

Endothelin Receptor Blockade Prevents Augmented Extracellular Matrix Component mRNA Expression and Capillary Basement Membrane Thickening in the Retina of Diabetic and Galactose-Fed Rats

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Endothelins (ETs) are a family of vasoactive peptides that have mitogenic properties and have also been associated with altered long-term nuclear signaling. We have previously shown that mRNA levels for ET-1, ET-3, and their receptors are upregulated under hyperhexosemic conditions. In this study, an endothelin antagonist was used to assess the effects of endothelin blockage on the production of two basement membrane transcripts, fibronectin and collagen $\alpha 1$ (IV). The microvascular basement membranes were analyzed using ultrastructural morphometry. Streptozotocin-induced diabetic rats, galactose-fed rats (30% galactose in diet), and nondiabetic, non-galactose-fed control rats were studied after 1-month and 6-month follow-up. Simultaneously, similar animal groups were treated with a general ET receptor blocker (bosentan, $100 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) and investigated. Semiquantitative reverse transcription-polymerase chain reaction for fibronectin and collagen $\alpha 1$ (IV) was conducted after 1 month of follow-up with comparison to β -actin housekeeping gene using slot blot hybridization and densitometry. Basement membrane thickness was assessed after 6 months of follow-up in diabetic rats, using the orthogonal intercept method. After 1 month of follow-up, increased fibronectin and collagen $\alpha 1$ (IV) mRNA were present in the retina of diabetic and galactosemic animals, and the bosentan-treated groups exhibited mRNA levels similar to the control animals. After 6 months of follow-up, diabetes and galactose feeding induced basement membrane thickening, which was partially prevented by bosentan treatment. The above findings indicate that increased production of extracellular matrix proteins leading to thickening of microvascular basement membrane, secondary to hyperhexosemia, may be mediated via augmented ET production. *Diabetes* 49:662–666, 2000

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BM, basement membrane; ECM, extracellular matrix; ET, endothelin; PCR, polymerase chain reaction; PKC, protein kinase C; RT, reverse transcription; STZ, streptozotocin.

One of the defining structural alterations that occur in the retinal vasculature in diabetic retinopathy is the thickening of basement membranes (BMs) (1,2). The exact mechanisms responsible for the thickening of vascular BM are not known, but the key initiating factor appears to be hyperglycemia (1). Evidence from in vitro studies of vascular cells cultured under hyperhexosemic conditions has implicated an increased production of extracellular matrix (ECM) components, such as fibronectin, laminin, and collagen $\alpha 1$ (IV) (4–6), as one of the mechanisms in the production of BM thickening. The increased production of ECM components has been shown to occur early in the retina of galactose-fed rats and in humans with diabetes (7–9), whereas BM thickening occurs after several months in diabetic rats.

Recently, many studies have provided evidence to suggest that increased levels of diacylglycerol leading to protein kinase C (PKC) activation are intricately involved in the development of diabetic vascular complications (10). Utilization of a PKC- β general inhibitor (LY333531) in diabetic rats prevented increased transcription of fibronectin and collagen $\alpha 1$ (IV) in glomeruli (11). PKC activation has been associated with the activation of vasoactive factors such as vascular endothelial growth factor and endothelin-1 (ET-1) (10,12). Hence, it is conceptually possible that some of the actions of PKC in hyperhexosemia are mediated via these vasoactive factors.

Endothelins (ETs) are a family of 21 amino acid peptides with diverse functions, including vasoactivity and mitogenesis (13,14). ETs consist of peptides that include ET-1, ET-2, and ET-3 (15). Receptors for these peptides have been well classified and include ET_A, ET_B, and ET_C (16,17). Originally discovered as endothelial products, the ETs have since been found in various cell types and tissues, including ocular and neuronal tissues (14,18). In addition to being potent vasoactive molecules, ETs also possess mitogenic properties (19).

We and others have shown that ETs in the retina are upregulated in diabetes, including demonstrating increased immunoreactivity of ET-1 and ET-3 as well as increased ET and ET receptor gene expression in retinal tissues in chronically diabetic BB/W rats (18,20–22). We have further shown that galactose feeding- and diabetes-induced early vasoconstriction in the retina was prevented by an ET receptor antagonist (23,24).

Previous studies have provided evidence of a regulatory link between ET and the ECM, with ET-1 increasing the

expression of ECM component mRNA (25). In addition, a specific ET_A receptor antagonist prevented increased mRNA production of ECM components and growth factors in glomeruli of diabetic rats (26). In the current study, we have investigated the hyperhexosemia-induced augmented ET system and its role in the alterations of the ECM component mRNA expression of retinal capillaries and in retinal capillary BM thickening. In particular, we have employed a general ET receptor antagonist, bosentan, to elucidate the effect of ET blockade on fibronectin and collagen $\alpha 1$ (IV) mRNA production after 1 month of follow-up, and we have assessed the effect of ET blockade on BM thickening after 6 months of follow-up in streptozotocin (STZ)-induced diabetic and galactose-fed rats.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats weighing ~200 g were obtained from Charles River Canada (St. Constant, PQ, Canada) and were randomly assigned to each of five groups. One group of rats was fed regular rat food containing 30% D-galactose (PMI Feeds, St. Louis, MO) with water ad libitum. Galactose-fed rats were further randomized into two groups: 1) untreated galactose-fed and 2) galactose-fed on bosentan treatment. Diabetes was induced by a single intravenous injection of STZ (65 mg/kg body wt, in citrate buffer). Diabetic rats were randomized into two groups: 1) poorly controlled diabetics and 2) poorly controlled diabetics on bosentan treatment. Nondiabetic control animals received an injection of the same volume of citrate buffer. The presence of hyperglycemia was confirmed by blood glucose estimation (Surestep blood glucose meter; LifeScan, Burnaby, BC, Canada). Age- and sex-matched animals were used as nondiabetic, nongalactosemic controls. All animals were monitored daily with respect to urine volume, urine glucose, and ketones (Uriscan Gluketo; Yeong Dong, Seoul, Korea). Diabetic animals received small daily doses of insulin to prevent ketosis. Bosentan was obtained from Hoffman-La-Roche (courtesy of Dr. M. Clozel, Allschwil, Switzerland). Bosentan is a potent ET_A and ET_B receptor blocker. Bosentan was administered by daily oral gavage at a dose of 100 mg/kg body wt per day. Randomly selected animals from each group were killed after 1 and 6 months of follow-up. The animals were anaesthetized with sodium-pentobarbital (50 mg/kg i.p.) and killed by cardiac puncture, with blood collected for glycated hemoglobin measurement (Glycotest; Pierce, Rockford, IL). After 1 month of follow-up, upon death, the retina from the right eye of each animal was snap-frozen in liquid nitrogen. After 6 months of follow-up, upon death, the left eyes were fixed in 2.5% gluteraldehyde in phosphate buffer (pH 7.3) for electron microscopic analysis.

RNA isolation. TRIZOL reagent (Canadian Life Technologies, Burlington, ON, Canada) was used to isolate RNA. Briefly, after homogenization, RNA was extracted with chloroform followed by centrifugation. RNA was recovered from the aqueous phase by precipitation with isopropyl alcohol and suspended in diethyl pyrocarbonate water. Quantitation of RNA was performed by determining the absorbance at 260 and 280 nm.

First-strand cDNA synthesis. First-strand cDNA synthesis was performed using a Superscript-II system (Canadian Life Technologies). RNA was added to oligo (dT) primers, denatured at 65°C for 10 min, and quenched on ice for 10 min. Reverse transcription was carried out by the addition of Moloney murine leukemia virus-reverse transcriptase and dNTPs at 42°C for 50 min in a total reaction volume of 20 μ l. The reaction was terminated by a 15-min incubation at 70°C.

Polymerase chain reaction. The amplification was carried out using protocols similar to those of Roy and Lorenzi (7) with modifications. For fibronectin, primer 1 (sense, 5'-GAGAGATCTGGAGGTCAT-3') and primer 2 (antisense, 5'-GGGTGACACCTGAGTGAA-3') were used. The primer pairs for fibronectin encompass the alternatively spliced exon known as EIIIA, resulting in a truncated 582-bp product (without EIIIA) and an 852-bp product (with EIIIA). For collagen $\alpha 1$ (IV), primer 1 (sense, 5'-CAGGCTCTGCTCAGA-3') and primer 2 (antisense 5'-CCTCGTGGAGCAGAA-3'), with a predicted product size of 212 bp, were used. Simultaneously, a housekeeping gene (β -actin) was amplified in a separate set of tubes using the same reverse transcription (RT) product, 5'-TGGTGGTATGGGT CAGAAGG-3' and 5'-ATCCTGTCAGCGATGCCTGGG-3' primer sets using the same cycling parameters with a predicted product size of 813 bp. Reactions were performed in 30- μ l volumes containing polymerase chain reaction (PCR) buffer, 1.5 mmol/l $MgCl_2$, 250 μ mol/l dNTP mix, 1 μ mol/l of each primer, 2.5 U Taq polymerase, and 4 μ l of the RT product. The initial cycle was carried out for 3 min at 94°C for denaturation, 1 min at 60°C for annealing, and 3 min at 72°C for extension. Subsequent cycles of PCR were performed using the following conditions: denaturation, 45 s at 94°C; annealing, 45 s at 54°C; and extension, 1 min at 72°C and 7 min for final extension. The linearity of the PCR was established by analyzing

PCR product with a variable amount of template and variable cycle numbers. In this study, we used 30 cycles of amplification for all three genes. The amplification products were analyzed by electrophoresis on a 2.5% agarose gel in 1 Tris acetate buffer followed by staining with ethidium bromide and visualization under ultraviolet light.

Southern hybridization. The specificity of the amplifications was confirmed by Southern blot after the PCR product was transferred from the gel onto nylon membranes following denaturation and neutralization. Hybridizations were performed with biotinylated amplification product-specific oligo probes (fibronectin: 5'-CTCATCAGTTGGGAACCC-3', which hybridizes with both spliced variants; collagen $\alpha 1$ (IV): 5'-GTCTCTGTTGGTCCCCGCGC-3'; β -actin: 5'-CTGACCTGAA GTACCCATT-3'). The detection was carried out using a nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) system (Sure blot; Oncor, Gaithersburg, MD).

Quantitation. Quantitation was performed by serial dilution slot-blot hybridization and densitometry of the products from the upstream of amplification onto nylon membranes. Hybridization was carried out using the biotinylated oligo-probes, as described above, and the detection was carried out using an NBT/BCIP system. The blots were analyzed by a Hewlett-Packard 4C scanner using Mocha software (Jandel Scientific, San Rafael, CA). The densitometric values were expressed as arbitrary units per micrograms of total RNA, and the ratio of β -actin to the specific gene(s) was obtained.

BM thickness measurement. The tissues from all animals were processed for electron microscopy following standard procedures (27,28). Toluidine blue-stained 0.5- μ m sections were used for light microscopic orientation. Ultra-thin sections (50–90 nm) on copper grids (200 mesh) were stained with uranyl acetate and lead citrate and then analyzed by electron microscope. At least 10 horizontally cut capillaries from the outer plexiform and inner nuclear layer of each sample were photographed and stored on compact disc and were subsequently analyzed using Mocha software (Jandel Scientific). Orthogonal intercept measurement method was used to generate 15 random lengths for each vessel (29–31). Harmonic means were taken for each vessel, and an average was calculated for each of the 10 vessels analyzed for each sample.

Statistical analysis. The data are expressed as means \pm SE and were analyzed using one-way analysis of variance followed by multiple Student's t test with Bonferroni correction.

RESULTS

Diabetic animals with and without bosentan treatment showed reduced body weight gain, hyperglycemia, glucosuria, and elevated glycated hemoglobin levels. Galactose-fed animals with and without bosentan treatment showed reduced body weight gain, euglycemia, and elevated glycated hemoglobin levels (Table 1). Diabetic and galactose-fed animals had >2% reducing sugar in the urine (approximately >111 mmol/l).

Collagen $\alpha 1$ (IV), β -actin, and fibronectin mRNA were detected in retinas of all animal groups. Amplification for collagen $\alpha 1$ (IV) and β -actin were demonstrated as single bands at 212 bp and 813 bp respectively, whereas the fibronectin product consisted of three bands: 582-bp product (EIIIA⁻), 852-bp product (EIIIA⁺), and an intermediate band of ~650 bp, which represents heteroduplex DNA consisting of one strand of each product mentioned above.

Analysis of RNA by semiquantitative RT-PCR showed that after 1 month of diabetes and galactose feeding, retinal mRNA for fibronectin and collagen $\alpha 1$ (IV) were significantly elevated (Fig. 1). Bosentan-treated animals did not have increased production of fibronectin and collagen $\alpha 1$ (IV) mRNA levels compared with controls, and their production was significantly less than that of their respective nontreatment groups, i.e., diabetics and galactose-fed ($P < 0.05$) (Fig. 1).

After 6 months' follow-up, the diabetic and galactose-fed rats had increased BM thickening compared with controls ($P < 0.01$) (Fig. 2). Although BM thickness was higher in the bosentan-treated diabetic rats and the bosentan-treated galactose-fed rats than in normal controls, it was significantly less than in the untreated diabetic and galactose-fed groups ($P < 0.01$).

TABLE 1
Clinical data of animal groups after 1 month and 6 months of follow-up

	Control	Diabetic	Diabetic + bosentan	Galactosemic	Galactosemic + bosentan
1 Month					
n	9	8	6	8	6
Body weight (g)	400.01 ± 9.66	352.14 ± 8.64*	333.5 ± 9.6*	356.7 ± 5.64*	364.7 ± 9.6*
Blood glucose (mmol/l)	5.74 ± 0.12	21.54 ± 1.28*	21.89 ± 1.44*	5.20 ± 0.28	5.42 ± 0.44
Glycated hemoglobin (%)	5.64 ± 0.3	10.54 ± 0.9*	10.28 ± 1.6*	9.25 ± 0.6*	9.0 ± 0.4*
6 Months					
n	8	8	8	8	8
Body weight (g)	631.8 ± 23.51	495.2 ± 15.2*	484.9 ± 9.8*	542.2 ± 17.2*	529.2 ± 9.2*
Blood glucose (mmol/l)	6.62 ± 0.4	23.0 ± 2.0*	21.97 ± 1.2*	7.26 ± 0.4	7.06 ± 0.3
Glycated hemoglobin (%)	4.9 ± 0.9	11.4 ± 0.4*	11.2 ± 0.3*	10.8 ± 0.4*	10.2 ± 0.2*

Data are means ± SE. *Significantly different compared with the control group.

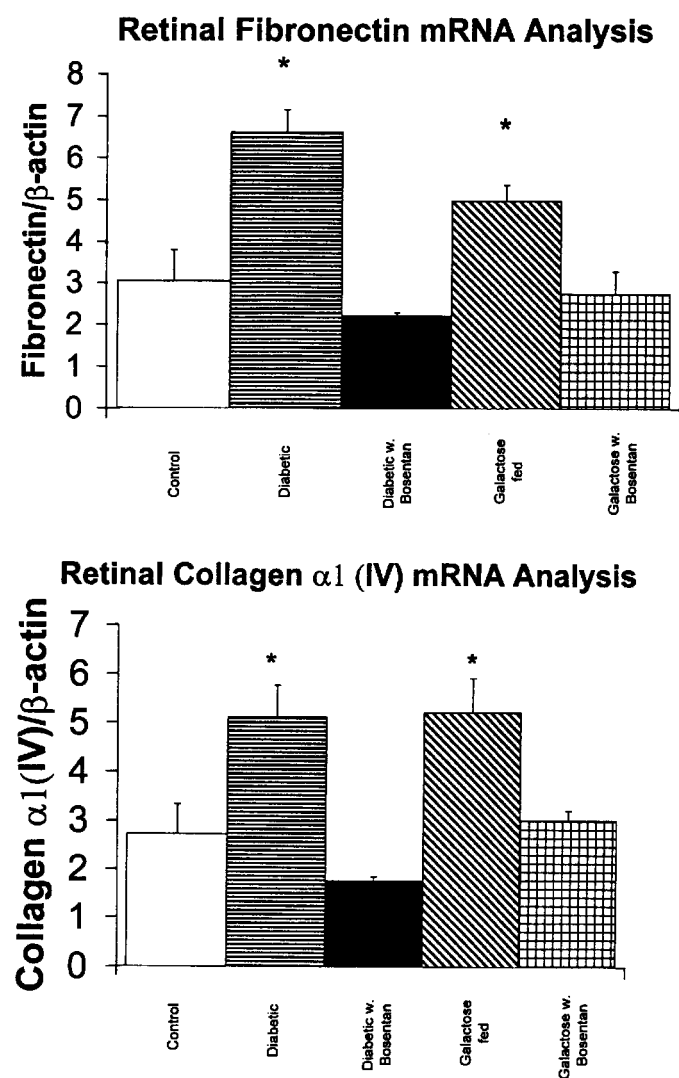


FIG. 1. mRNA analysis of retinal fibronectin and collagen α 1 (IV) by semiquantitative RT-PCR from the retina of diabetic (n = 8), bosentan-treated diabetic (n = 6), galactose-fed (n = 8), bosentan-treated galactose-fed (n = 6), and control (n = 9) animals after 1 month of follow-up. The data are expressed as the ratio of housekeeping gene β -actin, which did not alter in various groups. *Significantly different from controls.

DISCUSSION

This study demonstrates that treatment with the ET receptor blocker bosentan was effective in preventing the increased production of mRNA of two main components of the ECM—fibronectin and collagen α 1 (IV)—and subsequent capillary BM thickening in the retina of hyperhexosemic rats. We have previously demonstrated that in the retina of both diabetic and galactosemic rats, ET-1, ET-3, and their receptors ET_A and ET_B mRNA are upregulated and that treatment with a general ET receptor blocker was able to ameliorate alterations of retinal blood flow found in both diabetic and galactose-fed rats (23,24). Although some of the vasoactivity effect of ETs has been studied in animal models, in diabetes few studies exist that investigate other possible effects of ETs in vivo. Previous in vitro studies have shown that ET-1 induces mesangial cell proliferation (32) and can increase the production of ECM components such as laminin and collagen α 1 (IV) (25). Further evidence of a role for ET-1 stimulating ECM component production was supplied in a study that utilized a specific ET_A antagonist to successfully prevent increased production of collagen α 1 (I), α 1 (III), and α 1 (IV), as well as laminin B1 and laminin B2, in diabetic rat glomeruli (26).

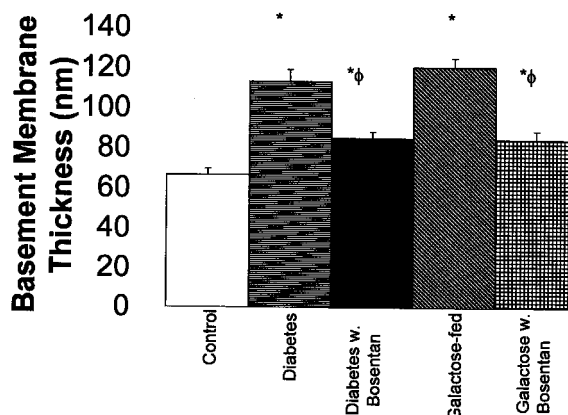


FIG. 2. BM thickness of retinal capillaries from diabetic (n = 8), bosentan-treated diabetic (n = 8), galactose-fed (n = 8), bosentan-treated galactose-fed (n = 8), and control (n = 8) animals after 6 months of follow-up. The data are expressed in nanometers. *Significantly different from controls; ϕ significantly different from respective non-bosentan-treated group.

Further evidence for an active role of ETs in a hyperhexosemic milieu was provided by the morphological studies on BM thickening in 6-month hyperhexosemic rats. ET receptor blockade by bosentan was able to prevent hyperhexosemia-induced BM thickening. The exact mechanism of BM thickening in diabetes is not known. BM thickening, although a common finding in diabetes, has not been causally related to vascular dysfunction in most organs (33). Data from the present study suggest that ET production may be a key component in the generation of hyperhexosemic-induced structural changes in diabetes.

Bosentan is a potent ET receptor blocker, and it has been demonstrated to block ET receptors in several organs (34). It is well tolerated and has been shown to prevent ET-mediated functional and structural changes in several other conditions both experimentally and clinically, including heart failure and cerebral vasospasm (34). Some side effects of bosentan have been reported in clinical trials (35). However, they appear to be due to ET receptor blockade in other organs. Several mechanisms in hyperhexosemic dysmetabolism may upregulate ET expression in the retina. ET-1 expression is known to be mediated by PKC, and activation of PKC may be one of the factors leading to increased ET-1 production (10). ET-1, on the other hand, is a PKC activator through induction of activation protein-1 regulatory sites (33). Therefore, a positive feedback mechanism may be one of the consequences of increased production of ET-1, increasing PKC levels and leading to further augmentation of ET-1 activation (34). Other mechanisms, such as reduced NO synthesis in diabetes, may also stimulate ET-1 production (35). We have previously shown that treatment with bosentan reduces ¹²⁵I-ET-1 binding in the retina of diabetic rats (23). Insulin is a stimulator of ET-1 production (36,37). However, in the present study and in earlier studies from our laboratory, we have demonstrated identical changes in diabetes and galactose-fed rats; hence, the data suggest that ET-1 activation is secondary to hyperhexosemia (24). Increased ET production may lead to increased ECM protein synthesis via proto-oncogenes such as *c-fos* and *c-jun* (19,38). Such mechanisms, although demonstrated in the kidneys of diabetic rats, have not been demonstrated in the retina. Hence, ET-mediated increased ECM transcript production in hyperglycemia appears to be a generalized phenomenon. We have found similar alterations in ECM transcript production in the heart and kidneys of diabetic animals (S.Che., T.E., D.X.D., K. Mukerjee, S.Cha., unpublished data).

The present data suggest the important role of the ET system in the pathogenesis of diabetes-induced changes in the retina. The effect of ET blockade in the development of diabetic retinopathy may be a potential adjuvant therapeutic mechanism that needs to be confirmed by further studies in larger animal models.

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