Endothelins and Microvascular Endothelial Responses in Diabetes

[1,2]Subrata Chakrabarti and [1]Zia Ali Khan

[1]*Department of Pathology, University of Western Ontario, London, Canada*
[2]*Department of Microbiology and Immunology, University of Western Ontario, London, Canada*

Diabetes is the leading cause of blindness, renal failure, and limb amputation in the North American population [1, 2]. In spite of improvements in therapeutic modalities, this disorder accounts for significant morbidity and mortality in diabetic patients. Long-standing diabetes leads to structural and functional alterations in both micro- and macrovasculature. The most devastating complications in terms of morbidity are, however, of microvascular origin. The determinant of these complications is sustained hyperglycemia, which leads to biochemical and structural anomalies in the eye, kidney, heart, and peripheral nerves. Microvascular endothelial damage may be a key factor in the pathogenesis of chronic diabetic complications. Endothelins, by virtue of multifunctional capability and widespread tissue distribution, may affect function and structure of microvasculature in several target organs of diabetic complications. Early events in small vessel disease include functional deficits such as blood flow alteration and increased vascular permeability [2]. These early events are essentially reversible with adequate blood glucose control. With progression, however, structural remodeling of microvessels takes place that entails thickening of capillary basement membrane (BM), loss of capillary pericytes, and microaneurysm formation. Later stages may also lead to neovascularization in some organs such as the retina. This review will outline the role of endothelins in microvascular complications of diabetes with emphasis on putative mechanisms of vascular endothelial cell damage.

Endothelins

Endothelins (ETs) are by far the most potent vasoactive peptides identified to date. These peptides exert vasoregulatory action by interacting with cell surface receptors on vascular endothelial and smooth muscle cells [1, 3]. Three structurally similar isoforms of ETs have been identified, ET-1, ET-2, and ET-3. These 21-amino-acid peptides are produced by a number of tissues with vascular endothelium being the major source. ET-1 appears to be the predominant isoform that is constitutively expressed in the vascular endothelium. ETs are regulated primarily at the transcriptional level and a number of stimulators have been identified that upregulate ET expression. These ET inducers include growth factors, cytokines, and various physiochemical factors [1]. In addition to transcriptional regulation, ET production may be regulated via destabilization of mRNA species.

ET gene products undergo two steps of enzymatic cleavage to generate biologically active ET peptides. These peptides perform vasoregulatory action by interacting with specific cell surface receptors, ET_A, ET_B, and ET_C. Among the ET receptors, only ET_A and ET_B receptor types are expressed in mammals. These receptors are coupled to phospholipase C via G proteins [1, 3]. ET_A receptors are localized primarily on vascular smooth muscle cells and are involved in sustained slow-onset vasoconstriction. Activation of ET_A receptors results in calcium influx via phospholipase C-mediated diacylglycerol (DAG) and inositol trisphosphate (IP_3) production. Elevated intracellular calcium and DAG-mediated protein kinase C (PKC) activation lead to myosin light chain kinase (MLCK) phosphorylation and smooth muscle cell contraction. ET_B receptors are involved in generation of nitric oxide (NO) by endothelial cells and thus regulate vasodilation. Endothelial-derived NO activates guanylate cyclase in smooth muscle cells and causes vasodilation by decreasing intracellular calcium levels. The net vascular effect would, therefore, depend on

ET concentration, relative density of ET receptor types, and the vascular tissue.

Mechanism of ET Alteration in Diabetes

Alteration of ETs has been demonstrated in both type I and type II diabetes. Although a number of studies can be cited that provide contradictory reports of plasma ET levels in diabetic patients, it should be noted that these peptides act in both an autocrine and paracrine fashion. Therefore, plasma levels may not provide an adequate assessment of their biological activity [1]. In both animal and human diabetes, use of ET antagonists may be more revealing in terms of the consequences of ET alteration. We and others have demonstrated that in endothelial cells and in several target organs of diabetic complications, ETs are upregulated and mediate structural and functional alterations [1].

The mechanisms by which sustained hyperglycemia leads to upregulation of ETs include activation of PKC, augmented polyol pathway and pseudohypoxia, oxidative stress, elaboration of growth factors, and alteration of vasoactive factors such as NO. Mechanisms and consequences of ET alteration in diabetes are diagrammed in Figure 1. We will briefly describe the possible mechanism by which these biochemical anomalies may lead to alteration of the ET system in diabetes.

PKC Activation

PKC activation has been demonstrated in both diabetic micro- and macrovasculopathy [2]. High glucose levels can induce de novo synthesis of DAG and activation of PKC. PKC has been implicated in mediating several important vascular functions such as regulation of blood flow, vascular permeability, expansion of extracellular matrix, and in the elaboration of various growth factors and cytokines. Studies have demonstrated an interactive relationship between PKC and ETs. We have previously demonstrated inhibition of high glucose-induced ET upregulation by both general (chelerythrine) PKC inhibitor and specific (LY379196) PKCβ inhibitor [4]. PKC activation may also regulate several other growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor, epidermal growth factor, insulin-like growth factor, and fibroblast growth factor. Elaboration of these growth factors may also mediate PKC-induced ET alteration in diabetes.

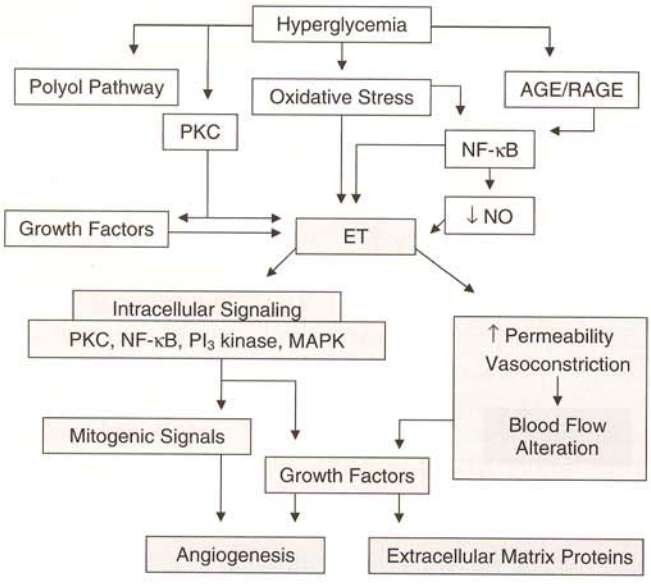

Figure 1 Putative mechanisms and consequences of ET alteration in diabetes. A schematic outlining various hyperglycemia-induced pathways leading to upregulation of endothelin levels is shown in the upper panel. Some of the major effects of increased ET levels are also presented. (see color insert)

Polyol Pathway

Polyol pathway has been implicated in several chronic diabetic complications [1]. High intracellular glucose levels overwhelm the glycolytic pathway and lead to enzymatic conversion of glucose to sorbitol via aldose reductase (AR). Sorbitol is subsequently metabolized to fructose by sorbitol dehydrogenase. AR activity requires oxidation of NADPH whereas sorbitol dehydrogenase requires reduction of NAD^+. Therefore, increased flux through the polyol pathway leads to alteration of NADH:NAD^+ and NADPH : $NADP^+$. Such imbalance in redox state may cause endothelial dysfunction secondary to hyperglycemia [1]. Interestingly, the augmented polyol pathway may reduce NO synthesis, which also requires NADPH. Impairment of the NO system may lead to ET alteration, as NO has been shown to regulate ETs.

Nonenzymatic Glycation

Glucose and other reducing sugars such as glucose 6-phosphate, trioses, and fructose may react nonenzymatically with amino groups of proteins. Advanced glycation end products (AGEs) may also be produced from glycating dicarbonyl compounds such as 3-dioxyglucosone, methylglyoxal, and glyoxals. AGEs were first thought to mark senescent proteins for degradation. However, in recent years, numerous AGE receptors (RAGEs) have been identified. Binding of AGEs to AGE receptors may mediate intracellular signaling and cause upregulation of growth factors such as ET-1 and VEGF [1]. In addition, AGE formation has been shown to reduce NO, which would further lead to ET alteration.

Oxidative Stress and NO

Increased oxidative stress due to glucose autoxidation, AGE/RAGE interaction, and NO generation has been implicated in the pathogenesis of diabetic complications. In

cultured endothelial cells as well as several target organs of diabetic complications, NO synthase mRNA has been shown to be upregulated. However, diabetic patients exhibit impaired endothelium-dependent relaxation. Several theories have been proposed to reconcile these contradictory results. It is interesting to note that concurrent with increased NO synthase expression is increased production of free radicals. Activation of various lipoxygenase enzymes, secondary to hyperglycemia, may promote scavenging of NO by superoxide anions. This interaction yields highly reactive peroxynitrite and hydroxyl radicals. Sequestration of NO by superoxide anions could also contribute to reduced NO bioactivity and availability leading to upregulation of ETs. Increased oxidative stress has also been demonstrated to mediate PKC activation, AGE formation, augmented polyol pathway and sorbitol accumulation, and NF-κB activation in endothelial cells. Such anomalies may further alter the ET system in diabetes. Recently, poly (ADP-ribose) polymerase (PARP), an enzyme well known for polymerizing ADP-ribose in DNA backbone synthesis, has been implicated in ET upregulation. Use of various PARP inhibitors was shown to prevent diabetes-induced alteration of ETs in kidney tissues [5]. Whether diabetes-induced PARP activation leads to ET upregulation in other target tissues, such as the retina, remains to be determined.

Hyperinsulinemia

Insulin represents another factor leading to ET alteration which could be of significance to microvascular complications in type II diabetes. Insulin has been shown to upregulate ET peptide and receptor expression in vascular endothelial and smooth muscle cells [1]. Administration of insulin also increases plasma ET levels in both humans and animals. In addition, hyperinsulinemia has been linked to accelerated macroangiopathy in diabetic patients. The role of insulin, however, in ET alteration and microangiopathy still remains to be determined.

Other Factors

Several other factors may be of importance in augmented ET-1 expression in diabetes. A large number of studies indicate the role of angiotensin II and transforming growth factor-β (TGF-β) in the development of diabetic micro- and macroangiopathy. Angiotensin II is mitogenic for smooth muscle cells and can lead to increased extracellular matrix (ECM) protein synthesis. Recent reports indicate that angiotensin II possibly mediates mitogenic and fibrogenic effects via ET system. Angiotensin II has also been shown to increase synthesis and secretion of ET-1 from vascular endothelial cells. In addition, an interactive relationship between TGF-β and ET has been established. These findings suggest multiple signaling pathways leading to alteration of ET-1 in chronic diabetes.

ETs and Microcirculatory Flow Alterations

Hemodynamic alterations in diabetes are believed to arise as a result of hyperglycemia-induced metabolic abnormalities and elaboration of vasoactive factors including ETs. There is great heterogeneity in findings from microcirculation studies in humans. Study of nailfold microcirculation has revealed elevated as well as reduced blood velocity in diabetic patients when compared to healthy subjects. Reduced blood velocity has also been observed in gastric mucosal blood flow studies. Much of the inconsistency in such studies can be attributed to duration of diabetes, interstudy variability, and limitations of techniques used for measurement of blood flow.

In parallel to in vivo blood flow studies, ex vivo measurement of blood vessel responsiveness to ETs has produced conflicting results. Depending on the relative distribution of ET_A and ET_B receptors in the vascular bed, responsiveness has been shown to be attenuated as well as exaggerated. Limited animal model studies suggest that diabetes leads to alteration of ET responsiveness. Based on existing evidence, however, diabetes is believed to cause vasoconstriction and reduced blood flow early in target organs of chronic complications. These hemodynamic alterations are mediated by increased vasoconstrictors such as ETs and reduced vasodilators including NO. Administration of ET-1 in humans has demonstrated reduced coronary and renal blood flow and increased vasoconstriction. Furthermore, we have demonstrated increased diabetes-induced vasoconstriction in the retina which was normalized with ET receptor antagonist [6].

ETs in Microvascular Endothelial Dysfunction

Endothelial cell dysfunction is increasingly being realized as the unifying mechanism of development and progression of chronic diabetic complications. Endothelial cells are critical for a complex array of functions such as providing a barrier between blood and tissues, maintaining growth and phenotypic characteristics of smooth muscle cells, balancing pro- and anti-inflammatory changes, and fluidity of blood. Alteration of endothelial function, therefore, may affect one or more of these properties. In diabetic microangiopathy, endothelial dysfunction is exhibited as increased permeation, vasoconstriction, and increased synthesis of ECM proteins. Hyalinosis of arterioles and capillaries in diabetes suggests accelerated loss of microvascular endothelial cells and increased ECM deposition. Endothelial degeneration, together with pericyte loss, may bring about a proliferative response and successive elaboration of BM proteins. ETs are implicated in several parameters of microvascular endothelial dysfunction. Administration of ET antagonists has been shown to prevent increased permeability, vasoconstriction, and BM protein expression.

Permeability

We have previously demonstrated that ETs regulate vascular endothelial permeability. Such increased permeability was normalized by treatment with ET receptor antagonist and PKC blocker [4]. The mechanisms by which ETs regulate endothelial permeability are not fully understood. Increased permeability may be arbitrated through endothelial cell contraction. Administration of calcium has been shown to cause phosphorylation of MLCK and cell contracture in endothelial cells [7]. Augmented ET expression by high glucose levels could increase endothelial permeability through interaction with ET_B receptor that is G protein coupled and increases calcium via augmented IP_3. In addition to cell contracture, ETs could also increase permeability via an MLCK-independent retraction mechanism. In such a process, ET-mediated PKC activation is of great significance. PKC has been shown to phosphorylate actin-linking proteins, talin and vanculin, producing intercellular gaps and increased permeability [8].

Mitogenic Responses

ETs are potent mitogens for vascular endothelial cells. The mitogenic property of ETs was first demonstrated in the early 1990s by DNA synthesis assays. Administration of ET-1 was shown to induce DNA synthesis in brain capillary endothelial cells. It has been demonstrated that selective ET_B receptor antagonist can prevent endothelial cell proliferation and migration. In addition to endothelial cells, ETs exhibit mitogenic property toward smooth muscle cells. One interesting difference between the signaling pathways for endothelial and smooth muscle cell proliferation is the involvement of ET receptor type. It has been demonstrated that mitogenic signals are mediated through respective predominant receptor type, that is, for endothelial cells, ET_B, and for smooth muscle cells, ET_A.

In addition to several in vitro studies, ex vivo and in vivo studies also demonstrate mitogenic effects of ETs. Several biochemical pathways may mediate such proliferative signals. ET-induced tyrosine phosphorylation of proteins, such as Src, focal adhesion kinase, and janus kinase, may be involved in these mitogenic responses. In addition, PKC-dependent activation of mitogen activated protein kinase (MAPK) family members may be important in the transduction of mitogenic signals.

ECM Protein Upregulation

A structural hallmark of diabetic microangiopathy is increased capillary BM thickening. Increased expression and decreased degradation of ECM proteins is believed to be critical in BM thickening. The major fibrogenic proteins involved in upregulation of ECM proteins are ETs, TGF-β, and angiotensin II. We have previously demonstrated that high glucose concentration in endothelial cells and hyperglycemia in diabetes leads to upregulation of ECM proteins, fibronectin (FN) and collagen, via ET alteration. Furthermore, recent studies suggest that TGF-β and angiotensin II may also cause increased expression of ECM proteins through ETs. Studies from our laboratory demonstrate that ETs activate NF-κB and AP-1 in target organs and in cultured microvascular endothelial cells leading to FN upregulation [9]. It should be noted, however, that parallel activation of PKC and MAPK family by ETs may also be involved in increased FN expression.

In addition of direct upregulation of ECM proteins such as FN, ETs may also regulate composition of ECM. Recently, we have demonstrated that ETs regulate preferential expression of oncofetal FN, a splice variant of FN [10]. Oncofetal FN is exclusively expressed in proliferating tissues such as embryos and tumors and has recently been proposed to be a marker of tumoral angiogenesis. We have also shown that ET-mediated oncofetal FN is involved in microvascular endothelial cell proliferation. The mechanism by which ET-mediated oncofetal FN regulates cellular proliferation is still obscure. However, recent studies from our laboratory suggest a potential role of oncofetal FN in VEGF expression.

ETs in Organ-Specific Microvascular Alterations in Diabetes

Diabetic Retinopathy

Diabetic retinopathy (DR) predominantly affects the vascular components of the retina. Early in the disease course, diabetes causes functional alterations such as reduced retinal blood flow [2]. With sustained hyperglycemia structural changes such as capillary BM thickening, loss of pericytes, and breakdown of intracellular endothelial cell junctions occur.

Retinal tissue is a rich source of ET-1 and ET-3 [1]. We and others have shown increased mRNA and protein expression of both ET-1 and ET-3 in retinas of diabetic animals in association with retinal vasoconstriction [1]. Blockade of ET receptor mediated signaling and ECE1, enzyme involved in ET peptide processing, prevents retinal vasoconstriction and associated structural changes.

With respect to structural changes, we have demonstrated that ET receptor blockade with dual ET_A and ET_B antagonist, bosentan, prevents diabetes-induced upregulation of FN and collagen alpha-1 (IV) mRNA, and increased capillary BM thickening in animals [1]. ETs could also arbitrate later stages of DR as selective ET_B receptor antagonists can prevent endothelial cell proliferation and migration, two fundamental steps in the process of angiogenesis. In a few recent studies, vitreous ET-1 levels were found to be significantly elevated in patients with proliferative DR as compared to nondiabetic subjects [10].

Diabetic Nephropathy

Diabetic nephropathy (DN) remains the most common cause of renal failure. Sustained hyperglycemia leads to

glomerular hyperfiltration and microalbuminuria. With progression, patients develop overt macroalbuminuria and reduced glomerular filtration rate. Pathological features of DN include mesangial matrix expansion, thickening of glomerular capillary BM and tubulointerstitial fibrosis.

ETs may regulate renal blood flow and glomerular filtration. Recent studies from our laboratory have demonstrated increased expression of ET-1, ET-3, ET_A, and ET_B in the diabetic rat [1]. Increased ET-1 mRNA and increased renal ET-1 clearance in association with proteinuria has been demonstrated in human diabetes. Furthermore, treatment of diabetic animals with ET receptor antagonist has been shown to prevent microalbuminuria.

Studies in rat mesangial cells have implicated ETs in regulating ECM protein production. Diabetes-induced increased expression of ECM proteins and other fibrogenic growth factors has been shown to be completely blocked by treatment with an ET_A receptor antagonist and dual ET_A and ET_B receptor antagonist.

Diabetic Cardiomyopathy

Diabetic cardiomyopathy is a prominent cardiac complication that involves structural and functional changes in both cardiomyocytes and capillary endothelial cells. Pathological features of diabetic cardiomyopathy include myocyte hypertrophy and/or necrosis, interstitial and perivascular fibrosis, and capillary BM thickening.

ETs have been shown to be produced by both cardiomyocytes and endothelial cells. We have previously demonstrated upregulation of ET-1 along with ET_A and ET_B receptor expression in heart tissues of diabetic rats. Such alterations were associated with focal apoptosis of cardiomyocytes, scarring of the myocardium, and increased expression of ECM proteins. Inhibition of ET receptor signaling completely prevented these structural abnormalities. Furthermore, a duration-dependant alteration of chronotropic and inotropic responses to ET-1 has been demonstrated in isolated atria of diabetic rats. Recently, we have demonstrated that ET-1 may interact with sodium–hydrogen exchanger-1 (NHE-1) in mediating diabetes-induced structural and functional changes. NHE-1 may act as the downstream mediator in the development of ET-mediated functional and structural changes in diabetic myocardium.

Diabetic Neuropathy

Diabetic neuropathy is one of the most prevalent complications of chronic diabetes. The pathogenesis of diabetic neuropathy involves chronic hyperglycemic insult to both neurovasculature and neuronal parenchyma. Studies in STZ-induced diabetic rats have established a role of ETs in impairment of endoneurial blood flow. In addition, reduced NO production in the vasculature of the peripheral nerve has been demonstrated, which may further augment ET expression. ET receptor antagonism has been shown to prevent impairment of endoneurial blood flow in diabetic animals.

Neuronal parenchymal damage is believed to be due to impaired nerve conduction velocity. Impaired nerve conduction velocity has been associated PKC activity and could possibly be mediated via ETs. We have demonstrated increased immunoreactivity of ET-1 and ET-3 in peripheral nerves in diabetes. Furthermore, inhibition of ET receptor-mediated signaling has been shown to prevent early nerve conduction velocity deficits in STZ-induced diabetic rats.

Concluding Remarks

Experimental and clinical studies over the past few years indicate that ETs are of significance in several human diseases. Their predominant expression in vascular tissues and their multifunctional nature do indeed suggest that alteration of ETs may be involved in diseases affecting both the micro- and macrovasculature. In both animal and human diabetes, ETs have been shown to be upregulated. Hyperglycemia-induced biochemical anomalies such as PKC activation, nonenzymatic glycation, oxidative stress, augmented polyol pathway, and elaboration of various growth factors and cytokines may contribute to alteration of ETs. ETs, in turn, may regulate other vasoactive factors and growth factors leading to changes in both hemodynamic and structural parameters. A schematic outline of ET alteration and its consequences has been depicted in Figure 1. In support of a central role of these multifunctional peptides in diabetes-induced pathogenetic changes, it has been shown that ET-receptor antagonists prevent structural and functional abnormalities in all target organs of chronic diabetic complications in animal models. Based on the available data, ET antagonism may have a potential role in the treatment of these complications.

Glossary

Basement membrane: A ubiquitous supportive tissue that underlies an epithelium or endothelium. This tissue contains macromolecules such as collagen, fibronectin, laminin, and sulfated proteoglycans.

Extracellular matrix: A meshwork-like substance found within the extracellular space. It provides a supporting structure for cells and regulates cellular events.

Neovascularization: The development of new blood vessels. Neovascularization is an important event in tissues where circulation has been impaired by trauma or disease.

Vascular permeability: The property of the vasculature to be pervaded by water and large molecular weight proteins.

Vasoactive factors: Factors that exert an effect on the caliber of blood vessels. These factors include endothelins, angiotensin II, nitric oxide, and prostacyclins.

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lins in diabetic complications. Special emphasis is on the possible mechanisms of endothelin alteration in diabetes and the effects of such alteration in target organs of chronic diabetic complications.

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Capsule Biography

Dr. Chakrabarti is a professor in the Department of Pathology at the University of Western Ontario, and a pathologist at the London Health Sciences Centre, Canada. His laboratory primarily focuses on structural and functional alterations in diabetic microangiopathy. His work is supported by grants from the Canadian Diabetes Association, Canadian Institutes of Health Research, Heart and Stroke Foundation of Ontario, and Lawson Health Research Institute.

Mr. Zia A. Khan is a graduate student in the Department of Pathology at the University of Western Ontario, Canada.