Free Radicals, Diabetes and Endothelial Dysfunction

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Short title: Diabetic endothelial dysfunction

Key words: Endothelium, endothelial dysfunction, nitric oxide, NAD(P)H oxidase, reactive oxygen species, diabetes, free radicals

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Introduction

Diabetes mellitus, a metabolic disorder characterised by high levels of blood glucose, is associated with several vascular complications. Although insulin treatment, oral medications, dietary regulations and exercise can delay the development of diabetic microangiopathy [1], the development of macroangiopathy cannot be prevented solely by glycaemic control [2]. Diabetic retinopathy and nephropathy leading to blindness and renal failure are the hallmarks of microangiopathy. However, diabetic-macroangiopathy refers mainly to an accelerated form of atherosclerosis. This in turn affects both the coronary and cerebral vasculature, thus increasing the risk of myocardial infarction, angina pectoris and cerebrovascular accidents. Indeed, coronary heart disease and peripheral vascular disease are the leading causes of morbidity and mortality in diabetes mellitus [3]. Diabetes mellitus in humans [4,5] and animal models of diabetes [6,7] are associated with impaired endothelium-dependent relaxation i.e. endothelial dysfunction. The term "endothelial dysfunction" in fact refers to impairment of many significant functions of the endothelium including anti-inflammatory and anti-proliferative characteristics as well as vasodilatation [8,9]. However, in many scientific publications it is solely used to describe impaired endothelium-derived vascular relaxation that may develop secondary to hypertension, atherosclerosis or hyperglycaemia. In this review the term endothelial dysfunction, a surrogate marker for the development of diabetic macroangiopathy, will be used in the same context. Several factors including increased synthesis of vasoconstrictor agents through the cyclooxygenase (COX) pathway [10] and dysregulation of the gene encoding endothelial type of nitric oxide synthase (eNOS) [11,12] in endothelium have been proposed to account for this defect in diabetes. However, in recent years, reduced bioavailability of nitric oxide (NO), the most important endogenous vasodilator agent, due to excessive synthesis/release or diminished destruction of reactive oxygen species (ROS) [13-15] has been implicated in the pathogenesis of this defect. The purpose of this review is therefore to summarise the mechanisms whereby vascular cells produce NO and ROS, to examine molecular and pharmacological mechanisms underlying the pathogenesis of diabetic endothelial dysfunction with particular reference to reactions between ROS and NO, and finally to discuss the reversal of diabetic endothelial dysfunction.

Vascular endothelium

The endothelium, once considered a simple monolayer of cells covering the entire inner surface of all the blood vessels, has recently been established as a strategically-located multifunctional organ. It lies between circulating blood and the vascular smooth muscle and

plays many pivotal roles in the regulation of vascular tone and endothelial integrity as well as in the maintenance of blood fluidity and homeostasis. To perform such a wide range of functions, the endothelium synthesises or releases several vasoactive substances, including the vasodilators NO, prostacyclin and endothelium-derived hyperpolarising factors (EDHFs) and the vasoconstrictors angiotensin II and endothelin-1. Under physiological conditions, the endothelium acts as an inhibitory regulator of vascular contraction, leukocyte adhesion, vascular smooth muscle cell growth and platelet aggregation [16]. However, the characteristics of the endothelium change in response to local or systemic changes such as trauma, hyperglycaemia or dyslipidaemia and dysfunction of endothelium is considered present when normal organ function can no longer be preserved either in the basal state or in response to any given physical, humoral or chemical stimuli.

Nitric Oxide (NO)

NO is generated along with L-citrulline from the cationic amino acid L-arginine by a class of enzymes known as nitric oxide synthases (NOSs) in the presence of molecular oxygen and NADPH [17,18]. NOSs contain both flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and require several co-factors including tetrahydrobiopterin (H_4B) and reduced glutathione for activity [19,20]. Three isoforms of NOSs have so far been identified all of which are the products of separate genes which share approximately 60% homology at amino acid level [21]. NOSs are divided into two classes with regard to the nature of their expression and requirement of Ca^{2+} for their enzymatic activity. Both endothelial type (eNOS or NOS3) and neuronal type (nNOS or NOS1) NOS are constitutively expressed and Ca^{2+}-dependent while the inducible type (iNOS or NOS2) is expressed in response to several stimuli including cytokines and does not require Ca^{2+} for its activity. It is important to note in this context that, although NOS3 is constitutively expressed, many patho-physiological stimuli regulate its expression. Indeed, chronic fluid shear stress [22], exercise [23] and sex hormones [24] elicit an increase in NOS3 gene expession while tumor necrosis factor α [25] and hypoxia [26] downregulate its expression at mRNA and/or protein levels. The current data on the molecular regulation of NOS3 in diabetic animals [11,12] and in endothelial cells grown under hyperglycaemic conditions suggest a defect in its gene regulation [27]. NOS3 is expressed in abundance in cardiac myocytes and coronary microvascular endothelial cells and is therefore considered as the main source of NO within the vascular endothelium [28].

Endothelium-derived NO is known to be the most potent endogenous vasodilator in the body. It is synthesised and released by the endothelium in response to a wide range of chemical, physical and humoral stimuli including thrombin, hormones, local autacoids, alterations in oxygen tension and shear stress [29,30]. After synthesis NO is released into the subendothelial space and vascular lumen where it directly causes the underlying vascular smooth muscle to relax by binding to the heme moiety of soluble guanylate cyclase, thereby increasing the production of intracellular cyclic 3'-5'-guanosine monophosphate (cGMP) [31] [Fig. 1]. Endothelial secretion of NO counterbalances the direct vasoconstrictive effects of norepinephrine, serotonin, angiotensin II and endothelin on the vascular smooth muscle [32]. NO has also been shown to reduce oxygen consumption [33] and plays a critical role in the pathogenesis of atherosclerosis due to its inhibitory effects on platelet aggregation [34], leukocyte adhesion [35], DNA synthesis [36] and vascular smooth muscle cell proliferation [37]. In addition to its roles mentioned above, NO plays a significant role in the regulation of blood pressure. Indeed, NOS3 gene knock out mice develop severe hypertension and blood vessels isolated from these mice do not relax when exposed to endothelium-derived vasodilators such as acetylcholine [38]. It has also been shown that the inhibition of NO synthesis leads to significant peripheral vasoconstriction and elevation of blood pressure [39,40] [Table 1].

Oxidative Stress in Diabetes

The term oxidative stress refers to a condition in which cells are subjected to excessive levels of molecular oxygen or its chemical derivatives called reactive oxygen species (ROS). Under physiological conditions, the molecular oxygen undergoes a series of reactions that ultimately lead to the generation of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and H_2O. Peroxynitrite ($OONO^-$), hypochlorus acid (HOCl), the hydroxyl radical ($OH^\cdot$), reactive aldehydes, lipid peroxides and nitrogen oxides are considered among the other oxidants that have relevance to vascular biology. In the vascular endothelium, increases in oxidant stress may arise due to several mechanisms [Table 2] and are associated with alterations in normal endothelial functions and are implicated in the pathogenesis of vascular complications in several disease states including diabetes mellitus (DM). However, the mechanisms underlying altered endothelium-dependent vascular relaxation in diabetes mellitus have been proposed to be multifactorial and seem to be dependent on the duration of hyperglycaemic state and vascular bed being studied. Indeed, ROS may enhance the sensitivity of the contractile elements to Ca^{2+} [41] and facilitate the mobilisation of cytosolic Ca^{2+} in vascular smooth

muscle cells [42]. ROS may modify endothelial function directly by activating several transcription factors leading to the upregulation of adhesion molecules to platelets and leukocytes and decreasing the bioavailability of NO or indirectly by increasing the formation of advanced glycation end products (AGEs) or increasing oxidation of low density lipoprotein.

O_2^- is considered to be the most important ROS that directly causes contraction of vascular smooth muscle cells [43]. It also rapidly scavenges NO within the vascular wall to reduce its biological half-life [44]. An increase in O_2^- levels has been reported in both diabetic rat aorta [14] and more recently endothelial cells grown under hyperglycaemic conditions [15]. The excess generation of O_2^- in diabetic vessels has been attributed to increased activity of several O_2^--generating enzymes including NOSs. Indeed, it has been suggested that NOSs are able to generate O_2^- in a Ca^{2+}-dependent manner particularly in the absence of substrate L-arginine and cofactor H_4B, both of which have been associated with diabetes [45,46]. However, compelling evidence suggests that NAD(P)H oxidase constitutes the main enzymatic source of endothelial and vascular O_2^- in other disease states associated with endothelial dysfunction such as hypercholesterolaemia [47] and hypertension [48]. It is noteworthy that a recent report has also linked endothelial dysfunction in the central retinas of an obese and non-insulin dependent diabetic BBZ/WOR rats to NADH-oxidase mediated oxidative injury [49]. NAD(P)H oxidase is a multicomponent enzyme system which catalyses one electron reduction of molecular oxygen to O_2^-. It is predominantly expressed in neutrophils and plays a pivotal role in non-specific host defence against pathogens by generating large (millimolar) quantities of O_2^- during the so-called respiratory burst [50]. The neutrophil enzyme is composed of a membrane-bound cytochrome b558 [p22-*phox* and gp91-*phox* (for phagocyte oxidase)], a small G protein either (*rac*1 or *rac*2) and several cytosolic components (p47-*phox*, p67-*phox* and p40-*phox*) [50,51]. On activation, the cytosolic components translocate to the plasma membrane where they tightly associate with the cytochrome *b*558 to create the active enzyme [52]. In current models, the full electron transfer activity of the neutrophil NADPH oxidase resides in the cytochrome *b*558, which is also critical for enzymatic stability as a whole. However, the presence of gp91-phox has not been demonstrated in vascular smooth muscle cells so far, despite the presence of a functional enzyme [53]. The endothelial and vascular smooth muscle cell NAD(P)H oxidases bear substantial similarities to neutrophil type enzyme including their non-mitochondrial location, in spite of some functional differences between them [54,55]. Namely, while endothelial and vascular oxidases appear to be constantly active, generating low levels of ROS and utilising NADH as a cofactor [55], phagocytic oxidase is activated in response to stimulation, generates high levels of ROS and preferentially uses

NADPH as a cofactor (hence the term NAD(P)H oxidase or NADH/NADPH oxidase) [56]. A recent report has indicated that these functional differences may be attributed (-at least in endothelial cells-) to different glycosylation patterns and mutations in NADPH binding as well as so called non-functional domains in endothelial oxidase [57]. Similar to endothelial cells, vascular smooth muscle cell function is also regulated by reactive oxygen species in both a paracrine and autocrine fashion. In vivo, smooth muscle cells produce O_2^- and H_2O_2 [48,58] and are exposed to free radicals released by circulating blood cells, inflammatory cells, and endothelial cells. Vascular smooth muscle cell-associated reactive oxygen species also derive mainly from an NAD(P)H oxidase. Indeed, an O_2^--generating NAD(P)H oxidase in pulmonary arteries that is modulated by hypoxia and is based on a cytochrome b558 electron transport system has recently been reported [54]. In support of this finding, another study has also shown that NAD(P)H-dependent O_2^- production in vascular smooth muscle cells is induced by angiotensin II and tumour necrosis factor-α [53]. The activity of vascular oxidase similar to endothelial cells is also inhibited by the flavoprotein inhibitor diphenylene iodonium (DPI) [55,59]. An identical enzyme has also recently been reported in the media or adventitia of rabbit aorta [60]. Taken together these data strongly indicate that an NAD(P)H oxidase is the major source of O_2^- in endothelial as well as vascular smooth muscle cells. Indeed, the contribution of other potential O_2^--generating enzymes including cyclooxygenase, xanthine oxidase, mitochondrial NADH dehydrogenase and NOSs to overall production of O_2^- in endothelial and vascular smooth muscle cells has been found to be minor, as selective inhibitors of these enzymes did not alter net production in either cell homogenates [55,60].

The increase in O_2^- levels could also be due to its decreased metabolism as opposed to its increased generation (or indeed both mechanisms may be responsible). Deficiency or inactivation of SOD enzymes (intracellular Cu/Zn- or Mn- and an extracellular Cu/Zn-containing isoforms) which dismutate O_2^- to hydrogen peroxide (H_2O_2) elevate O_2^- levels in intact blood vessels. SODs therefore may be critical in the pathogenesis of endothelial dysfunction in several pathological conditions including DM. However the exact role of SODs in the regulation of vascular tone and in the development of endothelial dysfunction is not known and the currently available related data are somewhat conflicting. Indeed, reports have suggested that SODs are both crucial [61,62] or ineffective [63,64] in the protection of NO in a variety of blood vessels. A recent study has demonstrated that the adenovirus-mediated transfer of Cu/Zn SOD gene did not improve vascular relaxation in diabetic rabbit carotid arteries [11] perhaps due to its inefficiency in increasing the amount of SOD in the tunica media in contrast

with the intima and the endothelium [65]. This may also be due to the fact that intracellularly localised Cu/Zn SOD may not be able to protect NO from O_2^- if the reaction between these two radicals takes place in the extracellular space.

It is important to note that the excess production of H_2O_2, mediated by the O_2^-/SOD pathway, also causes irreversible endothelial damage linked with diminished NO production, although it initially stimulates NO production [66]. Indeed, homocysteine-induced endothelial cell injury has been associated with H_2O_2 and has been reduced by the enzyme catalase [67]. Catalase, a H_2O_2 scavenger, catalyses the transformation of H_2O_2 to yield H_2O and oxygen. Several lines of data indicate that the activity of catalase, like SODs, is modulated by many stimuli and indeed is regulated to compensate for the biological requirements imposed by increased oxidative stress [68]. An in vivo study designed to investigate the expression of genes for Cu/Zn SOD and catalase in kidney tissue of rats with chemically induced controlled- or uncontrolled-diabetes has demonstrated a direct correlation between the levels of blood glucose and renal mRNA levels of both enzymes. However, while treatment of diabetic rats with a moderate dose of insulin normalised catalase mRNA levels, it did not have any effect on Cu/Zn SOD mRNA levels suggesting a different threshold of these genes to different glucose concentrations [69]. In support of these findings exposure of endothelial cells to high glucose concentrations has been shown to increase both the activity and the mRNA levels of catalase and Cu/Zn SOD implying a compensatory effect to neutralise increased free radical generation in vitro [70].

Glutathione peroxidase (GPx) localised to the cytoplasm is known to be another H_2O_2 scavenger. It has been shown that intracellular glutathione, a key aqueos phase antioxidant, levels are decreased in retinal pericytes grown under high (25 mmol/l) glucose concentrations coupled with the decrease in GPx activity [71]. Another study designed to investigate the link between increased oxidative stress and impaired free-radical scavenger function in endothelial cells exposed to high glucose concentrations has revealed a reduced GPx-dependent H_2O_2-degradation which may be associated with increased cellular damage elicited by H_2O_2 [72]. Indeed, high glucose-derived induction of oxidative stress has been reported in several cell lines including human endothelial cells [73] and porcine aortic vascular smooth muscle cells [74].

In addition to O_2^-, hyperglycaemia also stimulates the synthesis of NO via increased enzymatic activity of endothelial [75] and inducible [76] isoforms of NOS. However, the NO generated in diabetic vasculature is rapidly scavenged by omnipresent O_2^- to form peroxynitrite [$OONO^-$] at a rate of 6.7×10^9 ms^{-1} [77]. This rate is three times faster than the reaction

between O_2^- and SOD [78]. Hence, the formation of $OONO^-$ is a double-edged sword; on one hand potentially deleterious O_2^- is neutralised, on the other hand the most potent vasodilator NO is consumed and $OONO^-$ is produced as a result [79]. It is therefore easy to comprehend why $OONO^-$ itself has been suggested as both a toxic compound eliciting tissue damage as well as a protective molecule improving cellular and organ vitality. $OONO^-$ has been shown to increase insulin secretion, DNA damage and cell death in human and rat islets of Langenhars [80]. It has also been linked to attenuation of vascular responses in diabetic and preeclamptic human placentas [81]. A recent report has also demonstrated that $OONO^-$ contributes to the destruction of pancreatic islet beta-cells of NOD mice developing autoimmune diabetes, suggesting that $OONO^-$ may play a pivotal role in the initiation of insulin-dependent diabetes mellitus (IDDM) [82]. A recent in vitro study has also suggested that $OONO^-$ may mediate the apoptotic effects of high glucose on endothelial cells via NFκB activation since this induction of cell death was prevented by an antisense nucleotide to the p65 NFκB binding site [83]. $OONO^-$ has been shown to nitrosylate substrates such as tyrosine moieties within proteins thereby leading to organ malfunction [84]. $OONO^-$ is also known to cause lipid peroxidation [85] and depletion of important plasma antioxidants such as glutathione and cysteine [86]. Administration of $OONO^-$ impairs relaxation of isolated perfused rat heart [87] and when given systemically causes vascular dysfunction in rats via selective impairment of adrenoreceptors [88].

Contrary to its deleterious effects, $OONO^-$ also relaxes vascular smooth muscle either directly or indirectly by triggering intracellular second messenger pathways to increase cGMP levels. Although the presence of endothelium is not a prerequisite to this relaxation, it augments the overall relaxation [89]. Recent evidence has suggested that $OONO^-$ may actually preserve its beneficial properties under in vivo physiological conditions when thiol containing agents such as glutathione, albumin and cysteine are readily available to convert $OONO^-$ into nitrosothiols and other products with antiatherogenic characteristics. However, as a deficiency of glutathione and other antioxidant agents have been reported in both diabetic patients and several cell lines including pericytes grown under high glucose media it is tempting to speculate that $OONO^-$ will have no vasodilatory effect in diabetics [74,90].

It has also recently become apparent that free radicals advance endothelial dysfunction by promoting growth. Indeed, angiotensin II-induced hypertrophy has been linked to excessive generation of NAD(P)H oxidase-mediated ROS [53]. In the diabetic state, the high levels of glucose may adversely influence endothelial cell function by increasing the synthesis of

growth factors, in particular transforming growth factor β (TGFβ) and vascular endothelial growth factor (VEGF), and extracellular matrix components such as collagen and fibronectin. TGFβ stimulates the accumulation of matrix proteins such as collagens, fibronectin and proteoglycans both by enhancing their synthesis [91] and by reducing their proteolysis [92]. These effects of TGFβ are reversed by normalisation of blood glucose levels with insulin treatment [93] suggesting a significant role for TGFβ in the matrix alterations in microvessels. On the other hand, endothelial cells grown under hyperglycaemic conditions show decreased proliferation and fibrinolytic potential [94] and increased programmed cell death [95].

Cellular mechanisms for the development of diabetic endothelial dysfunction

Non-enzymatic glycation

Another mechanism that may account for hyperglycaemia-derived vascular cell dysfunction is the spontaneous formation of glucose adducts to basic amino acids [lysine and arginine] and other amine-containing molecules. Although these early non-enzymatic glycation products are reversible (like glycohaemoglobin), they later become irreversibly modified products of glucose called “advanced glycation end-products” or AGEs, via slow and complex processes including glycation, glycooxidation and auto-oxidative glycosylation [96]. Endothelial cells express receptors for AGEs [97] which facilitate their internalisation and transfer into the subendothelial space. AGEs may impair endothelium-dependent relaxation through glycosylation and oxidative modification of LDL which in turn directly inactivates or disrupts the formation of NO [98]. AGE induced modification of LDL also decreases the particle clearance [99] from the circulation thereby contributing to an expansion in LDL into endothelial cells.

Hyperglycaemia

Support for the concept of increased oxidative stress-mediated endothelial dysfunction in diabetes has derived from both in vitro and in vivo experiments which have suggested that hyperglycaemia is almost certainly the primary causal factor, mediated through several mechanisms, including alterations in the cellular redox state by an altered NADH/NAD^+ ratio, changes in the regulation of protein tyrosine kinases, dysregulation of protein kinase C and the accumulation of sorbitol.

Hyperglycaemia elicits an increase in the intracellular NADH/NAD^+ and a decrease in NADPH/$NADP^+$ ratios through hyperactivity of the sorbitol (polyol) pathway leading to a

cytosolic redox imbalance i.e. hyperglycaemic pseudohypoxia [100]. This is so called due to the fact that an increased NADH/NAD^+ ratio mimics the effects of tissue hypoxia. Aldose reductase is the first, and rate-limiting, enzyme in this pathway that catalyses the NADPH-dependent reduction of glucose to sorbitol which in turn is catalysed to fructose by sorbitol dehydrogenase [101]. The accumulation of sorbitol which increases intracellular osmolality is thought to account for polyol pathway-related changes. Extensive studies on aldose reductase have been conducted to elucidate a causal connection between the activity of this enzyme and diabetic complications. Various studies have reported a preventive role of aldose reductase inhibitors on the development of diabetes-like neuropathy [102], myopathy [103,104] and nephroptahy [104]. However, several lines of data revealed closer links between several metabolic alterations such as *myo*-inositol depletion [105], glycation [106], increased oxidative stress [107] and diabetic complications.

The cellular NADPH pool required for NO generation and to replenish antioxidant glutathione may also be depleted in diabetes by a hyperactive pentose phosphate pathway activity in endothelial cells [108], consequently leading to abnormalities in protein tyrosine kinase activation [109]. Activation of transcription factors by phosphorylation of tyrosine kinase plays significant roles in gene regulation of vascular cells to increase the production of extracellular matrix components discussed above.

Hyperglycaemia also alters several biochemical pathways including eicosanoids, protein kinase C (pKC) activity, long-chain fatty acids and ROS. Activation of several transcription factors by ROS plays a pivotal role in gene regulation and gene expression of vascular cells. Increased cellular uptake of glucose stimulates pKC activity which mediates endothelial and vascular smooth muscle cell functions through regulation of permeability, contractility, blood flow and basement membrane synthesis and has therefore been associated with several vascular abnormalities. pKC can modulate the actions of hormones, growth factors and ion channels such as the Na/proton antiport, a key regulator of intracellular pH, growth, differentiation and contractility. pKC activation in diabetes has been implicated in the increases in intracellular diacylglycerol through either de novo synthesis via increased glycolysis or membrane-associated phosphatidyl inositol 4,5-biphosphate. In a rodent model of insulin dependent diabetes mellitus an oral inhibitor of the pKC β isoforms ameliorated vascular dysfunction. pKC activity in addition to its aforementioned effects also activates peroxidase enzymes and the cyclooxygenase pathway thus causing overproduction of oxidative molecules [110-112].

Hyperglycaemia is also linked with the activation of coagulation system through its connections with some of the aforementioned mechanisms namely non-enzymatic glycation and AGE formation which may decrease antithrombin III activity and increase tissue factor activity respectively as well as increased oxidative stress [113-115]. It is highly likely that during later stages of diabetes loss of endothelial anticoagulant properties may further activate coagulation cascade. Higher levels of a number of coagulation factors such as endothelium-derived von Willebrand's factor, fibrinogen and PAI-1, in association with endothelial cell damage and micro- and macrovascular damage may also contribute to this procoagulant state [116-118].

Prevention and reversal of diabetic endothelial dysfunction

The close link between hyperglycaemia and endothelial dysfunction is supported by both in vitro and in vivo studies. The adverse effect of hyperglycaemia on vascular function in diabetes may be due to the consequences of the impaired L-arginine/NO pathway, oxidative stress and increased formation of AGEs via non-enzymatic glycosylation. Hence several therapies have been proposed for preventing and to a certain extent reversing endothelial dysfunction in diabetic state by directly targeting these pathogenetic mechanisms. It has been reported that plasma concentrations of basic amino acids (e.g. L-arginine, L-lysine and L-histidine) are reduced in diabetes and vascular rings obtained from diabetic rats show impaired endothelium-dependent vasodilatation. In vivo L-arginine treatment of streptozotocin-induced diabetic rats revealed an increase in the aortic relaxation to acetycholine and also prevented increases in plasma malondialdehyde levels, suggesting that diabetes-induced functional abnormalities occurring in rat aortas may in part result from L-arginine deficiency [119]. In support of this, it has previously been documented that the relaxation of vascular rings from diabetic animals to acetylcholine is potentiated by pretreatment with L-arginine (but not D-arginine). This again may imply the involvement of a decrease in L-arginine concentrations and/or a defect in the utilisation of L-arginine by NOS3 in the pathogenesis of endothelial dysfunction in diabetes [120]. Similar studies have also suggested a prominent role for H_4B availability in the regulation of NO production by diabetic endothelium, because 6-methyl-5,6,7,8-tetrahydrobiopterin improved the impaired endothelium-dependent vasodilatation in some vascular beds of diabetic animals [121].

Antioxidant defences may also be impaired in diabetes thereby contributing to net oxidative stress [122]. Indeed a variety of defects in serum antioxidant status has been reported in diabetic patients compared to healthy subjects [123,124]. Hence it has consequently been

suggested that diabetic patients might benefit from supplementation with antioxidant vitamins (vitamin C and vitamin E) to prevent free radical oxidation and endothelial dysfunction as a result [125]. Vitamin C deficiency in diabetes may occur as a result of excessive excretion or poor diet. An increased oxidation of vitamin C as a result of increased free radical synthesis [126] and hence increased generation of its oxidation product, dehydroascorbic acid (DHA) [127] or a decline in the regeneration of vitamin C from DHA may largely be responsible for this deficiency. The latter may be due to competitive inhibition of vitamin C transport across the cell membrane via structurally similar glucose [128]. A consistent beneficial effect of vitamin C has been reported in human subjects and several animal models of human diseases. Acute intra-arterial administration of vitamin C to patients with diabetes improves endothelium-dependent vasodilatation to methacholine but not the response to sodium nitroprusside, a NO donor or to a smooth muscle relaxant [129]. Similarly, physiological concentrations of vitamin C has been shown to reverse endothelial dysfunction in conduit arteries of patients with congestive heart failure [130] and angina [131] while intra-arterial infusion of supraphysiological concentrations of vitamin C has improved microvascular function in patients with hypertension and hypercholesterolaemia [132]. A recent report has also shown that vitamin C plays a significant role in the prevention of ROS production by scavenging O_2^- and apoptosis in the early stages of incubation of endothelial cells with high glucose [133]. The protective effects of antioxidants vitamin C and taurine have also been recently reported on renal injury in streptozotocin-induced diabetic rats in that these agents reduced albuminuria, glomerular hypertrophy, glomerular collagen and TGF-β1 accumulation [134]. Administration of vitamin E has also been shown to have similar effects to these antioxidants in the early phase of glomerular injury. However, studies of chronic treatment with vitamin E are still needed as chronic dietary supplementation of vitamin E to diabetic rats has been attended with higher mortality rates [135].

It is difficult to understand how vitamin C can act as an effective antioxidant in high risk (and high oxidative stress) patients considering very slow reaction rate between vitamin C and O_2^- compared to NO and O_2^- [136]. However, recent studies have suggested that vitamin C may improve the bioavailability of NO by regulating cellular redox state and also sparing intracellular glutathione from oxidation which may be important for NO in humans [137,138].

Vitamin E is the major lipid-soluble antioxidant, taken up by low-density lipoprotein (LDL) particles, which may improve endothelial function. Indeed, vitamin E supplements reduce the sensitivity of LDL to in vitro oxidation in healthy subjects as well as Type 2

diabetics [139] which suggests that endothelial function may be improved due to a reduction in the availability of oxidised LDL in diabetic vessels [140]. Vitamin E in addition inhibits glucose-induced protein kinase C β_{II} activation in vascular smooth muscle cells [141]. Protein kinase C β_{II} induction has been implicated in vasoconstrictive effects of several hormones such as angiotensin II [111]. It has been shown in animal models of diabetes that vitamin E treatment improves coronary and aortic vascular endothelial function and prevents diabetes-induced abnormalities [142,143] although the opposite has been reported in mesenteric arterioles [144]. In contrast to the consistent beneficial effects observed in animal models, the results in humans have been mixed. One randomised study, the Cambridge Heart Antioxidant Study (CHAOS), demonstrated a marked reduction in non-fatal myocardial infarction in patients randomised to treatment with 400-800 IU of vitamin E/day compared to patients receiving placebo [145]. However, many subsequent studies including the Heart Outcomes Prevention Evaluation (HOPE) have failed to confirm these findings and revealed no beneficial effect of vitamin E on the prevention of cardiovascular disease after 4.5 years of use [146].

The species differences and the phase of the disease may account for different results obtained from animal and human studies. Indeed, majority of the experimental studies investigated the bioavaliability of endothelium-derived NO or endothelium-derived vascular relaxation following treatment with vitamin E alone or in combination with vitamin C or β-carotene imminently after the onset of diabetes following streptozotocin or alloxan injections or initiation of hypercholesterolaemic diet. In contrast, the current human data have been obtained from patients with longstanding risk factors and/or proven coronary artery disease and peripheral vascular disease [147,148]. The vascular bed being studied may also have implications in inconsistent human results, namely, most of the studies carried out in human or animal conduit arteries have shown a beneficial effect while studies performed on human forearm i.e. microvessels have not revealed any beneficial effects [149,150].

Increased superoxide may however directly inactivate NO with the formation of the highly toxic oxidant, peroxynitrite. Endothelial dysfunction not only occurs in overt diabetes but can also be induced by simple exposure of isolated vessels to high glucose media in vitro [151]. Pretreatment of rat aorta with SOD produces significantly greater relaxations in aortic rings incubated in high glucose [14]. Likewise pretreatment with SOD plus catalase or an inhibitor of hydroxyl radical formation (DETAPAC) has been shown to improve endothelial dysfunction in aortic rings of streptozotocin-induced diabetic rats suggesting that vascular production of both O_2^- and hydroxyl radicals may contribute to endothelial dysfunction in this

model [152]. However, elevated ambient glucose concentrations in diabetes mellitus may result in glycosylation of native superoxide dismutase leading to impairment of its enzymatic activity [153].

In addition, the changes in intracellular cell signalling may impair appropriate activation of NOS in response to neurohumoral or mechanical stimuli. Indeed, several recent studies strongly indicate the involvement of the pKC pathway in vascular complications in diabetes. High concentrations of glucose strongly increase the intracellular levels of diacylglycerol which consequently lead to protein kinase C activation. In vitro hyperglycaemic endothelial dysfunction caused by incubation of vascular rings with high concentrations of glucose has been corrected with pKC inhibitors [154]. These in vitro observations have also been supported by in vivo studies demonstrating that therapy with pKC inhibitors ameliorated vascular complications in diabetic rats [112]. Although, the mechanisms underlying pKC-mediated endothelial dysfunction remain poorly understood, in vitro experiments have shown that NOS3 activity is diminished through phosphorylation of the NOS3 gene [155] and O_2^- production is enhanced by pKC activation [75].

A weak glutathione-related antioxidant defence, i.e. diminished enzymatic activities of glutathione peroxidase, glutathione reductase and in part glutathione transferase, is present in human atherosclerotic lesions [156] while intracoronary infusion of reduced glutathione improves endothelial vasomotor response to acetylcholine in human coronary circulation [157]. Furthermore, L-2 oxothiazolidine-4-carboxylic acid, which augments intracellular glutathione, improves endothelium-dependent relaxation in patients with coronary artery disease [158]. The instant improvement of NO availability following administration of antioxidants supports the role of ROS in the impaired endothelium-dependent relaxation in coronary artery disease and its risk factors and is consistent with the notion that the cellular redox state may be an important regulator of endothelium-derived NO.

In recent years gene therapy studies have been conducted to reverse the immune-mediated destruction of the pancreatic beta cells in case of type I diabetes. Insulin gene delivered via a retroviral vector to the liver improved fasting glucose levels in streptozotocin-diabetic rats, but had little effect on glucose levels after feeding [159]. However, this approach is at present far from application in humans because physiologic regulation of insulin production and release in response to blood glucose levels over minutes has not yet been accomplished.

Conclusions

The endothelium is an important locus of control of vascular functions. Several diseases including diabetes are associated with impaired endothelial function. Although, several factors including dysregulation of NOS gene, deficiencies of either substrate i.e. L-arginine or cofactor namely tetrahydrobiopterin for physiological NOS activity and excessive release of endothelium-derived vasoconstrictors such as prostaglandins have been implicated in the pathogenesis of impaired endothelium-dependent relaxation in diabetes, a single unifying mechanism has yet to emerge. However, several lines of evidence including the activation of transcription factors in particular NFκB, overexpression of growth factors and activation of protein kinase cascades suggest that in the initial stages of diabetes multiple pathways may converge to increase reactive oxygen species and a diabetes-induced oxidative stress [Fig. 2]. This may arise from enhanced generation of free radicals as a consequence of glucose autooxidation or pseudohypoxia. This may also arise from the overexpression of superoxide anion-generating enzymes as well as deficiency of free radical-metabolising enzymes. A form of oxidative stress as a direct consequence of interactions between NO and oxygen-derived radicals represents a common pathological mechanism in risk factors for atherosclerosis including hypertension, hypercholesterolaemia and diabetes.

The mechanisms whereby endothelial and vascular cells produce ROS are only presently coming to light and almost certainly will prove to be a focus for better-targeted future therapeutic strategies to reverse of endothelial dysfunction.

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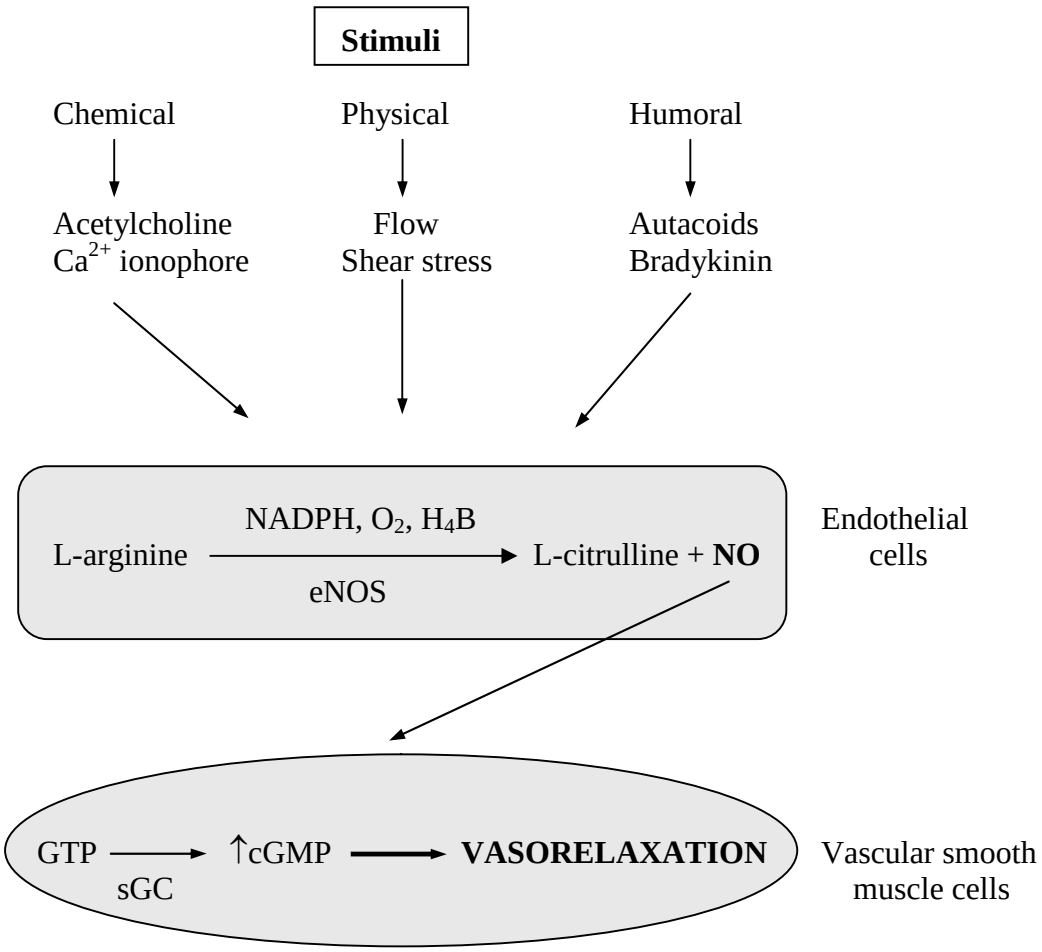
Figure legends

Fig. 1. Activation of endothelial cells by a variety of stimuli can stimulate the eNOS to convert amino acid L-arginine into NO and L-citrulline. NO in turn causes relaxation of underlying vascular smooth muscle cells by increasing the formation of cGMP from GTP by sGC. NO, nitric oxide; eNOS; endothelial nitric oxide synthase; GTP, guanosine triphosphate; sGC, soluble guanylate cyclase.

Fig. 2. Pathogenesis of endothelial dysfunction in diabetes mellitus through hyperglycaemia-induced oxidative stress. Hyperglycaemia elicits oxidative stress by directly impairing the cellular mechanisms (on the left) which in turn elicits endothelial dysfunction. Hyperglycaemia also induces the excess generation of NO and O_2^- through activation of NOSs and NAD(P)H oxidase respectively. O_2^- reacts with NO to produce $OONO^-$, another oxidant that increases oxidative stress and elicits endothelial dysfunction by promoting tissue injury. O_2^- is converted to H_2O_2 by SODs which not only increases oxidative stress but also generates endothelial dysfunction by modulating intracellular signalling and transcription factors. NO, nitric oxide; eNOS, endothelial NO synthase; iNOS, inducible NO synthase; O_2^-, superoxide anion; $OONO^-$, peroxynitrite; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; AGE, advanced glycation end products; GPx, glutathione peroxidase.


Stimuli
Chemical
Physical
Humoral
Acetylcholine
Ca2+ ionophore
Flow
Shear stress
Autacoids
Bradykinin
NADPH, O2, H4B
L-arginine
L-citrulline + NO
eNOS
Endothelial cells
GTP
sGC
↑cGMP
VASORELAXATION
Vascular smooth muscle cells

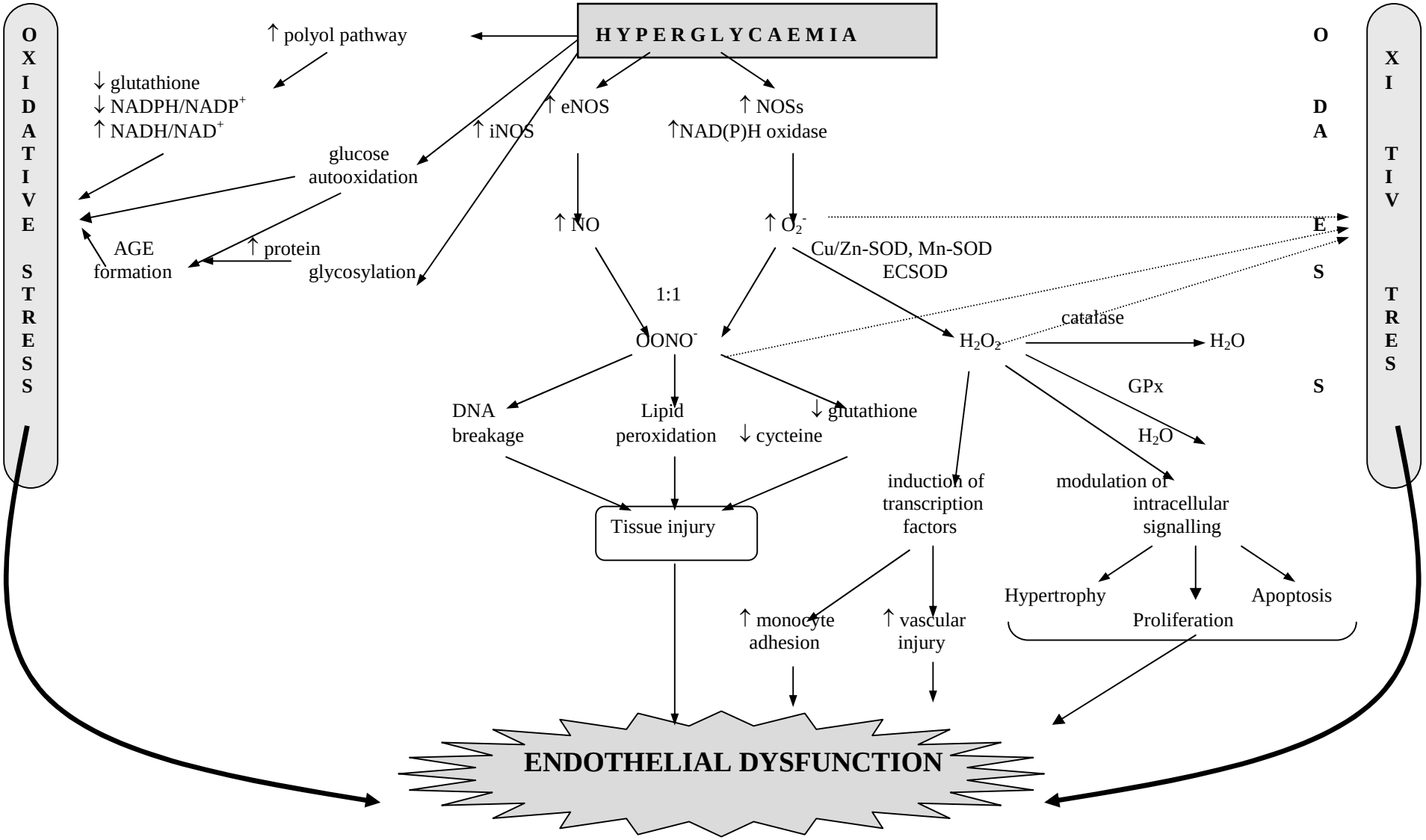

OXIDATIVE STRESS
HYPERGLYCAEMIA
↑ polyol pathway
↓ glutathione
↓ NADPH/NADP+
↑ NADH/NAD+
↑ iNOS
↑ eNOS
↑ NOSs
↑NAD(P)H oxidase
glucose autooxidation
AGE formation
↑ protein glycosylation
↑ NO
↑ O2-
Cu/Zn-SOD, Mn-SOD
ECSOD
1:1
OONO-
H2O2
catalase
H2O
GPx
H2O
DNA breakage
Lipid peroxidation
↓ glutathione
↓ cycteine
Tissue injury
induction of transcription factors
modulation of intracellular signalling
Hypertrophy
Proliferation
Apoptosis
↑ monocyte adhesion
↑ vascular injury
ENDOTHELIAL DYSFUNCTION
OXIDATIVE STRESS

Table 1. Functions of endothelium-derived NO

- Maintenance of normal vascular smooth muscle tone
- Inhibition of vascular smooth muscle cell proliferation
- Modulation of inflammatory and immune responses
- Regulation of endothelial integrity and vascular permeability
- Inhibition of leukocyte migration and adhesion
- Inhibition of platelet adhesion and aggregation
- Inhibition of LDL oxidation
- Suppression of endothelin production
- Regulation of blood pressure

Table 2. Potential causes of increased oxidative stress in diabetes mellitus

- Diminished expression/activity of eNOS and generation of NO,
- Overproduction of ROS in particular O_2^- by NOSs or NAD(P)H oxidase
- Impaired expression/activity of SODs
- Decreased antioxidant enzyme capacity i.e. catalase and glutahione peroxidase
- Reduced levels of antioxidants glutathione, α-tocopherol, ascorbate
- Enhanced protein glycosylation and AGE formation
- Enhanced glucose autooxidation
- Hyperactivity of the sorbitol (polyol) pathway