [178]. Additionally, it has been described that delivery of genes encoding antioxidant defense enzymes (e.g. superoxide dismutase, catalase, glutathione peroxidase, PON1 or HO1) or eNOS suppress atherosclerosis in animal models [49–51,66,97,123]. Similarly, delivery of genes encoding regulators of redox sensitive transcriptional factors (e.g. NF-kappa B, AP-1, and NF-E2-related factor-2) or reactive oxygen species scavengers has been successfully used in experimental studies [72,179].

Induction of endogenous antioxidant enzymes by activators of the NF-E2-related factor-2/antioxidant response element pathway may be an interesting approach to obtain sufficient levels of antioxidants and reduce oxidative stress [72,77]. Additionally, SIRT1-mediated inhibition of p66Shc (a key effector driving vascular memory in diabetes) may also contribute to the prevention of oxidative stress-induced endothelial dysfunction in vascular diseases [78]. Despite the promising results from basic science, the clinical applicability of these strategies has proven to be difficult and challenging.

3.2. Other therapeutic approaches

Novel therapeutic approaches designed to inhibit AGE formation and signaling (Fig. 11) [183,186], specifically directed to reduce inflammation [187,226] and restore the ox/redox balance in the endothelium may represent promising strategies to ameliorate vascular function in
Potential therapies also include: AMPK activators, PKC inhibitors, PARP inhibitors and rho-associated coiled-coil protein kinase (ROCK) inhibitors, among others.

AMPK is recognized as a key regulator of cellular energy status that has favorable effects on eNOS activity, insulin sensitivity, and mitochondrial function in a variety of cell types, including vascular cells. Therefore, pharmacological therapeutics that activate AMPK can be an important target in treating vascular complications in diabetes [137,219].

Inhibition of protein kinase C is another therapeutic approach. LY333531 (ruboxistaurin mesylate) has been shown to reduce oxidative stress and inflammation by blocking PKC-β isoform activation [188]. This inhibitory approach may also decrease vascular insulin resistance [133].

Furthermore, chronic treatment with the PARP inhibitors in rodent models has been demonstrated to improve endothelial dysfunction associated with aging [189,191]. In addition, pharmacological inhibition of PARP with PJ-34 restored endothelium-dependent vasodilation and reduced the levels of cytokines and inflammatory response [192,193]. Additionally, PARP-1 knockout protects against dyslipidemia-induced autonomic and vascular dysfunction in ApoE−/− mice [194]. PARP inhibitors are potential therapies for diabetic vasculopathy. Pharmacological catalytic decomposition of peroxynitrite with FP15 has been demonstrated to effectively eliminate peroxynitrite and prevent PARP activation both in vitro and in vivo [220,221], thereby improving cardiovascular function in various disease models.

Rho-associated coiled-coil protein kinases are potential targets for treatment in vascular disease as suggested by the use of specific inhibitors as fasudil. Treatment with fasudil was protective against vascular-injury-induced leukocyte recruitment in wild type but not eNOS KO mice [222]. In diabetic animal models, studies have demonstrated a significant correlation between increased RhoA activity and impaired vascular function [223]. Thus, testing of fasudil and newer more specific second generation ROCK inhibitors in a diabetic setting would be of great interest in an effort to limit vascular complications [95].

Overall it is important to identify new targets for therapy and develop new agents for clinical use.

### 3.3. Targeting vascular disease risk factors with nutritional therapeutics

Several nutritional agents such as lipoic acid, polyphenols, resveratrol, pomegranate, omega-3 fatty acids and bioavailable SOD have been shown to effectively improve and/or protect against endothelial dysfunction. Indeed, a comprehensive nutritional regimen can be adjoined with pharmacological approaches in order to target all of the risk factors that contribute to atherosclerosis.

Lipoic acid (LA) is a naturally occurring antioxidant that serves as a coenzyme in energy metabolism of fats, carbohydrates, and proteins. It can regenerate thioredoxin, vitamin C, and glutathione, which in turn can recycle vitamin E. LA reduces serum glucose levels in diabetic state. Potential therapies also include: AMPK activators, PKC inhibitors, PARP inhibitors and rho-associated coiled-coil protein kinase (ROCK) inhibitors, among others.

**Fig. 9.** Potential sites of therapeutic intervention in the L-arginine–NO-synthase–soluble guanylyl cyclase pathway. They are indicated by numbers (from 1 to 14). The schematic diagram shows an endothelial cell in yellow and a vascular smooth muscle cell in light pink. The numbers indicate: (1) L-arginine supplementation. (2) Inhibition of protein arginine N-methyltransferase type 1 (PRMT-1) to prevent the formation of asymmetric dimethyl-L-arginine (ADMA). (3) Increasing the expression and/or the activity of dimethylarginine dimethylaminohydrolase (DDAH) to increase ADMA degradation. (4) Inhibition of arginase-2 to preclude l-arginine metabolism. (5) Increasing the expression and/or activity of endothelial nitric oxide synthase (eNOS). (6) Stimulation of endothelium-derived nitric oxide release. (7) Enhancing the expression and/or activity of guanosine triphosphate cyclohydrolase (GCH1), to increase tetrahydrobiopterin synthesis (BH4), or direct supplementation with BH4 or with its precursors. (8) Enhancing the expression and/or activity of dihydrofolate reductase (DHFR), to increase BH4 regeneration. (9) Scavengers of reactive oxygen species (ROS). (10) Inhibition of the activity and/or expression of enzymes that generate ROS such as NADPH oxidases (NOX), cyclooxygenases (COX), lipoxigenases (LOX) or cytochrome P450 monooxygenases (P450). (11) Enhancing the expression and/or activity of enzymes that metabolized ROS such as superoxide dismutase (SOD) or glutathione peroxidase. (12) Activators of soluble guanylyl cyclase (sGC). (13) Activators of sGC. (14) Inhibitors of phosphodiesterase-5 (PDE-5). BH4, dihydrobiopterin; CAT-1, cationic amino acid transporters; CaV, voltage-activated calcium channel; cGMP, cyclic guanosine monophosphate; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; O2•−, superoxide anion; ONOO•, peroxynitrite; PKG, protein kinase G.
patients [195] and improves endothelial function in subjects with metabolic syndrome [196]. In type 2 diabetic animal models, we have previously shown a reduction of endothelial dysfunction after treatment with LA [21].

Different natural polyphenols have been shown to preserve endothelial function and prevent cardiovascular disease. Epidemiological evidence suggests a negative correlation between the consumption of polyphenol-rich foods (fruits, vegetables, and cocoa contained in chocolate) or beverages (wine, especially red wine, grape juice, green tea, among others.) and the incidence of cardiovascular disease [197–199]. Most polyphenols are mild antioxidants, some can reduce the activity of prooxidative NADPH oxidases, and others can stimulate antioxidative enzymes and eNOS [200–203]. The beneficial effects of silibinin on ADMA levels and endothelial dysfunction in db/db mice were recently described. The endothelium-dependent vasodilatation to ACh was impaired in db/db mice and was restored in the silibinin group, accompanied with a reduction of plasma and vascular levels of ADMA [128].

Several molecules with antioxidant properties (such as resveratrol, piceatannol, probucol, taurine) enhance dimethyl arginine dimethyl amino hydrolase activity, increasing ADMA catabolism [127].

The benefits of resveratrol include improvements in endothelial function [204–206]. Resveratrol seems to increase the number and activity of endothelial progenitor cells [205]. Resveratrol benefits the circulatory system by eliciting a decrease in the oxidation of LDL; by fostering decreases in platelet aggregation; and by promoting relaxation of arterioles [207]. Thus, resveratrol improves cardiovascular system by decreasing factors that contribute to the development of atherosclerosis and atherothrombosis [96,208].

Previous studies indicate that pomegranate and its extracts reduce oxidation and inflammation mainly through their effect on PON-1 activity, intervening at each step in the development of atherosclerosis [209–211].

Intake of omega-3 fatty acids might reduce Lp-PLA 2 levels and reduce the risk of vascular disease [212,213]. Omega-3 fatty acids serve as substrates for the conversion to a novel series of lipid mediators designated resolvins and protectins, with potent anti-inflammatory properties [187]. Studies have found that when omega-3 fatty acids were combined with rosuvastatin or other conventional therapies, the combination improved endothelial function [214,215].

Diminished levels of the antioxidant enzyme SOD have been linked with cardiovascular disease. Supplementation with GliSODin, a vegetal
SOD associated with gliadin, was effective in controlling the thickness of the carotid artery intima and media layers as measured by ultrasonography-B [216]. Previous studies have demonstrated the preventive efficacy of GliSODin at a preclinical stage in subjects with risk factors of cardiovascular disease.

4. Conclusions

Endothelial function is important for the homeostasis of the body and its dysfunction is associated with several pathophysiological conditions, including atherosclerosis, hypertension and diabetes. Understanding and treating endothelial dysfunction is a major issue in the prevention of vascular complications associated with all forms of diabetes mellitus.

Controlling a variety of risk factors causing inflammation and oxidative stress with combination therapy targeting intracellular mechanisms underlying metabolic alterations (such as inhibiting AGE formation and signaling and suppressing PKC activation) may simultaneously address multiple mechanisms underlying the pathogenesis of atherosclerosis. Since therapy addressing a single metabolic abnormality has not been effective, to reduce cardiovascular complications in type 2 diabetes may require simultaneous interventions within multiple metabolic and signaling pathways. Concurrent reduction of hyperglycemia, oxidative stress, inflammation and insulin resistance may be necessary to ameliorate the adverse effects that progress to diabetic vasculopathy in patients with cardiovascular risk factors.

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