# Towards Newer Molecular Targets for Chronic Diabetic Complications

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**Abstract:** Prior to the discovery of insulin, the major cause of death in the diabetic population was ketoacidosis. Although insulin and improved glycemic control have improved the longevity of diabetic patients, they still suffer from significant morbidity and mortality due to chronic secondary complications. Long standing diabetes leads to structural and functional alterations in both the micro- and macrovasculature. These complications, involving the retina, kidney, and peripheral nerves, as well as cardiovascular system, severely compromise the quality and expectancy of life. Large scale clinical trials have identified hyperglycemia as the key determinant for the development of such complications. Therapeutic modalities have been developed to target glucose-induced alterations, such as protein kinase C activation, augmented polyol pathway activity, non-enzymatic glycation and oxidative stress to ameliorate chronic complications. However, clinical trials targeting these biochemical alterations have failed to show significant beneficial effects. The plethora of biochemical anomalies that govern the development of chronic diabetic complications may therefore be subject to cross-interaction and complex interplays. Studies in both animal and human diabetes have, however, showed alteration of several vasoactive effector molecules such as endothelins. These molecules may be instrumental in mediating diabetes-induced structural and functional deficits at both the early and late stages of the disease. This review will discuss the current mechanistic understanding of chronic diabetic complications and will explore the potential novel therapeutic interventions.

**Keywords:** Diabetes, aldose reductase, protein kinase C, advanced glycation end products, oxidative stress, endothelin, fibronectin.

## 1. INTRODUCTION

Diabetes mellitus (DM) affects about 6% of the North American population [1]. A recent report estimated that 8.2% of adult population worldwide has impaired glucose tolerance, a condition which proceeds DM [2]. DM is classified based on etiology, the two major types being type 1 (insulin-dependent diabetes mellitus; IDDM) and type 2 (non-insulin-dependent diabetes mellitus; NIDDM). Prior to the discovery of insulin, the most common cause of death in the diabetic population was ketoacidosis. Even after the introduction of insulin and the development of various therapeutic modalities, diabetic patients suffer from significant morbidity and mortality due to chronic complications [1-4]. Once DM is present, the risk of developing secondary complications significantly increases. These complications include coronary and cerebrovascular disorders, peripheral arterial disease, nephropathy and retinopathy [5,6].

Long standing DM leads to structural and functional alterations in both micro- and macrovasculature [5,6]. The most devastating complications in terms of morbidity are, however, of microvascular origin. These microvascular complications are predominantly present in type 1 DM [3,7]. Type 2 diabetic patients, however, exhibit more pronounced macrovascular complications, which manifest as atherosclerosis, myocardial infarction and peripheral arterial disease [8]. These late complications, which severely compromise the quality and expectancy of life, have hyperglycemia as the determinant [3-8]. Sustained hyperglycemia leads to biochemical and structural anomalies in the eye, kidney, heart, and peripheral nerves. In this review, we focus on molecular alterations, which include signaling molecules, in an attempt to identify new targets of adjuvant therapy.

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## 2. PATHOPHYSIOLOGY OF CHRONIC DIABETIC COMPLICATIONS

### 2.1 Functional Alterations

The earliest pathological changes in diabetic complications are essentially hemodynamic in nature [5-7,9]. These aberrant hemodynamic parameters are believed to result from hyperglycemia-induced metabolic abnormalities and alteration of vasoactive factors, including endothelins (ETs) (Fig. **1**) [5,9]. Altered expression of ETs may lead to increased vasoconstriction and reduced blood flow in diabetes [9]. Increased activity of ET has been demonstrated in resistance arteries of diabetic patients [10]. Vasodilator responses in diabetic subjects can also be improved with ET receptor antagonism [10]. Furthermore, responsiveness to ET has been shown to be unaltered in DM [11]. These *in vivo* findings argue against a deficit in vascular responsiveness to ET and indicate that altered expression of ET and associated vasoconstriction may initiate vascular dysfunction.

Studies involving nailfold microcirculation, as an index of blood flow alteration, have shown both elevated and re-

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duced blood velocity in diabetic patients [12-14]. Furthermore, reduced blood flow velocity has also been shown in the gastric mucosal blood flow test [14,15]. These studies suggest that ET alteration may cause reduced blood flow in DM. However, there is heterogeneity in findings from microcirculation studies which can be attributed to differences in duration of the disease and limitations of the blood flow measurement techniques.

Consistent with increased vasoconstriction, which has been demonstrated in animal models of chronic DM, are findings which suggest impaired endothelium-dependent vasodilation in both type 1 and type 2 DM [16-18]. It is plausible that concurrent reduction in vasodilators like nitric oxide (NO) may contribute to the hemodynamic abnormality in DM (Fig. **1**) [9]. It is, therefore, postulated that blood flow alterations are mediated by increased ETs and reduced NO [5-7,9,19,20]. Administration of ET-1 in humans has demonstrated reduced coronary and renal blood flow and increased vasoconstriction [21-23]. We have also demonstrated DM-induced increase in vasoconstriction in the retina, which was normalized with ET receptor antagonist [24]. Furthermore, we have recently shown that treatment of diabetic animals with the NO donor, molsidomine, prevents DM-induced vasoconstriction, as assessed by reduced resistivity index in the retina [25].

Vascular defects in DM may also lead to other functional alterations. In diabetic neuropathy, reduced nerve perfusion and endoneural hypoxia may play a significant role in the pathogenesis of nerve dysfunction [26,27]. Chronic DM may lead to deterioration of nerve mylenation and structure and cause reduced nerve conduction velocity (NCV) and amplitude [26,27]. With the progression of the disease, nerve fibers may also be lost [26,27]. Pharmacological therapies, which have shown beneficial effects in terms of nerve function, have indicated vascular targets for all the beneficial effects [28-30]. Vasodilator therapy produced improved nerve function in both animal models of the disease and human DM [28]. Furthermore, treatment of diabetic animals with an ET receptor antagonist prevents the DM-induced NCV deficit and reduced endoneural blood flow [31,32].

Functional alterations in diabetic complications also include increased permeability, which manifests as macular edema and exudate formation in the retinopathy and as albuminuria in nephropathy [25,33]. Renal complications include microalbuminuria, an early stage present in both type 1

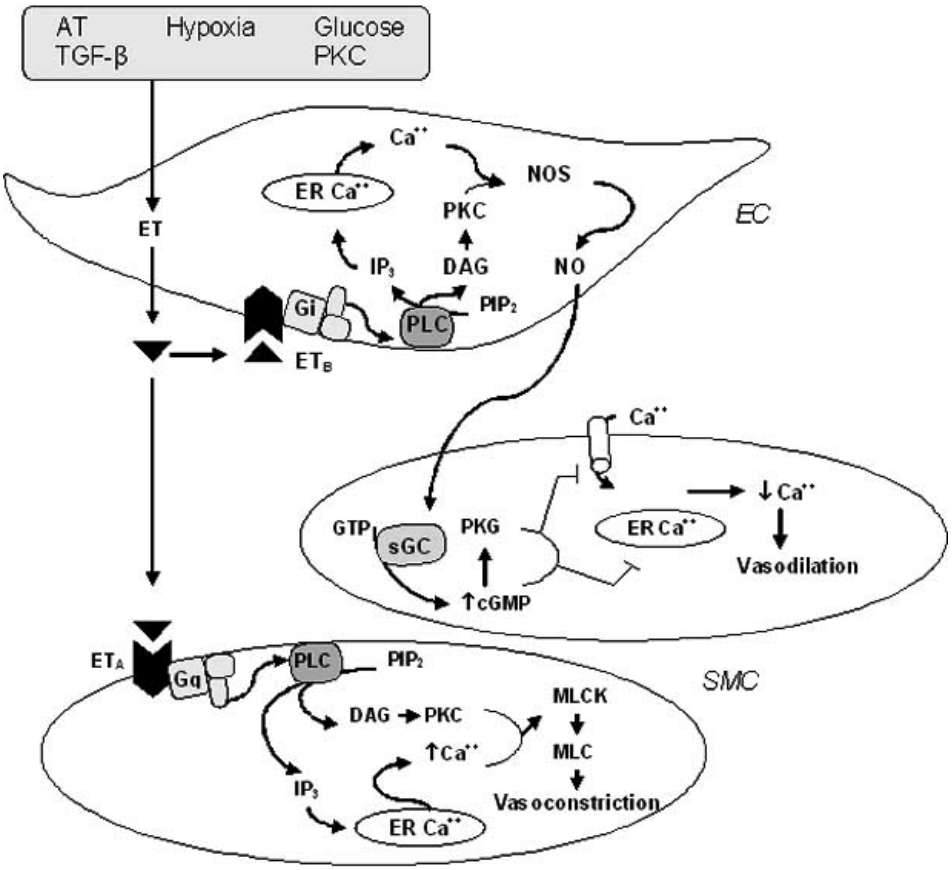


**Fig. (1).** Vasoregulation by constrictors and dilators. Diabetes leads to imbalance between ET and NO causing increased vasoconstriction and impaired endothelium-dependent vasodilation [AT = angiotensin; cGMP= guanosine 3',5'-(cyclic)phosphate; DAG = diacylglycerol; EC = endothelial cell; ER = endoplasmic reticulum; $ET_A$ and $ET_B$ = ET receptors A and B; G = G-protein; $G_i$, $G_q$ = G; GC = guanylate cyclase; $IP_3$ = Inositol triphosphate; MLC = myosin light chain; MLCK = MLC kinase; $PIP_2$ = phosphatidylinositol bisphosphate; PKG = protein kinase G; PLC = phospholipase C; sGC = soluble GC; SMC = smooth muscle cell; TGF-β = transforming growth factor-β].

and type 2 DM [33-35]. A large scale study has shown an association between microalbuminuria and increased glomerular basement membrane thickening [36]. Furthermore, inhibition of angiotensin signaling by losartan, an angiotensin II receptor antagonist, reduced urinary albumin excretion and increased effective renal plasma flow [37,38]. Interestingly, increased ET expression and clearance in association with proteinuria in diabetic animals has been demonstrated [39].

### 2.2 Structural Alterations

One early event in the development of diabetic angiopathy is vascular endothelial cell damage and loss (Fig. **2**) [40,41]. 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 regulating blood fluidity. This cellular defect in DM may occur due to increased oxidative stress, toxicity from high levels of glucose, and other factors. In the retina, DM has also been shown to cause pericyte damage and loss [42], which may result in increased vasoconstriction and permeability [42,43]. As discussed earlier, ET-mediated vasoconstriction has been shown in both human and animal DM [9]. ET alteration in DM may also serve as a surrogate marker of endothelial dysfunction. In addition to vasoconstriction, increased permeability may also contribute to non-perfusion and ischemia in target organs of late complications [44,45]. The exchange between blood and tissues represents an important function of endothelial cells. Loss of such control can compromise the integrity of the irrigated organ. Permeability studies in type 1 and hypertensive type 2 diabetic patients have revealed increased albumin flux through the endothelium [46]. The cellular and molecular mechanisms are still obscure, in part, due to our inability to adequately assess vascular permeability. It is, however, believed that a dysfunction in the transfer property of the endothelium occurs, which when precipitated with occlusion and vasoconstriction, leads to under- or non-perfusion of target tissues.

Tissue ischemia, in conjunction with cellular damage, may trigger a reparative response; a finding which is well documented in retinal microcirculation [40,41,47]. This reparative mechanism entails alteration of various growth factors, such as transforming growth factor-β and vascular endothelial growth factor (VEGF) [47]. However, these responses may be damaging as activation of vascular cells and augmentation of growth factors may lead to increased extracellular matrix (ECM) deposition, basement membrane (BM) thickening, and further aggravation of ischemia (Fig. **2**). Increased ECM protein expression and re-duplication of capillary BM thickening is among the most consistent features of diabetic angiopathy [48-50]. We have demonstrated that DM leads to increased capillary BM thickening in all target organs of diabetic complications, namely the retina,

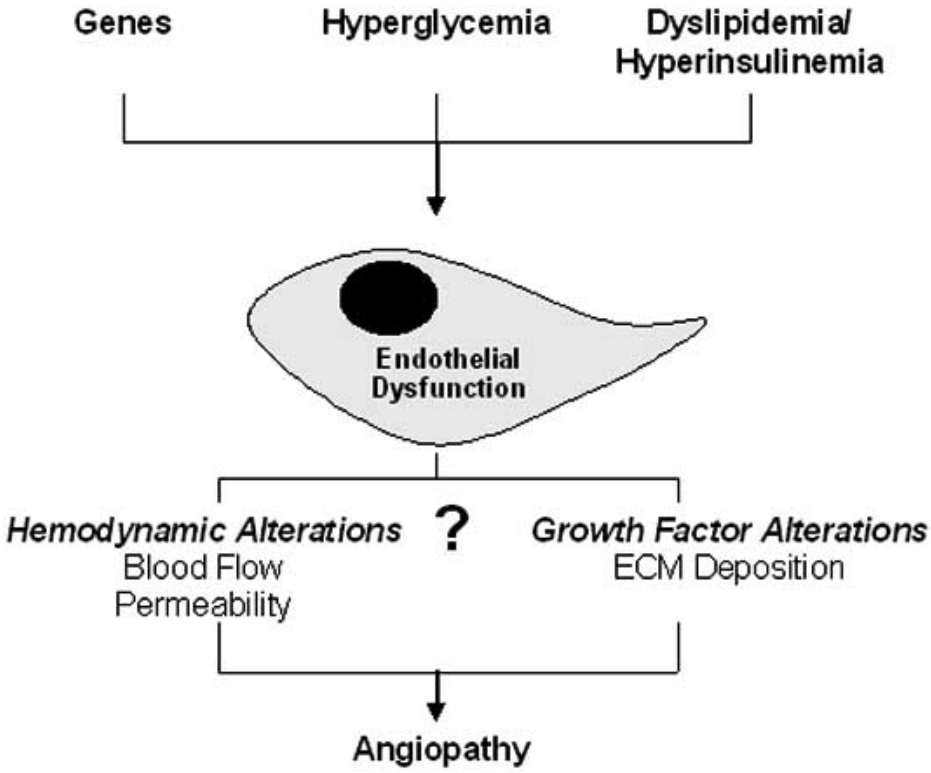


**Fig. (2).** Endothelial dysfunction is the common precursor of diabetic angiopathy. Hyperglycemia in concert with environmental and genetic factors leads to endothelial activation and loss of normal cell function. The early manifestation of endothelial dysfunction is blood flow alteration, increased permeability, and non-perfusion. In response to ischemia, target tissues elaborate growth factors and lead to a proliferative response which increase ECM protein expression and in certain targets, such as the retina, can cause neovascularization.

kidney, heart and aorta [51,52]. Furthermore, we have shown that inhibition of ET receptor signaling prevents DM-induced BM thickening in all target organs of late complications [51,52]. Capillary BM thickening can result from increased production and decreased degradation of BM components, such as fibronectin (FN) and collagen. The signals for increased BM thickening are becoming clearer, but the complex interactions between these stimuli are not very well understood. High glucose levels can increase mRNA expression of collagen and FN in both vascular endothelial cells and mesangial cells [53-56]. BMs of diabetic animals have been shown to contain increased collagen α1 (IV), β and γ chains of laminin and FN [57]. These changes are brought upon as early as 8 weeks following onset of DM [57]. In addition to augmentation of collagen α1 (IV) and FN, upregulation of other ECM proteins such as tenascin has been found in retinal vessels of diabetic patients and animals [58,59].

## 3. BIOCHEMICAL BASIS OF COMPLICATIONS

A large number of studies in both humans and animal models focused on elucidating the mechanisms of diabetic vascular disease. Although experimental and animal studies, together with clinical assessments, have provided information on the pathology of the late complications of DM and the underlying mechanisms, the exact pathogenesis is not yet fully understood. A clearer understanding of the mechanistic details is important for the development of specific therapies. Two large scale clinical trials, the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS), have alluded to hyperglycemia as the determinant of chronic diabetic complications in both type 1 and type 2 DM, respectively [3]. High levels of glucose cause many functional abnormalities in the vasculature and lead to structural tissue changes. The mechanisms that mediate these adverse effects include nonenzymatic glycation processes, aberrant aldose reductase (AR) enzyme activation, and alterations of various signal pathways such as protein kinase C (PKC) pathway [4-7]. This aberrant biochemical status results in endothelial cell dysfunction and development of angiopathy. Although high levels of glucose appear to be a major mechanism, other factors such as hyperinsulinemia and lipid dysmetabolism may play important role in diabetic complications. We will discuss the potential mechanism by which chronic DM may lead to vascular disease.

### 3.1 Aldose Reducatse (AR) and the Polyol Pathway

Under normoglycemic conditions, only a small proportion of glucose is metabolized via the polyol pathway. However, when intracellular glucose levels overwhelm the glycolytic pathway, AR activity is increased. Augmented AR activity has been shown to be an important mechanism in the pathogenesis of chronic diabetic complications [60,61]. Increased activity of AR leads to sorbitol accumulation, as AR is the rate limiting enzyme in the polyol pathway [61]. Sorbitol may also cause cellular damage, since intracellular accumulation of this impermeable metabolite leads to osmotic stress and alteration of myo-inositol [61,62]. Interestingly, we have shown that supplementation of myo-inositol attenuates increased diabetes-induced BM thickening in the retina [63].

In the augmented polyol pathway, some of the sorbitol is metabolized to fructose by the proceeding sorbitol dehydrogenase reaction. Metabolism of glucose to sorbitol via the AR requires NADPH [60,61]. In addition, augmentation of polyol pathway may also cause depletion of $NAD^+$ through sorbitol dehydrogenase. Therefore, increased flux of glucose through the polyol pathway produces a state of redox imbalance [64,65]. Various enzyme systems, including antioxidant reduced glutathione enzyme, require NADPH cofactor and thus, may exhibit impaired activity. Furthermore, increased NADH production, via augmented polyol activity, may increase advanced glycation end product (AGE) formation. NADH is a co-factor for glyceraldehyde 3-phosphate dehydrogenase, which produces glyceraldehyde 3-phosphate, a precursor of AGE-forming methylglyoxal [66].

Recently, polymorphism in AR gene has been shown to be linked to increased susceptibility of microvascular complications in DM [67-69], suggesting a role of AR inhibition as a therapeutic modality. AR inhibition has also shown some beneficial effects in diabetic neuropathy and nephropathy [70,71]. However, several clinical trials have been performed that involve the inhibition of AR without any conclusive results. In a randomized trial with AR inhibitor sorbinil, no significant changes were observed between the placebo and sorbinil treatment groups [72]. However, the number of microaneurysms in the retina increased at a slower rate with sorbinil treatment. These somewhat beneficial effects were only significant at midpoint but not at the maximum follow-up of the study. These findings indicate that AR inhibition may only be beneficial in tissues that exhibit significantly/quantitatively higher AR activity levels such as in diabetic neuropathy. In fact, somewhat positive results have been obtained in diabetic patients suffering from neuropathy [73]. However, in strictly vascular terms, lowering AR activity does not seem to be sufficient in reducing glutathione, increasing oxidized glutathione and preventing triose phosphate oxidation [73].

### 3.2 Protein Kinsae C (PKC) Activation

Hyperglycaemia has been shown to increase *de novo* synthesis of diacyl glycerol (DAG), the endogenous activator of PKC [74,75]. PKC-mediated activation of numerous substrate proteins is important in regulating vascular cell function. PKC may mediate endothelial cell permeability, pericyte loss, and expression of various angiogenic factors, which are implicated in diabetic retinopathy [74-77]. In addition, PKC may affect the activation of vasoactive factors such as ET-1 and NO [76,78], leading to blood flow alteration. DM has been shown to activate several PKC isoforms, including PKCα, βI, βII, γ, and δ [79,80]. However, PKC βI and βII have been shown to exhibit greater quantitative degree of activation in retina, heart and aorta [80]. It should be noted, however, that a lower degree of activation of one isoform may be of greater importance than increased activation of another isoform. The proliferative responses of endothelial cells cultured in high levels of glucose are mediated by atypical isoform of PKC, PKC τ [81]. These findings suggest

that multiple isoforms of PKC may mediate such wide array of vascular functions.

A significant role of PKC in various vascular changes in diabetic complications, particularly retinopathy, prompted the development of specific PKC inhibitors. The primary target of such an inhibitor would be the PKCβ isoforms, as these isoforms exhibited the greatest degree of induction in DM. Ruboxistaurin mesylate (LY333531), a highly specific PKCβ inhibitor, was developed in 1996 [82]. Since then, this PKC inhibitor has been used in various animal models and clinical trials to determine whether inhibiting PKCβ prevents DM-induced angiopathy [83-87]. After successful phase I tolerability and pharmokinetic trial, ruboxistaurin was shown to increase retinal blood flow in patients with minimal or no evidence of diabetic retinopathy [85-87]. Results from the phase III studies showed delay of the occurrence of moderate visual loss in patients with moderately severe to severe non-proliferative diabetic retinopathy at only 24 months [88]. However, at both 12 and 36 month ruboxistaurin treatment periods, the results were not significantly different as compared to placebo. In terms of other targets of chronic diabetic complications, results are not yet available, thus limiting any indication of the beneficial effects of PKC inhibition.

### 3.3 Advanced Glycation End (AGE) Products

Non-enzymatic glycation and generation of AGE products has been demonstrated as an important factor in the pathogenesis of chronic diabetic complications [89,90]. Glucose, glucose-6-phosphate, trioses, and fructose may all participate in non-enzymatic glycation of proteins [89-92]. AGEs may also be produced from strong glycating dicarbonyl compounds such as 3-dioxyglucosone, methylglyoxal and glyoxals [93]. AGEs accumulate in tissues over time during normal aging; however, DM leads to acceleration of this process [89-96]. Furthermore, both AGEs and their receptors (RAGEs) have been localized to the target organs of diabetic complications [91,94,95,97]. The activities and functions of the receptors *in vivo* remain unclear, although some of them are well characterized. These receptors are found on many cells including endothelial and smooth muscle cells and may mediate extracellular signals in the respective cells [89-91,94,95,97]. Aminoguanidine, a specific inhibitor of non-enzymatic glycation, has been shown to inhibit the development of retinopathy in diabetic dogs [98].

The mechanisms by which AGEs may cause pathologic changes include alteration of protein function, interference with ECM function, and elaboration of cytokines. Production of AGEs can also generate reactive oxygen species, oxidative stress, and activation of nuclear factor-κB (NF-κB) [93-95,99]. In vascular endothelial cells, AGE formation may affect gene expression of thrombomodulin, and ET-1, and modify growth factors such as VEGF and basic-fibroblast growth factor (bFGF) [100,101]. In addition, AGE can form cross-links with collagen in the ECM, reduce arterial compliance and alter gene expression of several important intracellular molecules [102]. In further support of a role of AGEs in vascular cellular dysfunction are studies which demonstrate that exogenous administration of AGEs can lead to pericyte loss in the retina without any metabolic abnormality [103]. In contrast to contractile cells, retinal endothelial cells proliferate in response to glycated BMs [104], which could be of importance in advanced stages of diabetic retinopathy such as neovascularization.

### 3.4 Oxidative Stress

Increased oxidative stress has been implicated in both the early and late pathogenic changes in all target organs of diabetic complications [5,105,106]. Hyperglycemia-induced

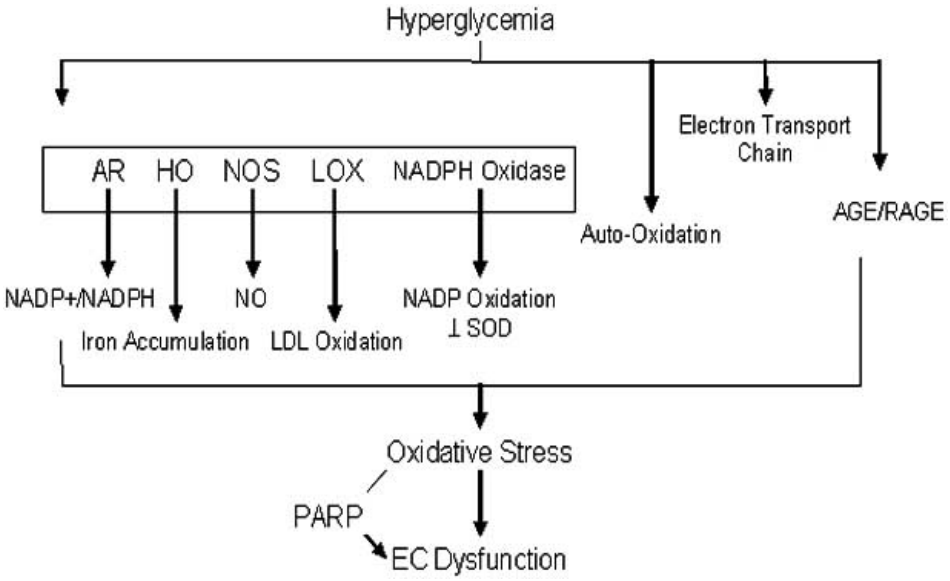


**Fig. (3).** Putative mechanisms causing hyperglycemia-induced oxidative stress. High glucose levels directly increase ROS production by auto-oxidation, increased glycolytic pathway, and modified proteins. Hyperglycemia may also increase ROS indirectly by increasing the activity of various enzymes that lead to oxidative stress and vascular endothelial dysfunction [SOD = superoxide dismutase].

oxidative stress could be increased via a number of pathways (Fig. **3**). These indications lead to the hypothesis that increased oxidative stress, via mitochondrial superoxide overproduction, may represent a unifying mechanism of chronic diabetic complications [5]. Oxidative stress is certainly important, given the results of recent studies; however, it may not account for beneficial effects observed with other seemingly unrelated pathway inhibitors, including ET antagonists [9]. Structural studies are necessary to confirm the role of oxidative stress in diabetic complications. Traditional scavengers are not very efficient in preventing superoxide production [107]; on the other hand, compounds like bentofotamine have been shown to be effective in preventing changes in diabetic retinopathy [107]. Regardless of these findings, oxidative stress does seem to play a significant role in the development and progression of diabetic complications. High levels of glucose increase the production of ROS directly by auto-oxidation [5,108]. Such reaction could yield both superoxide and hydrogen peroxide. Hyperglycemia may also increase ROS by increased flux through the mitochondrial electron transport chain [109]. As electrons are transferred through the mitochondrial complexes (complex I, III, and IV), a proton gradient is created. With increase in the gradient, the electrochemical potential difference augments superoxide production [5,109]. Inhibition of complex II and uncoupler of oxidative phosphorylation, which prevents proton gradient generation, has been shown to abolish glucose-induced ROS production in cultured endothelial cells [109]. Furthermore, mitochondrial superoxide overproduction has been linked to induction of plasminogen activator inhibitor-1, which prevents ECM protein degradation [110], suggesting an upstream localization of oxidative stress in DM-induced structural changes.

Another potential mechanism of hyperglycemia-induced ROS generation is via modified proteins. Auto-oxidation of protein-bound amadori products and AGE/RAGE interaction has been shown to increase production of ROS and activation of redox-sensitive transcription factor, NF-κB [99]. In addition to the mechanisms outlined above, alteration of various enzymes may also mediate DM-induced ROS generation (Fig. **3**). Elaboration of various factors such as angiotensin may increase the activity of NADPH oxidase and result in cell death by superoxide [111,112]. NADPH oxidase, which carries out oxidation of NADPH, increases xanthine oxidase activity and may also inhibit superoxide dismutase. Increased flux of glucose through the AR pathway may also contribute to oxidative stress via imbalance between redox factors. Increased $NADP^+$ to NADPH ratio may severely comprise the ability of anti-oxidant enzyme systems and result in oxidant injury in vascular cells. All these direct and indirect mechanisms of ROS production may precipitate the complications of DM by damaging protein, lipids and DNA. Oxidative DNA damage has been shown to increase the activity of a novel protein, poly (ADP-ribose) polymerase (PARP) [113,114]. This augmented activity of PARP may lead to depletion of $NAD^+$, reduced ATP formation and electron transport, and may cause direct endothelial dysfunction [115-117]. Inhibition of PARP prevents DM-induced impairment of endothelium-dependent vasodilation [116,117].

Studies in human DM have shown impaired endothelium-dependent relaxation in the vasculature. However, we and others have shown increased expression of NO producing enzymes, endothelial NO synthase (eNOS) and inducible NO synthase (iNOS) in target organs of diabetic complications [118,119]. These contradictory results suggest that impaired vascular relaxation in DM is due to altered bioavailability of NO [120]. Numerous studies show efficient interaction between NO and superoxide yielding peroxynitrite (Fig. **4**) [121,122]. The particular arrangement of eNOS, caveolin-1 and a scavenger receptor, CD36, could offer some explanation for the efficient scavenging of NO in DM [123]. CD36 has been shown to be expressed in microvascular endothelial cells [124]. The prototyopical ligand of this receptor is modified lipoprotein molecule such as oxidized low density lipoprotein (ox-LDL). We have shown upregulation of CD36 in target organs of diabetic complications and cultured endothelial cells exposed to high levels of glucose [123,125]. Furthermore, high glucose causes increased uptake of ox-LDL and oxidative stress in endothelial cells [123]. It is possible that CD36 alteration could increase oxidative stress and NO scavenging via the eNOS-caveolin-CD36 axis. Recent evidence has also shown that certain lipoxygenase enzymes (LOX) induce oxidation of LDL and may contribute to sequesteration of NO [126]. Peroxynitrite may irreversibly modify both intracellular and secreted proteins and result in impaired function and oxidative stress through a mechanism similar to AGE/RAGE interaction. Furthermore, peroxy radicals carry out lipid oxidation, formation of lipid hydroperoxides, and may also increase alkyl radicals. Hydroperoxides may also form aldehydes (malondialdehyde) and damage endothelial cell membranes.

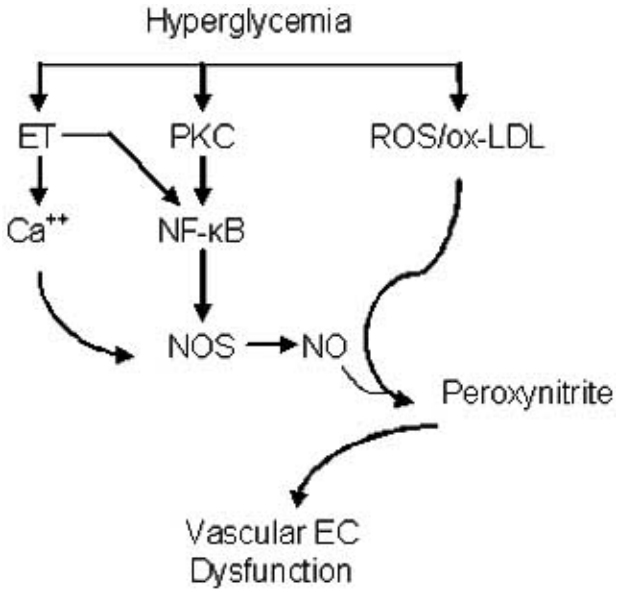


**Fig. (4).** Mechanisms of endothelial-dependent impaired vasodilation in diabetes. Glucose-induced aberration of ET and PKC may regulate NOS at both transcriptional and posttranslational level. NO generated from altered NOS is sequestered by oxidative stress reducing NO availability.

A potential new therapeutic target to decrease oxidant injury may be a stress-responsive protein system, the heme oxygenase (HO) system [118,127]. We have recently demonstrated that DM leads to increased expression and activity of HO in the heart [118]. Such alteration was shown to mediate increased iron accumulation and oxidant injury in cardiomyocytes. We have further demonstrated that attenuation of HO system by a highly selective inhibitor, tin-protoporphyrin IX, prevents DM-induced oxidative stress [118]. In addition, we have shown that inhibition of HO in a non-diabetic system may also decrease oxidative stress [118]. These findings suggest that HO alteration may represent a novel mechanism of increased oxidative stress and could provide another target for the development of therapeutic modalities.

Regardless of the mechanism of production, ROS are physiologically quenched by endogenous anti-oxidant enzyme systems. These enzymes include superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. It seems plausible that in parallel to increased ROS production, DM leads to impairment of oxidant scavenging system. Antioxidant defense activities have been shown to be impaired in both galactosemic and diabetic animals [129,130]. In cultured smooth muscle cells, superoxide dismutase expression level has been demonstrated to be increased in response to high levels of glucose [131]. A similar response in other contractile cells like pericytes has not shown any glucose-induced upregulation [132]. Whether high levels of glucose in vascular endothelial cells cause a similar alteration remains to be determined.

### 3.6 Imbalance between Vasoactive Factors

#### *3.6.1 Alteration in Early Events*

DM-induced increased vasoconstriction and impaired vasodilation is well documented as an early functional alteration that may lead to endothelial dysfunction. The most potent vasoconstrictor ET and vasodilator NO have been shown to exhibit a state of imbalance in all target organs of diabetic complications [5,9,16-20,24,25]. In terms of ET alteration, overwhelming number of studies show increased expression and functional consequence in the vasculature. Only a few studies, aimed at determining whether serum ET levels correlate with disease state, have produced conflicting results [133,134]. However, it is well accepted that ET alteration is of great significance in the tissue microenvironment. Therefore, serum levels may not necessarily reflect the importance of ET alteration in DM. We have demonstrated that DM-induced ET upregulation causes vasoconstriction in the retinal vasculature [24]. Inhibition of ET-receptor signaling by bosentan completely prevented such vasoconstriction. In addition to blood flow regulation, we have also demonstrated increased ET-mediated vascular permeability which was normalized by bosentan [135].

At the other spectrum of vasoregulation, DM may also lead to alteration in the NO pathway. A substantial number of studies in both diabetic patients and animal models have shown impaired endothelium-dependent vasodilation in the vessels [16-18]. These findings suggest that chronic DM may, in addition to ET-mediated vasoconstriction, cause impairment in NO function. Such NO pathway alteration may be mediated at the level of NO synthesis or stability. NO is produced by NOS isozyme family in both calcium-dependent and -independent manner [136,137]. Vascular endothelial cells predominantly express two isozymes, calcium-dependent eNOS and calcium-independent iNOS. Although, eNOS is constitutively expressed in endothelial cells, it is also subjected to regulation at the transcriptional level [120,138-140]. High levels of glucose increase the expression of eNOS and iNOS in cultured endothelial cells [141]. Various stimuli, such as growth factor alteration and intracellular protein kinase activation, may also lead to eNOS upregulation [142]. Furthermore, the activity of eNOS may be regulated posttranslationally by phosphorylation [120]. PKC and protein kinase B (PKB) have recently been shown to phosphorylate eNOS at the serine residues and lead to increased activity [142-144]. In addition, we have demonstrated increased mRNA expression of both eNOS and iNOS in the heart tissues of diabetic animals [118]. We and others have also demonstrated VEGF upregulation leading to increased permeability in the retina [25]. A recent study has also provided evidence of co-regulation and localization of inducible NOS and VEGF in retinas of diabetic patients [119]. These studies indicate that DM may upregulate NOS expression but may alter NOS activity and NO stability by another mechanism. Interestingly, we recently demonstrated DM-induced upregulation of caveolin-1 [123], a scaffolding protein which renders NOS inactive [145]. It is possible that DM leads to impaired NOS function, in part, via increased expression of caveolin-1. Conversely, a postulate put forth to reconcile the contradictory reports of NO alteration suggest that NO stability may also be subjected to regulation. The basis of such a notion underlies efficient scavenging of NO by free radicals as discussed earlier.

#### *3.6.2 Alterations in Late Events*

In addition to functional hemodynamic alterations, ETs are implicated in the regulation of other endothelial parameters (Fig. **5**) [9]. Inhibition of ET-receptor signaling has been shown to prevent high glucose-induced permeability and expression of ECM proteins, collagen and FN [53-55,135]. The mechanisms by which ETs may regulate permeability and ECM protein expression may entail activation of PKC [135]. Interestingly, ET-mediated increased permeability and ECM protein expression has been shown to be attenuated with PKC blocker, chelerythrine [135]. ET may increase PKC activation via G protein-coupled ET receptor type B ($ET_B$) (Fig. **1**,**5**) [9,146]. Activation of these $ET_B$ receptors, which are predominantly expressed on endothelial surface, causes phospholipase C-mediated DAG synthesis and activation of PKC [9,146]. PKC may then phosphorylate talin and vinculin producing intracellular gaps and increased permeability [147]. It should be noted, however, that endothelial permeability may also be arbitrated directly by $IP_3$-mediated increased intracellular calcium levels. Administration of calcium has been shown to cause endothelial cell contracture [147].

ETs may also regulate late structural changes in target organs of chronic diabetic complications. We previously demonstrated that high levels of glucose in endothelial cells cause increased expression of collagen and FN [53-55]. These changes can be normalized by inhibition of ET recep-

tor signaling, PKC and mitogen activated protein kinase (MAPK). Furthermore, our studies in animal models of chronic DM have shown that ETs may lead to increased ECM protein expression, via activation of transcription factors NF-κB and activating protein-1 (AP-1) [51]. These findings were also confirmed in culture endothelial cells exposed to high levels of glucose and ET-1 peptide [53,55]. It is interesting to note that ETs may also regulate composition of ECM. We recently demonstrated that ET alteration in DM leads to alternative splicing of FN in the vitreous of patients with proliferative diabetic retinopathy and retinal and aortic tissues of diabetic animals [54]. Such FN alternative splicing produces the embryonic variant of the ECM protein, oncofetal FN. The significance of these oncofetal variants was elucidated in cultured endothelial cells. Specific degradation of oncofetal FN was shown to reduce endothelial cell proliferation and VEGF expression [54,148]. These findings suggest that ETs may also regulate late pathogenetic changes in target organs of diabetic complications and may arbitrate increased endothelial proliferation and angiogenesis in target organs such as the retina.

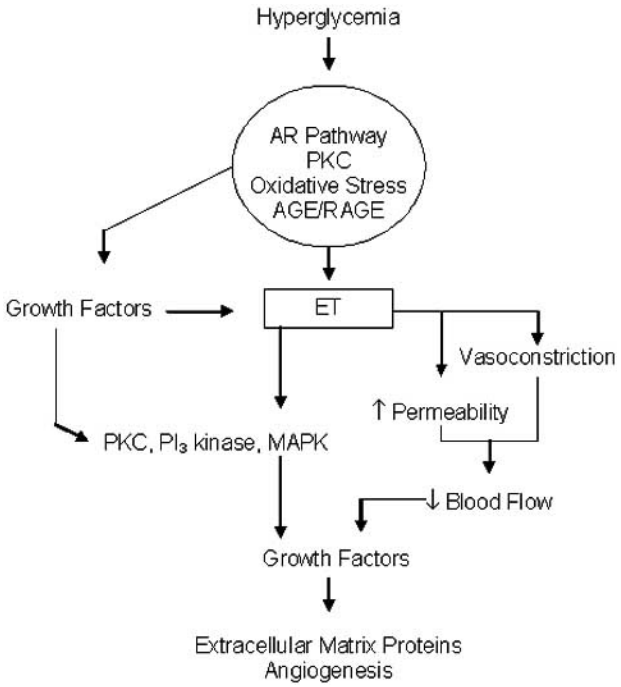


**Fig. (5).** Mechanisms of ET alteration and functional consequences. High glucose-induced biochemical anomalies increase ET expression. Altered ET levels may then lead to both early (blood flow and permeability) and late (ECM deposition) changes in target organs of chronic diabetic complications.

### 3.7 Other Signaling Molecules

In addition to PKC and NF-κB, other signaling molecules may mediate glucose-induced structural alterations in the vasculature. Recent studies suggest an important role of MAPK pathway in diabetic complications [55, 149,150]. We have shown that ET-mediated increased BM protein expression in cultured endothelial cells may be arbitrated *via* increased activity of MAPK pathway [55]. MAPK mRNA expression also correlates with glomerular lesions in diabetic nephropathy [151]. In cultured renal tubular cells, MAPK has also been shown to regulate ECM protein expression and hypertrophy [152]. Furthermore, increased activity of p38, a member of MAPK pathway, has been implicated in reduced NCV in diabetic neuropathy [153] and reduced contractility of cultured cardiomyocytes [154].

One of the most interesting developments in the elucidation of glucose transducers of diabetic complications has been the identification of novel protein kinases. Using cultured vascular endothelial cells, we have recently shown an important role of significantly homologous protein kinase family members, PKB [155] and serum- and glucocorticoid-regulated kinase-1 (SGK-1) [156] in increased ECM protein expression. We have shown that inhibition of PKB and SGK-1 by dominant negative transfections and small interfering RNA-mediated gene silencing leads to complete abolishment of glucose-induced FN expression, respectively [155,156]. These studies provide yet more targets which could be exploited in the development of therapeutic modalities.

## 4. POTENTIAL THERAPEUTIC TARGETS

Evidence accumulated to date suggests that microvascular and macrovascular anomalies are a consequence of diabetic dysmetabolism. Good glycemic control is associated with delaying the onset and preventing the progression of the complications. Chronic DM leads to the development of late complications by aberration of various signaling pathways that ultimately result in endotheliopathy. However, the relative contribution of these metabolic and biochemical changes in producing complications of DM remain uncertain. Animal models and cultured vascular cells fail to fully recapitulate the magnitude of hyperglycemia's adverse effects. Inhibitors of many signaling pathways such as AR, PKC, and oxidative stress have shown some promise in animal models but show no significant normalization of hyperglycemia-induced pathogenic changes in diabetic patients. Such contradictory results in animal models and human DM could be due to the complexity in convergence and divergence between different signaling pathways, the susceptibility to endothelial dysfunction, and vascular state at the time of intervention among others. Current knowledge suggests that there may be no well established unifying mechanism that underlies the development of chronic diabetic complications. Therefore, a combinatorial therapy targeting both early and late pathogenic changes may represent the best strategy towards combating the development and the progression of these complications. Various molecules that may be exploited as therapeutic targets are summarized in Table **1**. Factors that are involved in both early and late stages are of particular importance. These factors, which include vasoactive factors ET/NO and oxidative stress, may be important in preventing blood flow alteration and cellular dysfunction. The best strategy, based on current knowledge, therefore, is a combination therapy targeting various biochemical anomalies in order to ameliorate the functional and structural deficits arbitrated by chronic DM.

**Table I. Therapeutic Targets for Chronic Diabetic Complications**

| Target | Intervention and Outcome |
|---|---|
| **Primary Targets** | |
| ***Oxidative stress*** | Increase NO availability |
| | Restore equilibrium between ROS production and scavenging |
| | Prevent growth factor alteration and PKC activation |
| ***PKC Activation*** | Prevent permeability, vasoconstriction, and EC proliferation |
| ***AR Activity*** | Prevent redox imbalance |
| ***AGE/RAGE Interaction*** | Prevent AGE formation and RAGE signaling, Prevent oxidative stress |
| **Other Potential Targets** | |
| ***ET Expression*** | Reduce vasoconstriction, permeability, and ECM deposition |
| ***NO Synthesis*** | Increase NO synthesis |
| ***PARP Activity*** | Prevent impairment of endothelium-dependent vasodilation. Prevent EC dysfunction |
| ***NF-κB Activation*** | Prevent pro-inflammatory cytokine expression and ECM deposition |
| ***NADPH Oxidase Activity*** | Prevent superoxide production |
| ***HO Activity*** | Prevent iron accumulation and oxidative stress |
| ***Caveolin-1 Expression*** | Prevent eNOS inactivation |
| ***Other Protein Kinases*** | Reduce signaling through glucose transducers, PKB, $PI_3$ kinase, and SGK-1 |
| ***VEGF*** | Prevent EC proliferation and unregulated angiogenesis for retinopathy |
| ***Oncofetal FN*** | Prevent EC proliferation and unregulated angiogenesis for retinopathy |

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## ABBREVIATIONS

ACE-1 = Angiotensin converting enzyme-1

AGE = Advanced Glycation End Products

AP-1 = Activating protein-1

AR = Aldose reductase

AT = Angiotensin

bFGF = basic-fibroblast growth factor

BM = Basement membrane

cGMP = Guanosine 3',5'-(cyclic)phosphate

DAG = Diacyl glycerol

DCCT = Diabetes Control and Complications Trial

EC = Endothelial cells

ECM = Extracellular matrix

ER = Endoplasmic reticulum

eNOS = Endothelial nitric oxide synthase

ET = Endothelin

$ET_A$ = Endothelin receptor type A

$ET_B$ = Endothelin receptor type B

FN = Fibronectin

G = guanine-nucleotide-binding protein/G protein

HO = Heme oxygenase

IDDM = Insulin-dependent diabetes mellitus

iNOS = Inducible nitric oxide synthase

$IP_3$ = Inositol triphosphate

LDL = Low density lipoprotein

LOX = Lipoxygenase

MAPK = Mitogen activated protein kinase

MLC = Myosin light chain

MLCK = Myosin light chain kinase

NAD = Nicotinamide-adenine-dinucleotide

NADH = Reduced nicotinamide-adenine-dinucleotide

NADP = Nicotinamide adenine dinucleotide phosphate

NADPH = Reduced nicotinamide adenine dinucleotide phosphate

NCV = Nerve conduction velocity

NF-κB = Nuclear factor-κB

NIDDM = Non-insulin-dependent diabetes mellitus

NO = Nitric oxide

NOS = Nitric oxide synthase

Ox-LDL = Oxidized low density lipoprotein

| | | |
|---|---|---|
| PARP | = | Poly (ADP-ribose) polymerase |
| $PI_3$ Kinase | = | Phosphatidyl inositol 3-kinase |
| $PIP_2$ | = | Phosphatidyl inositol bisphosphate |
| PKB | = | Protein kinase B |
| PKC | = | Protein kinase C |
| PLC | = | Phospholipase C |
| RAGE | = | Receptors for AGEs |
| ROS | = | Reactive oxygen species |
| SGK-1 | = | Serum- and glucocorticoid-regulated kinase-1 |
| SOD | = | Superoxide dismutase |
| TGF-β | = | Transforming growth factor-β |
| VEGF | = | Vascular endothelial growth factor |

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