Therapeutic Targeting of Endothelial Dysfunction in Chronic Diabetic Complications

Zia A. Khan[1,2] and Subrata Chakrabarti[1,*]

[1]*Department of Pathology, University of Western Ontario, London, Ontario, Canada,* [2]*Vascular Biology Research Program and Department of Surgery, Children's Hospital Boston, Harvard Medical School, Boston, MA, USA*

Received: March 3, 2006; Accepted: March 6, 2006; Revised: April 5, 2006

Abstract: In an ever-increasing population of patients with diabetes, morbidity and mortality due to the secondary complications require prompt identification of the underlying mechanisms. Aberration in the vascular endothelial cell function may be a key element in the development and progression of the chronic diabetic complications. We present the hypothesis that preservation and restoration of the endothelial cell function may potentially be the most efficient means of targeting the adverse effects of hyperglycemia.

Keywords: Endothelial cells, diabetes, microangiopathy, macroangiopathy, basement membrane, extracellular matrix, fibronectin, oxidative stress, protein kinase C, aldose reductase, nitric oxide, endothelin, vasoactive factors, cardiomyopathy, advanced glycosylation end products.

INTRODUCTION

Diabetes is estimated to affect 6% of the North American population [1]. After the discovery of insulin, morbidity and mortality associated with diabetes are presented primarily due to the secondary vascular complications. These complications include microangiopathy (retinopathy, nephropathy, neuropathy, and cardiomyopathy) and macroangiopathy (accelerated atherosclerosis) [2, 3]. Macroangiopathic changes may affect the coronary artery, the carotid artery, as well as peripheral arteries. Hyperglycemia has been identified as the key mediator of target organ dysfunction in diabetes [4, 5]. This review will explore the hypothesis that vascular endothelial cell dysfunction is the origin of chronic diabetic complications and represents an important therapeutic target.

ENDOTHELIAL CELLS – THE PRIMARY TARGET OF HYPERGLYCEMIC DAMAGE

Endothelial cells (ECs) represent the primary target of glucose-induced adverse effects in the vasculature. The vascular ECs and smooth muscle cells (SMCs) incorporate glucose via facilitative diffusion [6]. In contrast to the ECs, the contractile cells exhibit autoregulated glucose transport [6]. Thus, increased levels of glucose accumulate in the ECs but not SMCs. *In vitro* studies have shown that exposure of ECs to glucose leads to activation of the ECs, which is reflected by increased production of the extracellular matrix proteins (collagen and fibronectin), as well as procoagulant proteins (von Willebrand Factor; vWF), and shows decreased cellular activities (proliferation and migration) [7-12]. Increased insulin levels, a concurrent event in type 2 diabetic patients, may also modulate several of these factors [13-15]. Thus, all of the normal EC functional properties (Table 1) may be affected by glucose and/or insulin in diabetes, making EC dysfunction a key pathological element in the development of chronic diabetic complications [16, 17].

EC dysfunction may not be uniform in all organs in the patient with diabetes. The mechanism by which chronic diabetes leads to impairment of blood supply in some target organs while inducing unregulated angiogenesis in others such as the retina, remain undetermined. Selectively targeting vasculature in the target organs suggests the importance of two key elements, the tissue microenvironment and the intrinsic properties of the targeted ECs. Until 1960s, the EC lining was considered to be merely a non-thrombogenic cellular barrier. However, Florey and colleagues observed the notion that EC lining is dynamic, metabolically relevant for tissue function and represents more than an inert cell sheet [18]. The variability in the ECs is evident at the level of cell size, cell shape, antigenicity, function, and susceptibility to pathological states. For example, Weible-Palade bodies (site of vWF) exhibit differential patterning in the vasculature, being highest in number in the ECs close the heart as compared to other vascular beds [19-21].

A. VASOACTIVE FACTORS – THE EARLY MEDIATORS

The important role of ECs in microvascular complications of diabetes is well established [22]. Macrovascular atherogenesis also exhibits an important EC component [23, 24]. In atherosclerosis, ECs are important for the initiation (increased antigeinicity/ permeability) and perpetuation of atherosclerotic process (retention of atherogenic proteins/ leukocytes) [25-27]. EC dysfunction in diabetes can be defined (classically) as impaired endothelium-dependent vasodilation (EDV) [28-30]. In diabetes, impairment of EDV has been shown to precede the structural changes in the vasculature and represents an early functional alteration caused by high glucose levels [31-36]. Acute exposure to high levels of glucose in healthy individuals shows impairment of EDV [37, 38]. The mechanism of such acute

*Address correspondence to this author at the Department of Pathology, University of Western Ontario, London, Ontario N6A 5C1, Canada; Tel: (519) 685-8500; Ext: 36350; Fax: (519) 661-3370; E-mail: subrata.chakrabarti@schulich.uwo.ca

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Table 1. EC Function – and Their Mediators Altered in Diabetes

EC Function	Mediators
Hemodynamic/Vascular Tone	*Vasoconstriction*
	Angiotensin II
	Endothelin-1
	Thromboxane A_2
	Prostaglandins H_2 and $F_{2\alpha}$
	Vasodilators
	Bradykinin
	Nitric Oxide
	Prostaglandin I_2
EC Growth and Proliferation	*Proliferation of ECs*
	Basic-Fibroblast Growth Factor
	Endothelin-1
	Epidermal Growth Factor
	Hepatocyte Growth Factor
	Insulin-like Growth Factor (1 and 2)
	Nerve Growth Factor
	Oncofetal Fibronectin
	Platelet Derived Growth Factor
	Transforming Growth Factor-β
	Tumor Necrosis Factor-α
	Vascular Endothelial Growth Factor
	Inhibition of EC proliferation
	Angiostatin
	Endostatin
	Nitric Oxide
	Pigment Epithelium Derived Factor
	Prostaglandin I_2
Anti-thrombogenic/-fibrinolytic	*Pro-thrombogenic/fibrinolytic*
	Plasminogen Activator Inhibitor-1
	von Willebrand Factor
	Anti-thrombogenic/fibrinolytic
	Nitric Oxide
	Tissue type-Plasminogen Activator
	Thrombomodulin
Inflammation	*Pro-inflammatory*
	E-Selectin
	Intracellular Adhesion Molecules-1
	Vascular Adhesion Molecule-1
	Anti-inflammatory
	Nitric Oxide

effects, although not fully determined, may involve increased expression of endothelin-1 (ET-1) and decreased bioavailability of nitric oxide (NO). Augmented ET levels lead to vasoconstriction and reduced blood flow in diabetes [39]. Increased activity of ETs has been shown in the resistance arteries of diabetic patients [40]. Moreover, administration of ET-1 in humans reduces coronary and renal blood flow and increases vasoconstriction [41-43]. Improvement of the vasodilator responses has been noted in diabetic patients administered an ET antagonist [40]. We have also reported that diabetes-induced retinal capillary vasoconstriction is normalized with an ET receptor antagonist (Bosentan) [44].

Acetylcholine-induced paradoxical vasoconstriction in atherosclerotic coronary arteries has suggested an important role of nitric oxide synthase (NOS) enzymes [45]. Acute exposure of ECs to glucose decreases NO generation by agonists including bradykinin and A23187 [46]. These effects were shown to be the direct result of high glucose levels. Purified endothelial NOS (eNOS) when assayed in the presence of glucose also shows a significantly lower level of NO production [46]. We have shown that the treatment of diabetic animals with NO donor, molsidomine, prevents diabetes-induced vasoconstriction in the retina [47]. Recent studies have also documented restoration of EC function by overexpression of eNOS [48, 49].

The mechanisms of vasoactive factor alteration and scavenging are intimately related to increased oxidative stress. Increased glucose-induced oxidative stress is an early change in the ECs. The pathways which increase oxidative stress are multifactorial (Fig. 1). Acute exposure to high ambient glucose causes glucose auto-oxidation [50]. As mentioned earlier, eNOS has been reported to reduce the production of NO in the presence of glucose [46]. A number of other pathways also induce oxidative stress including, protein kinase C activation, polyol and hexoseamine pathways, oxidized-low lipoprotein, and heme oxygenase pathway [51].

B. GROWTH FACTORS – THE LATE MEDIATORS

We have recently proposed that a balance exists between fibrosis and neovascularization in diabetic complications [22]. The balance is shifted towards EC proliferation and neovascularization in diabetic retinopathy (and possible nephropathy) and towards scar formation/fibrosis in diabetic cardiomyopathy and neuropathy. Understanding the differences between such contrasting effects of diabetes on the target organs is critical. Tissue microenvironment, intrinsic EC differences, as well as the presence of other risk factors (hyperlipidemia, hyperinsulinemia) may be involved in the process. Current molecular understanding of the changes in the target organs shows the involvement of the same players such as oxidative stress and protein kinase C.

In the retina, the oxygenation state regulates various growth factors that promote angiogenesis in order to meet the oxygen demands of the tissue [52]. However, unregulated expression of these growth factors and induction of other pro-angiogenic factors, which may not necessarily be regulated by tissue oxygenation, lead to uncontrolled retinal neovascularization and blindness in the diabetic patients. Likewise, early structural changes in diabetic nephropathy include increased glomerular filtration rate

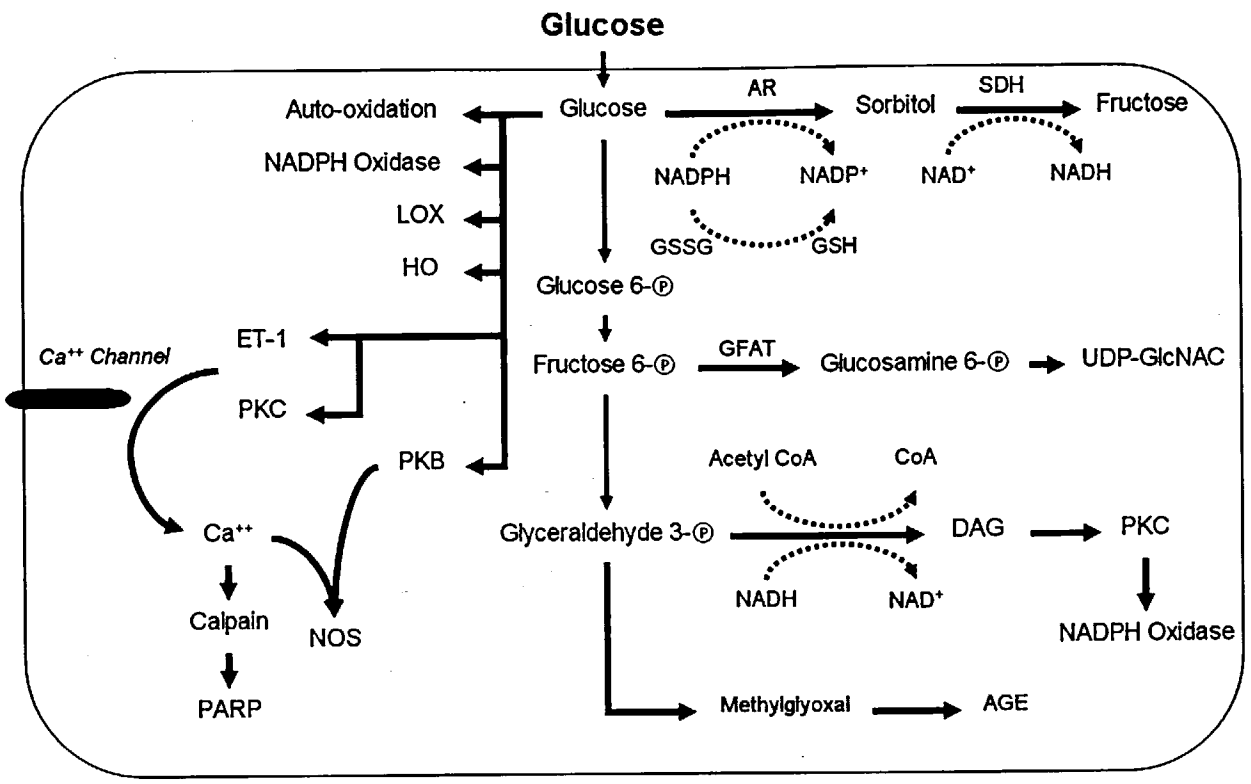

Fig. (1). Proposed mechanisms of glucose-induced biochemical changes in the ECs. High glucose levels lead to activation of polyol pathway, hexoseamine pathway, protein kinase B and C, AGE formation, and PARP activation. In addition, glucose may increase oxidative stress indirectly by activating lipoxygenase and heme oxygenase enzyme systems. These biochemical changes may culminate in increased ET-1 activity, reduced NO availability, and EC dysfunction. [AR, aldose reductase; SDH, sorbitol dehydrogenase; GSSG, oxidized glutathione; GSH, glutathione; GFAT, glutamine:fructose-6-phosphate amidotransferase; UDP-GlcNAC, UDP-*N*-acetylglucosamine; DAG, diacylglycerol; PKC, protein kinase C; AGE, advanced glycosylation end product; LOX, lipoxygenase; HO, heme oxygenase; PKB, protein kinase B; NO, nitric oxide; PARP, poly(ADP-ribose) polymerases].

which may, in part, be credited to increased surface area [53]. Studies have reported an increase of 30-50% in surface area per glomerulus and 20-30% increase in the number of capillaries in diabetic rats [53]. However, in contrast to the retina [22, 54] and possible kidney [53], diabetes leads to impairment in the neovascularization of the heart. It has been reported that diabetes causes reduced expression of VEGF and its receptors in the myocardium [55]. In addition, collateral vessel formation, which represents an adaptive mechanism to reduce tissue ischemia, has also been reported to be impaired in the diabetic patients [56-58]. One possible explanation could underlie defective VEGF signaling in monocytes, which are important for the process of arteriogenesis [57]. Activated monocytes have been reported in ischemic tissues [59, 60]. Monocytes migrate in response to VEGF through the activation of VEGFR1 (Flt-1). *In vitro* assays have shown that monocytes isolated from diabetic patients fail to migrate in response to VEGF [61]. Taken together, these findings suggest that reduced expression of growth factors and growth factor receptors and impaired signaling may in concert lead to inadequate neovascularization in the myocardium. Further studies investigating the contribution of 'angiogenesis' and 'arteriogenesis' in the heart are required before diabetic cardiomyopathy may be adequately targeted.

C. EXTRACELLULAR MATRIX – THE LASTING MEMORY

One important question is what determines the mechanisms of chronic alteration of vasoactive factors/ growth factors, and intracellular signaling molecules? Extracellular matrix (ECM) may offer some explanation (Fig. 2). ECM provides a scaffold for the organization of the vascular cells. The role of ECM in EC biology spans from proliferation, migration and stabilization. Binding of the EC surface integrins to the ECM regulates cell survival/ apoptosis, growth, as well as cytoskeletal changes [62]. The heterogeneity in the ECM, therefore, is important in EC function. Growth factor-induced changes in the ECs are also matrix-dependent [63]. Moreover, the neutralizing effect of angiostatic proteins (thrombospondin, endostatin, etc.) on growth factor-induced proliferation and migration also depends on the ECM [64].

ECM changes during tumorigenesis include increased expression of fibronectin (FN), collagen and tenascin-C [65, 66], whereas, laminin and collagen (IV) levels are decreased.

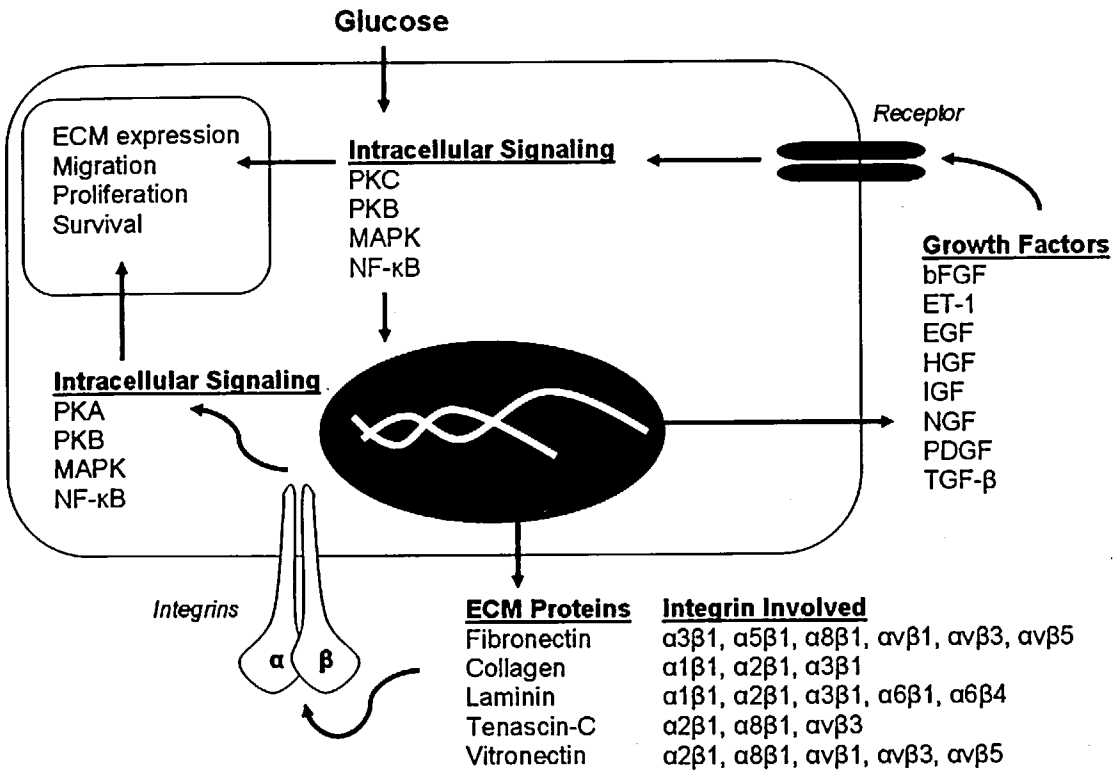

Fig. (2). Effect of glucose on growth factor and ECM protein expression. High levels of glucose increase expression of various growth factors which alter the ECs and cause increased ECM protein expression. ECM proteins which are altered in diabetes and the corresponding integrin receptors are also presented. [MAPK, mitogen activated protein kinase; NF-κB, nuclear factor-κB; bFGF, basic-fibroblast growth factor; EGF, epidermal growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β].

These ECM protein changes are thought to invoke a proliferative response in the vascular ECs during angiogenesis [63]. A similar scenario is evident in the retinal vascular development [67]. In the retina, FN has been reported to be expressed in the active zones of vascularization [67]. Moreover, pericytes and laminin in the basement membranes (BM) appear with EC differentiation and vessel maturation. Could differential temporal/spatial expression of FN and laminin dictate increased angiogenesis in the retina while causing impaired vessel formation in the heart? Retinal BMs of diabetic animals have been shown to contain increased collagen α1 (IV), β1 and γ chains of laminin and FN [68]. These changes are brought about as early as 8 weeks following the onset of diabetes [68]. In addition, upregulation of tenascin has been reported in retinal vessels of diabetic patients and animals [69, 70]. Other BMs in diabetic patients exhibit somewhat similar ECM protein profile [71-74]. In the rat myocardium, diabetes increases laminin expression at 2 months following onset [75]. Two simplistic theories can be formulated to explain these findings; a) proliferating ECM proteins outweigh differentiating ECM proteins, and b) differential proteolytic processing of ECM proteins may produce fragments, which confer an 'angiogenic' phenotype in the ECs. In support of such a notion, we have recently shown increased expression of an embryonic splice variant of FN (ED-B$^+$ FN/oncofetal FN) in the vitreous samples of proliferative diabetic retinopathy [76]. Targeted inhibition of ED-B$^+$ FN is associated with decreased EC proliferation and tube morphogenesis [77]. It is also plausible that FN, which undergoes extensive proteolytic processing [78], produces angiogenic fragments [79, 80].

Another important factor which may arbitrate the differential EC responses in retinopathy as compared to cardiomyopathy may be the presence of abundant cardiac fibroblasts. In the heart, cardiomyocytes constitute about 70% of the volume but only 30% in terms of cell number [81, 82]. ECs outnumber the cardiomyocytes by a ratio of 3:1 [81, 82]. A significant number of cardiac fibroblasts are also present in the heart, which may be responsible for the cardiac ECM deposition in addition to the cardiac ECs. Angiotensin II is able to cause cardiac fibroblast proliferation and collagen deposition [83, 84]. Studies have also indicated that angiotensin II exerts the action on cardiac fibroblasts through the induction of ET-1 [85, 86]. In addition to ECs, fibroblasts also express ET receptors [87]. *In vitro* studies reveal that isolated rat fibroblasts increase production of ECM components upon ET administration [87-89]. These changes can be readily blocked by ET

Table 2. Possible Pharmacological Targets for Restoration of EC Dysfunction in Diabetes

Targets	Effect
Vasoactive Factors	Vascular tone, cellular proliferation, ECM deposition
Angiotensin II	
ET-1	
NO	
Oxidative Stress	Vascular tone, growth factor expression
AGE/RAGE	
Aldose Reductase	
Heme Oxygenase	
Nuclear Factor-κB	
NADPH Oxidase	
PARP	
Protein Kinase C	
Others*	Cellular proliferation
ED-B' Fibronectin	
MAPK/ERK	
Protein Kinase B	
SGK-1	
VEGF	

*Increase/decrease in cardiomyopathy/retinopathy. [ED-B' FN, Extra domain-B containing fibronectin; SGK-1, serum- and gluococorticoid-regulated kinase-1;]

antagonists. It has further been shown that endocardial capillary ECs (in culture) show a 25% higher growth index as compared to other vascular ECs. These findings indicate that tissue microenvironment is an important regulator of impaired angiogenic response in the heart which could be mediated by increased ECM deposition. In clinical trials, ET antagonists have shown promise in short-term studies as indexed by improved cardiac index and pulmonary capillary pressure [90, 91]. However, long-term studies have yet to show beneficial effects [92].

Modification of ECM proteins may also represent an important event in chronic stimulation of biochemical pathways such as protein kinase C, and nuclear factor-κB. Glycation of ECM may decrease the turnover rate of the proteins and thus provide an efficient means of chronic signaling to the vascular ECs. Glucose reacts with proteins via Maillard reaction to produce advanced glycation end products (AGEs) [93] (Fig. 1). AGE products have been reported to cause NO scavenging [94]. Furthermore, AGE may interact with cell surface receptors (RAGEs) to increase cytokine/growth factor expression [95]. These findings support the notion that ECM is equally if not more important than growth factor alteration and may provide a more sustained signaling environment.

CURRENT & FUTURE DEVELOPMENTS

The hypothesis that diabetic complications arise as a result of EC dysfunction is increasingly being acclaimed. A number of biochemical variables are involved which dictate tailored therapy for the prevention as well as restoration of the EC function in diabetes. Acute effects of hyperglycemia may be solely mediated by metabolic changes (polyol pathway), vascular tone (vasoactive factors), and oxidative stress. Patented agents targeting some of these altered pathways have shown promise in terms of delaying the progression of the complications. However, the key elements which still require identification are the factors involved in the late stages of the disease. In addition, an important question which may provide the best therapeutic targets revolves around the elucidation of the molecular features that distinguish retinopathy and cardiomyopathy. Accumulating evidence indicates that ECM/BM changes (tissue environment), response of accessory cells, vasoactive factors, and oxidative stress may be involved. The restoration of the EC function should be the pre-requisite for therapy development (Tables 2 and 3). A possible avenue, which still requires investigation, may be the use of combinatorial therapy. For example, use of ET receptor antagonists (bosentan), NO

Table 3. Selective Agents for the Restoration of EC Dysfunction in Diabetes

Parameter/Targets	Agents	Patents [Reference]
Vascular Tone		
ACE Inhibitors	Enalapril	US4374829 [96]
	Lisinopril	US4374829 [96]
	Quinapril	US4344949 [97]
	Ramipril	US4587258 [98]
	Perindopril	US4508729 [99]
	Fosinoprol	US4337201 [100]
	Benazapril	US4410520 [101]

(Table 3) Contd...

Parameter/Targets	Agents	Patents [Reference]
ET Antagonists	A127722	US6573285 [102]
	ABT 627	US20050042172 [103]
	Bosentan	US20030176356 [104]
	BQ123	US6573285 [102]
	BQ 610	US20030176356 [104]
	BQ788	US20030176356 [104]
	FR 139317	US20030176356 [104]
	LU 135252	US20030176356 [104]
	PD 142893	US20030176356 [104]
	PD 14565	US20030176356 [104]
	PD 156707	US6573285 [102]
	RES 701-1	US 6855701 [105]
	SB 209670	US6573285 [102]
	TAK 044	US6573285 [102]
NO Prodrugs	AcOM-DEA/NO	US5366997 [106]
	DETA/NO (NOC-18)	US5155137 [107]
	DPTA/NO (NOC-19)	US5155137 [107]
	MAHMA/NO (NOC-9)	US5155137 [107]
	PAPA/NO (NOC-15)	US5155137 [107]
	PROLI/NO	US6379660 [108]
	SPER/NO	US5155137 [107]
	V-PYRRO/NO	US5366997 [106]
Oxidative Stress		
AGE Blockers/Breakers	Benfotiamine	US3064000 [109]
	ALT-711	
	Thiazolium compounds	US6790859 [110]
ARI	Pyridazinone	US6849629 [111]
PKCβ Inhibitor	Arxxant	To be filed
NADPH Oxidase	Apocynin	US5902831 [112]
	Diapocynin	US5990137 [113]
PARP	3-AB	WO9307868A1 [114]
	NU 1025	WO9307868A1 [114]
	6-(5H)-Phenanthridinones	WO9911624A1 [115]
	4-Iodo-3-nitrobenzamide	WO9911624A1 [115]
	GPI-6150	WO9911645A1 [116]
	DPQ	WO991649A2 [117]
	Benzimidazole-4-carboxamides	WO9704771A1 [118]
	2-Aryl-1H-benzimidazole-4-carboxamides	WO0026192A1 [119]
	4-Aryl-phthalazones	WO0236576A1 [120]
	PJ34	WO0142219A2 [121]

donors (molsidomine or other prodrugs), and oxidative stress inhibitors (may comprise of PKC, NADPH oxidase, AGE breakers, and PARP inhibitors) may provide defense against both early and late phases of the vascular disease. Targeting the EC lining in the target organs would provide the most efficient therapeutic tool for the treatment of chronic diabetic complications.

ACKNOWLEDGEMENTS

The authors acknowledge grant supports from the Canadian Diabetes Association in honor of the late Glenn W Liebrock, the Canadian Institutes of Health Research, and the Lawson Health Research Institute.

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