Endothelins: Regulators of Extracellular Matrix Protein Production in Diabetes

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Fibronectin (FN), a key extracellular matrix protein, is upregulated in target organs of diabetic angiopathy and in cultured cells exposed to high levels of glucose. FN has also been reported to undergo alternative splicing to produce the extra domain-B (ED-B) containing isoform, which is exclusively expressed during embryogenesis, tissue repair, and tumoral angiogenesis. The present study was aimed at elucidating the role and mechanism of endothelins (ETs) in FN and ED-B FN expression in diabetes. We investigated vitreous samples for ED-B FN expression from patients undergoing vitrectomy for proliferative diabetic retinopathy. Our results show increased FN and ED-B FN expression in the vitreous of diabetic patients in association with augmented ET-1. Using an antibody specific to the ED-B segment of FN, we show an increase in serum ED-B FN levels in patients with diabetic retinopathy and nephropathy. We further examined retinal tissues, as well as renal and cardiac tissues, from streptozotocin-induced diabetic rats. Diabetes increased FN and ED-B FN in all three organs, which was prevented by ET antagonist bosentan. To provide insight into the mechanism of glucose-induced and ET-mediated ED-B FN upregulation, we assayed endothelial cells (ECs). Inhibition of mitogen-activated protein kinase with pharmacological inhibitors and protein kinase B with dominant negative transfections prevented glucose- and ET-1–mediated FN and ED-B FN expression. Furthermore, treatment of cells exposed to high levels of glucose with ET antagonist prevented the activation of all signaling pathways studied and normalized glucose-induced ED-B FN expression. We then determined the functional significance of ED-B in ECs and show that ED-B FN is involved in vascular endothelial growth factor expression and cellular proliferation. These studies show that glucose-induced and ET-mediated FN and ED-B FN expressions involve complex interplays between signaling pathways and that ET may represent an ideal target for therapy in chronic diabetic complications. Exp Biol Med 231:1022–1029, 2006

Key words: basement membrane; diabetic complications; endothelins; extracellular matrix; fibronectin

Introduction

Despite the fact that insulin was introduced nearly 70 years ago to sustain life for people with diabetes, little progress has been made in developing preventive therapies for the chronic diabetic complications. Long-standing diabetes leads to both structural and functional anomalies in the vasculature (1–3). These secondary complications include retinopathy, nephropathy, cardiomyopathy, peripheral vascular disease, cerebrovascular disorders, and atherosclerosis. The single most important instigator of these complications is hyperglycemia (4, 5). Various biochemical changes have been attributed to mediate the adverse effects of high levels of glucose. These molecular events include activation of protein kinase C (PKC), augmentation of oxidative stress, glycation and modification of proteins, and increased glucose flux through the polyol pathway (3, 6, 7). Among the vast array of molecular changes, the single most consistent theme in the development of chronic diabetic complications seems to be the involvement of endothelial cell (EC) dysfunction (3, 8, 9).

The earliest changes during the onset of chronic diabetic complications are hemodynamic and dictated by the EC state (3, 6, 8). Reduced blood flow and increased permeability have been described in various animal models of chronic diabetic complications (3). These changes are essentially brought on by an imbalance between the vasoconstrictors and the vasodilators. Elaboration of the vasoconstrictor, endothelins (ETs), has been well established in animal models of the disease and human diabetes (3, 10). Beneficial effects of ET antagonism have also been shown (3, 11). A recent report indicates a possible

The authors acknowledge grant supports from the Canadian Diabetes Association in honor of the late Glenn W Liebrock, the Canadian Institutes of Health Research (MOP 43841), and the Lawson Health Research Institute.

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Received September 29, 2005.
Accepted November 14, 2005.

1535-3702/06/2316-1022$15.00

advantageous effect of ET receptor inhibition in reducing hyperglycemia and the onset of vascular injury in the diabetic NOD (non-obese diabetic) animals (12). The involvement of ETs in EC dysfunction spans various parameters including permeability, vasoconstriction, and extracellular matrix (ECM) expansion (3, 13, 14). Administration of ET antagonists has been shown to prevent all of these EC functional changes (3, 8).

ECM alterations and basement membrane (BM) thickening are structural hallmarks of diabetic complications (15–17). These connective tissues comprise an insoluble meshwork of proteins that assemble into a sheetlike structure by cell surface anchors and receptors (18–20). The importance of these connective tissues was realized when several vascular diseases were found to be associated with defects in the components of these structures (21). Furthermore, the presence of organ-specific molecules in the BMs suggests a significant role in modulating cell behavior and maintaining the tissue microenvironment. Increased ECM deposition and BM thickening in diabetic patients was first documented in the landmark study by Siperstein and colleagues in 1968 (15). Since these studies, major interest has been placed in elucidating the mechanism of BM thickening. We and others have shown increased ECM protein expression and BM thickening in all target organs of diabetic complications in animal models (3, 22–24). In the context of diabetic vasculopathy, the predominant proteins overexpressed are collagen and fibronectin (FN). Among the ECM proteins, FN displays a significant functional role in regulating cell behavior (25, 26).

Although FN molecules are products of a single gene, there is great heterogeneity in the population of FN molecules (26, 27). This diversity is the result of alternative pre-mRNA splicing. Three sites have been identified that undergo alternative splicing. These sites are termed extra domain-A, extra domain-B (ED-B), and type III connecting segment (26). Alternative splicing at the extra domain-A and ED-B regions is regulated in a tissue-specific and developmental manner. FN molecules containing the ED-B segment are highly restricted in terms of expression (26). It has been shown that ED-B FN is expressed only in proliferating tissues such as embryonic and tumor tissues (28, 29). Furthermore, it has been demonstrated that ED-B FN, which is virtually absent in normal adult tissues, is highly expressed in neoplastic blood vessels, suggesting a potential as an angiogenic marker (30, 31).

In the present study, we have determined whether diabetes causes upregulation of ED-B FN. We have also examined the role of ET-1 in ED-B FN expression. In addition, we have elucidated the signaling pathways that may cause ET-induced ED-B FN upregulation.

Materials and Methods

Human Vitreous and Serum Samples. Human vitreous samples were obtained from diabetic ($n = 18$; 7 females, 11 males; mean age ± SD, 58.1 ± 13.3 years) and nondiabetic ($n = 6$; 5 females, 1 male; mean age ± SD, 69.6 ± 8.91 years) patients undergoing vitrectomy for proliferative diabetic retinopathy and nondiabetes-associated ocular condition such as macular hole, respectively. The samples were pelleted by centrifuge and used for RNA isolation and real-time reverse transcriptase polymerase chain reaction (RT-PCR) (32). The vitreous is usually acellular but may contain hyalocytes, fibrocytes, and glial cells. However, during the course of proliferative diabetic retinopathy, the vitreous predominantly contains endothelial cells from the abnormal new blood vessels which grow in the plane anterior to the normal retinal vessels and extend into the vitreous.

Blood samples were obtained with consent of healthy volunteers ($n = 6$; 4 females, 2 males; mean age ± SD, 30.1 ± 8.1) and diabetic patients ($n = 20$; 12 males, 8 females; mean age ± SD, 58.8 ± 11.4) with known retinopathy or nephropathy, but not in dialysis, in collaboration with the department of Medicine, London Health Sciences Center, London. Serum from the blood samples was used for enzyme-linked immunosorbent assay. The antibody against ED-B FN has been characterized and shown to be specific to the ED-B region of FN (32). All human samples were obtained by approval of the Ethical Committee at University of Western Ontario and London Health Sciences Center, London, in accordance with the guidelines of the Declaration of Helsinki for research involving human tissues.

Animal Model of Diabetic Complications. Male Sprague-Dawley rats (Charles River Canada Ltd., St. Constant, Canada) weighing 200–250 g were made diabetic by a single intravenous injection of streptozotocin (65 mg/kg). Hyperglycemia was confirmed by blood glucose measurement (SurestepTM/Lifescan, Burnaby, Canada). Age- and sex-matched controls were given citrate buffer. Diabetic rats were divided into two groups, diabetics (DM) and diabetics on dual ET-receptor antagonist, bosentan (DM-B; oral gavage 100 mg/kg/day) (Courtesy of Dr. M. Clozel/Acetelion Ltd., Allschwill, Switzerland). After 3 months of treatment, rats were euthanized and tissues were obtained. All animal care adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

EC Cultures. Human umbilical vein endothelial cells (HUVECs; American Type Culture Collection, Rockville, MD) were cultured as previously described (32). For dose-response experiments, subconfluent cells were incubated with 5 m*M* (control), 15 m*M*, 25 m*M*, or 35 m*M* D-glucose for 24 hrs. After incubation, total RNA was extracted and subjected to real-time RT-PCR. The role of ET-1 in mediating the effects of high glucose was elucidated by treating cells in 5 m*M* glucose (CO) with 5 n*M* ET-1 (Peninsula Laboratories, Belmont, CA). In addition, we incubated cells in high glucose (HG; 25 m*M*) with 10 μ*M* dual ET receptor antagonist, bosentan. Other pharmacologic inhibitors were used at optimal doses (U0216, 10 μ*M*; PD098059, 50 μ*M*; ML-9, 100 μ*M*; chelerythrine, 1 μ*M*).

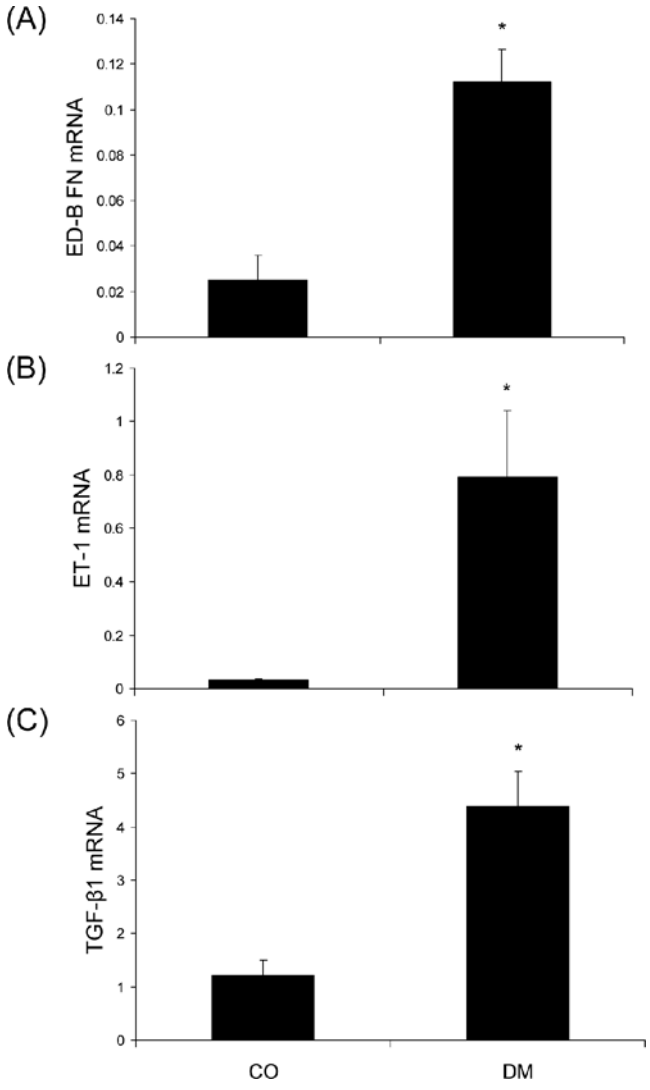

Figure 1. Real-time RT-PCR amplification of (A) ED-B FN, (B) ET-1, and (C) TGF-β1 in human vitrectomy samples showing upregulation of these transcripts in diabetes. ED-B FN is expressed as a ratio of ED-B FN:total FN; ET-1 and TGF-β1 mRNA levels are expressed as ratio of target transcript to 18S rRNA housekeeping gene; CO = controls ($n = 6$); DM = patients with proliferative diabetic retinopathy ($n = 18$); $^*P < 0.05$ compared with CO.

After 24 hrs of treatment period, total RNA was extracted and subjected to real-time RT-PCR. Gene silencing experiments were carried out as described by us previously (32).

Statistical Analysis. The data are expressed as mean ± SEM and were analyzed by ANOVA followed by Student's t test. Differences were considered significant at values of $P < 0.05$.

Results

ETs, Regulation of ECM Composition, and Cellular Proliferation. The emerging role of ETs in cellular proliferation is being realized (33–36). A number of human cancers exhibit increased ET-1 levels (35). ET-1 has been shown to increase cell growth in human cancer and

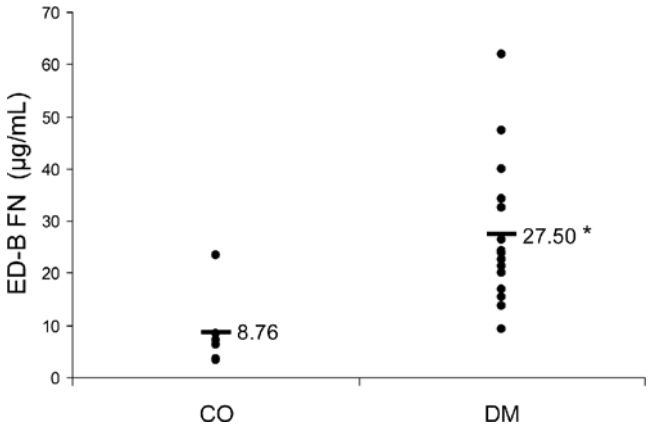

Figure 2. Elevated serum levels of ED-B FN were observed in diabetic patients as measured by enzyme-linked immunosorbent assay. CO = controls ($n = 6$); DM = diabetics ($n = 20$); $^*P < 0.05$ compared with CO.

suppress apoptosis of the cancer cells (37, 38). Studies in cultured cells and animal models indicate a role of ET in EC proliferation, migration and invasion, and neovascularization *in vivo* (35). ETs also increase the expression of vascular endothelial growth factor (VEGF) (39).

We have assayed for increased ED-B FN expression in vitrectomy specimens and serum samples from patients with proliferative diabetic retinopathy or nephropathy. Our results indicate that ED-B FN is upregulated in the vitreous from the diabetic patients as compared to the controls (Fig. 1). Interestingly, ET-1 and transforming growth factor–β1 (TGF-β1) levels were also elevated in the vitreous specimens suggesting an important association of these mitogens in ED-B FN expression. We next generated an antibody against the ED-B domain of human FN. The antibody was characterized and shown to be specific to ED-B–positive FN (32). Using this antibody, we assayed for serum levels of ED-B FN in diabetic patients. Our results indicate a 3-fold higher ED-B FN levels in diabetic patients (Fig. 2). Diabetic patients with established retinopathy or nephropathy showed a significantly higher serum ED-B level as compared with diabetics without documented microvascular complication (data not shown).

To elucidate such a regulatory role, we used a well-established model of chronic diabetic complications, the streptozotocin-induced diabetic rat. Our results show that 3 months of diabetes increased ED-B FN mRNA levels in the retina, kidney, and heart (Fig. 3). ET-1 and TGF-β transcript levels were also augmented in the diabetic animals. Treatment of diabetic animals with ET receptor antagonist, bosentan (courtesy of Dr. M. Clozel), prevented diabetes-induced ED-B FN expression and downregulated both ET-1 and TGF-β1.

We next determined the functional significance of ED-B FN reexpression in diabetes by using cultured ECs. We inhibited ED-B FN mRNA levels by gene silencing and assayed the ECs for molecular and morphologic changes. Our results indicate that specific inhibition of ED-B FN prevented both basal and high glucose-induced VEGF

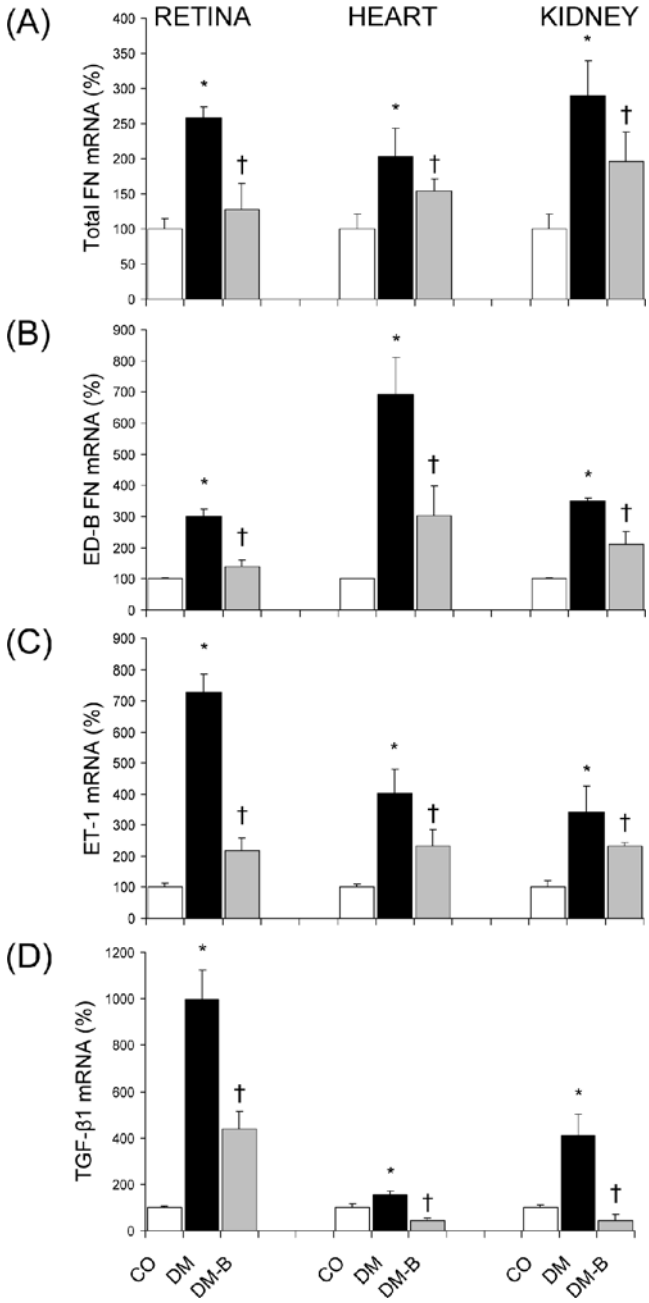

Figure 3. Real-time RT-PCR analysis of (A) Total FN, (B) ED-B FN, (C) ET-1, and (D) TGF-β1 in target organs of chronic diabetic complications. Total FN, ET-1, and TGF-β1 mRNA levels are expressed as a ratio of target transcript to 18S rRNA housekeeping gene; ED-B FN is expressed as a ratio of ED-B FN:total FN; CO = controls; DM = diabetics; DM-B = bosentan-treated diabetic animals; *$P < 0.05$ compared with CO; †$P < 0.05$ compared with DM; $n = 5$/group.

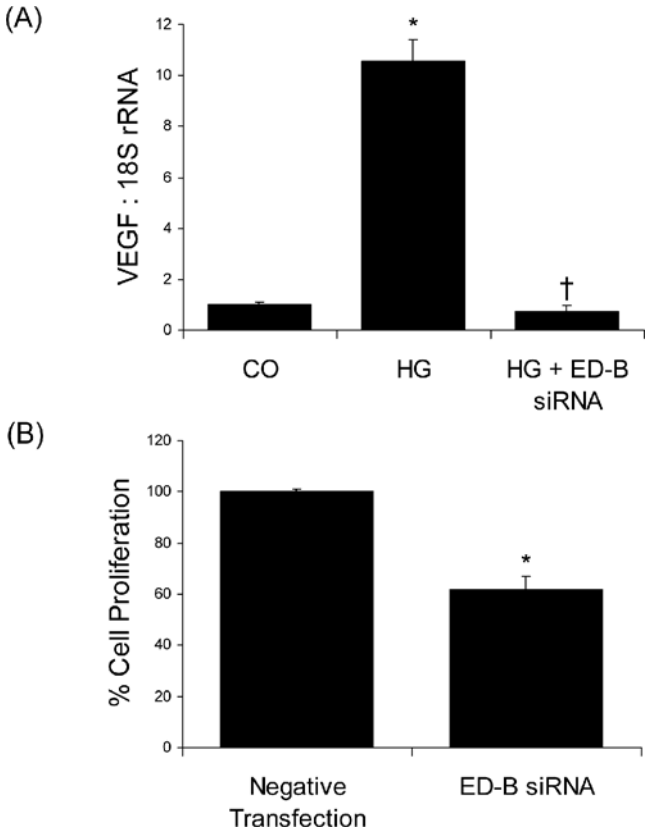

Figure 4. Quantitative analysis of VEGF mRNA expression (A) and ECs proliferation (B) after ED-B FN siRNA transfection in 25 m*M* glucose. $n = 5$/treatment; CO = 5 m*M* glucose; HG = 25 m*M* glucose; * $P < 0.05$ compared with CO; † $P < 0.05$ compared with HG.

expression in the ECs (Fig. 4A). Furthermore, functional assays showed that ED-B FN may mediate increased proliferation (Fig. 4B). Our results indicate that ET-mediated ED-B FN reexpression may underlie the proliferative and growth-promoting effects.

ETs and the Mechanism of ECM Regulation. Exposure of cultured vascular cells, ECs, and mesangial cells to high ambient glucose levels increases the expression of ECM proteins (13, 40). In the course of diabetic complications, these ECM/BM changes are brought on by elaborated growth factors and cytokines such as TGF-β, angiotensin II, and ETs (3). Several studies have indicated that most of the fibrogenic effects of growth factors such as TGF-β1 and angiotensin II may be mediated via ET signaling (41). Renal vascular sclerosis and collagen expression have been reported to be ameliorated by ET antagonism (42). ET antagonism by bosentan in animals treated with angiotensin II was associated with almost complete normalization of collagen promoter activity. Matrix expansion in the kidneys of diabetic animals by ACE inhibitor is also associated with reduced levels of circulating ET-1 (43). We have previously shown that diabetes-induced ECM protein expression and focal scarring in the heart is prevented by ET antagonism (23).

The signaling pathways involved in ET-induced ECM protein synthesis are not fully understood. ETs activate a number of intracellular signaling molecules including PKC, protein kinase B (PKB), and mitogen activated protein kinase (MAPK) (44). In the present study, we have investigated the signaling proteins which arbitrate the high glucose-induced and ET-mediated ED-B FN upregulation in cultured ECs. HUVECs were cultured in the presence of 5,

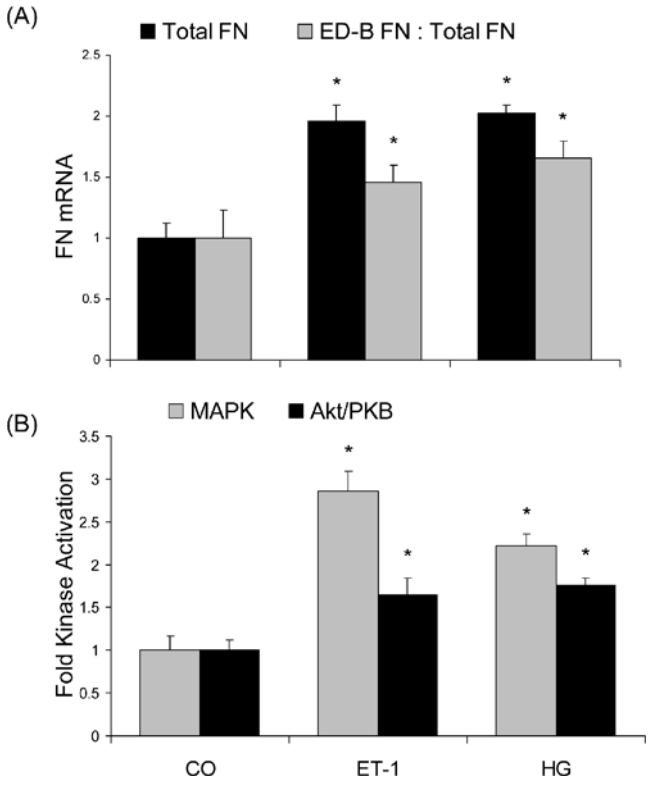

Figure 5. Effects of high glucose levels and ET-1 peptide on (A) FN and ED-B FN expression, and (B) MAPK and PKB activity. CO = 5 m*M* glucose; HG = 25 m*M* glucose; ET-1 = 5 n*M* ET-1 peptide; MAPK activity was assessed by ERK1/2 phosphorylation; PKB activity was determined by S473 phosphorylation; * $P < 0.05$ compared with CO.

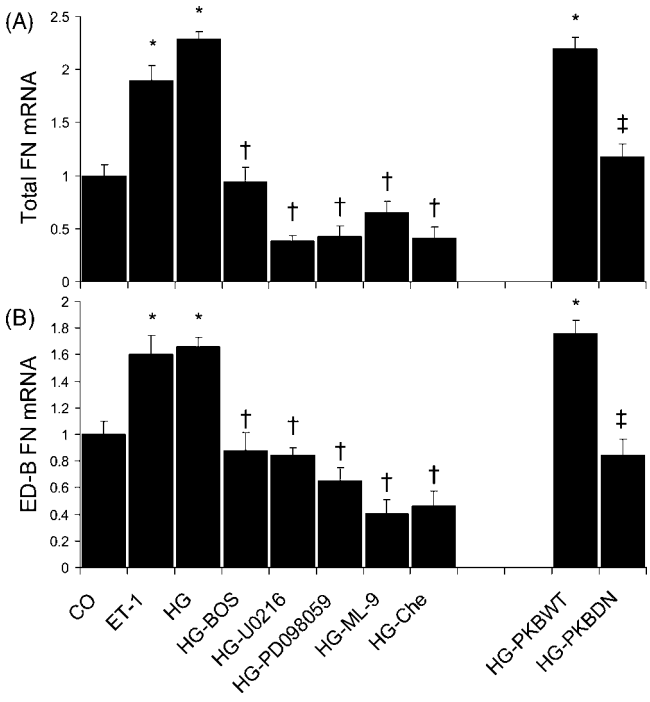

Figure 6. Real-time RT-PCR analysis of (A) total FN and (B) ED-B FN in ECs treated with inhibitors of various signaling pathways. HG-BOS = HG + 10 μ*M* ET antagonist bosentan; HG-U0126 = HG + 10 μ*M* MAPK inhibitor U0126; HG-PD098059 = HG + 50 μ*M* MAPK inhibitor PD098059; HG-ML-9= HG + 100 μ*M* PKB inhibitor ML-9; HG-Che = HG + 1 μ*M* PKC inhibitor chelerythrine; HG-PKBWT = HG + wild-type PKB transfection; HG-PKBDN = HG + dominant negative PKB; * $P < 0.05$ compared with CO; † $P < 0.05$ compared with HG.

15, 25, and 35 m*M* glucose and assayed for total and relative ED-B FN (ED-B FN:total FN) upregulation by real-time RT-PCR. Our results indicate that both total and relative ED-B FN mRNA levels were augmented in HUVECs exposed to 25 m*M* glucose for 24 hrs (Fig. 5A). Such upregulation of FN was also observed in cells treated with 5 n*M* ET-1 peptide. In parallel to the increased ED-B FN expression, high glucose and ET-1 peptide caused activation of PKB and MAPK (Fig. 5B). To determine the role of ET-1, we treated HUVECs exposed to high levels of glucose with the dual ET receptor antagonist, bosentan. ET receptor inhibition caused complete normalization of ED-B FN expression.

We next inhibited multiple signaling pathways in cells exposed to high levels of glucose to uncover the mechanistic basis. Inhibition of MAPK (PD098059 and U0126) and PKB (dominant negative transfections) prevented glucose-induced total and relative ED-B FN expression (Fig. 6). An important cross talk between these signaling pathways and PKC was also observed. Treatment of cells with PKC inhibitor chelerythrine caused significant reductions in activated MAPK, PKB, and ED-B FN expression (Fig. 6).

One of the most important findings of the study is inhibition of all glucose-induced signaling pathways studied and attenuation of ED-B FN upregulation with bosentan. These results indicate that ETs may represent the master molecular switch. In the context of diabetic complications, most of the effects of fibrogenic stimuli can be ameliorated by ET antagonism (3, 42).

Discussion

The present study demonstrates the upregulation of the ED-B FN in vitreous and serum samples from diabetic patients. Using a well-established model of chronic diabetic complications, we have shown that ED-B FN is upregulated in the retinal tissues of diabetic rats. This hyperglycemia-induced upregulation of ED-B FN was shown to be mediated via elaboration of mitogenic growth factor, ET-1, and its possible regulatory interaction with TGF-β1. Treatment of diabetic rats with a dual ET receptor antagonist, bosentan, was able to normalize diabetes-induced changes in the retina. We have extrapolated the studies to further demonstrate that upregulation of ED-B FN is not restricted to the retinal tissue. Other target organs of chronic diabetic complications include the heart and the kidney. We have demonstrated that these tissues also exhibit diabetes-induced preferential expression of ED-B FN. These findings suggest that such preference toward a particular isoform may represent a general phenomenon in hyperglycemia-induced vascular damage.

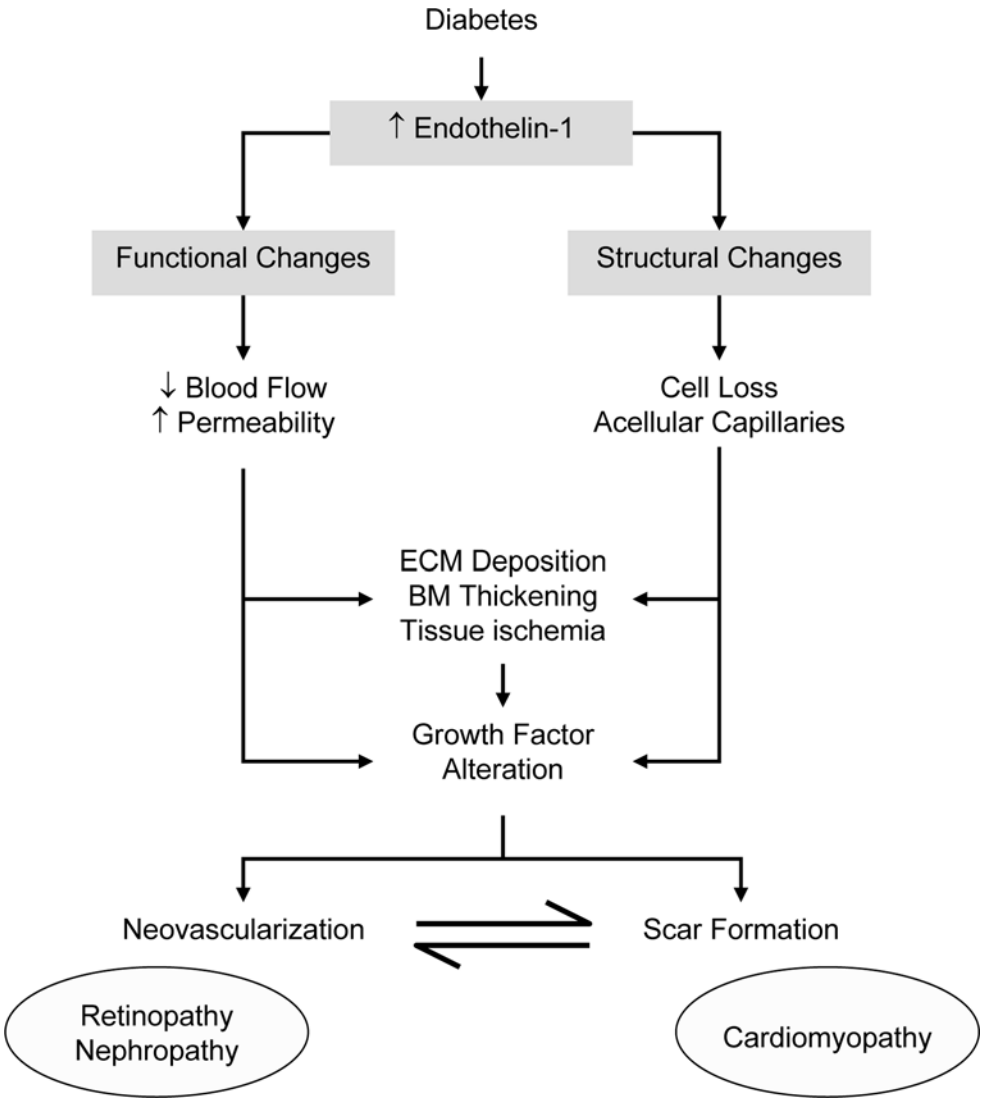

Figure 7. A schematic illustration showing the putative mechanisms of ET-induced changes in the vasculature and the development of diabetic complications.

Isoform switching of FN in the ECM may represent an important phenomenon in altering the behavior of vascular ECs. It is increasingly being realized that the ECM provides positional and environmental information (45). This information is important for proper tissue function; a phenomenon evident in studies which demonstrate heterogeneity in ECM composition and architecture in a tissue-specific manner (45). Signaling from the ECM would depend on the matrix composition (signal), the repertoire of receptors (transducers), and the types of cells (target). Connections from matrix through these receptors could determine the organization of cytoskeletal components and activation of signaling molecules (46, 47). The mosaic of FN species deposited may, therefore, be important in mediating signals to vascular cells and modulating their behavior. In support of such a notion are findings that indicate increased expression of an embryonic isoform of tenascin in promoting retinal endothelial migration and proliferation (48).

High levels of glucose have been shown to mediate proliferation of ECs (49). Our study suggests that ED-B domain, in part, mediates such proliferative effects. Targeted gene silencing of ED-B FN in ECs resulted in decreased proliferative capacity. We have also demonstrated that selective targeting and subsequent inhibition of ED-B FN production results in reduced VEGF expression.

The molecular mechanism by which high glucose-induced ET-1 expression leads to increased ED-B FN expression remains to be fully elucidated. PKC, phosphatidylinositol 3-kinase, and MAPKs may play a role in FN expression. Here, we provide evidence that ED-B FN expression in ECs is regulated by concurrent activation of various signaling pathways, including PKB, MAPK, and PKC.

In conclusion our results suggest that ETs may

represent the determining factor in ECM expression (Fig. 7). Our *in vivo* and *in vitro* findings suggest that inhibition of ETs is associated with almost complete attenuation of PKB, MAPK, PKC, transcription factor activity, and FN expression. These findings also suggest an important feedback mechanism for chronic activation of these signaling pathways in diabetic complications. ET expression has been reported to increase with augmented PKC activation. Interestingly, upregulated ETs appear to be necessary for continued activation of PKC and possibly other signaling proteins in the diabetic context. Therefore, ETs may provide an invaluable target for the development of therapeutic modalities for diabetic complications and other angiogenic and fibrotic diseases.

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