Diabetologia (1999) 42: 1228–1234

Diabetologia

Diabetes-induced vascular dysfunction in the retina: role of endothelins

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Abstract

Aims/hypothesis. Vasoactive factors like endothelins, by virtue of the microvascular regulation as well as by other effects, possibly play important parts in the pathogenesis of diabetic retinal microangiopathy. We investigated retinal vascular dysfunction in streptozotocin-induced diabetes and its relation with endothelins in short- and long-term diabetes.
*Methods***.** Diabetic rats with or without an endothelin receptor antagonist (bosentan) treatment were investigated after 1 month and 6 months of follow-up. Retinal blood flow was measured and compared with age- and sex-matched non-diabetic control animals. Retinal tissues were analysed for endothelin-1, endothelin-3, endothelin A and endothelin B mRNA. Distribution of endothelin-1 and endothelin-3 was investigated by immunocytochemistry and that for endothelin receptors by ligand binding and autoradiography.
Results. Diabetic animals showed hyperglycaemia, glycosuria, elevated glycated haemoglobin values and reduced body weight gain. Retinal blood flow showed an increased resistivity index, an indicator of vasoconstriction, after 1 month of diabetes which was prevented by treatment with bosentan. This functional change in diabetes was eliminated after 6 months of follow-up. The retina from the diabetic animals showed increased mRNA expression for endothelin-1, endothelin-3 and endothelin A after one month. In addition, endothelin B mRNA expression was increased after 6 months. Furthermore, endothelin-1 and endothelin-3 immunoreactivity and endothelin receptor concentrations were increased in the retina of diabetic rats.
Conclusion/interpretation. The results from this study indicate that the endothelin system is of importance in mediating retinal changes in diabetes although mechanisms of the endothelin system alteration as well as their effects might vary depending on the duration of diabetes. [Diabetologia (1999) 42: 1228–1234]

Keywords Endothelin-1, endothelin-3, endothelin A, endothelin B, retina, blood flow.

Endothelins (ETs) are a family of 21 amino acid peptides with diverse biological actions [1–5]. Alteration of ETs are of importance in several vascular dysfunctions [1–6]. ETs are produced by endothelium and other non-vascular cells [3, 6]. In the rat retina both ET-1 and ET-3 are present [7–9]. Others and we have previously shown that in the retina, ET-1 and ET-3 are present in the endothelium but not in the pericytes, as well as in the non-vascular cells such as Müller cells and ganglion cells [7–9]. We have also shown that in the retina of the long-term diabetic BB-rats, immunoreactivity and mRNA expression for both ET-1 and ET-3 as well as ET_A and ET_B receptor mRNA expressions were increased [7, 10].

Sustained hyperglycaemia might cause functional changes in target organs of diabetic complications

Received: 3 March 1999 and in final revised form: 21 May 1999

*Abbreviations***:** ET, endothelin; ETs, endothelins; ET-1, endothelin-1; ET-3, endothelin-3; RI, resistivity index; ET_A, endothelin A; ET_B, endothelin B; PKC, Protein kinase C; VEGF, vascular endothelial growth factor; NO, nitric oxide; STZ, streptozotocin; SV, systolic velocity; DV, diastolic velocity; RT-PCR, reverse transcriptase polymerase chain reaction.

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through a variety of mechanisms [11–15]. Protein kinase C (PKC), which is activated in the retina in diabetes, is a known up regulator of ET-1 and causes ET receptor induction [1–5, 14, 15]. ET-1, in turn, stimulates diacylglycerol (DAG) activity which further activates PKC [1–5]. In the aorta ET-1 has been shown to stimulate Na^+-K^+-ATPase activity by a PKC dependent pathway [16]. In kidneys from diabetic animals increased ET-1 synthesis and down regulation of ET receptors can be prevented by PKC inhibitors [17].

Short-term vasoactive properties of ET-1 up regulation could lead to vasoconstriction and alterations in blood flow in the retina and peripheral nerve in diabetes [18–20]. ETs might, however, also affect gene expression of other vasoactive substances such as vascular endothelial growth factor (VEGF), nitric oxide (NO) synthase and structural proteins [1–5, 21]. Nitric oxide inhibits the production and action of ET-1. On the other hand, ET-1 stimulates the production of NO [1–5]. In cultured endothelial cells a stimulatory interaction among ET-1, ET-3 and VEGF has been shown [21, 22]. In the kidneys of diabetic rats ET-1 inhibition results in reduced mRNA expression of extracellular matrix proteins [23].

To improve our understanding of the role of ETs in the retina in diabetes, we investigated diabetes-induced alteration of blood flow in the rat retina and studied the effects of the blockade of ET_A and ET_B receptors after short-and long–term follow-up. In addition, we investigated ET-1, ET-3, ET_A and ET_B with respect to their distribution and mRNA expression in the retina.

Subjects and methods

All animals were cared for in accordance with the Declaration of Helsinki regarding the guiding principles in the care and use of animals. The University of Western Ontario council on animal care committee formally approved all experimental protocols. Male Sprague-Dawley rats weighing approximately 200 g were obtained from Charles River Canada Ltd. (St. Constant, PQ, Canada). Diabetes was induced by a single intravenous injection of streptozotocin (STZ, 65 mg/kg of body weight, in citrate buffer). Age- and sex-matched non-diabetic control animals received an injection of the same volume of citrate buffer. Diabetic rats were randomized into a) a poorly controlled diabetic group and b) a poorly controlled diabetic group on bosentan treatment. Bosentan, a potent ET_A and ET_B receptors blocker, was obtained from Hoffman-La-Roche (courtesy of Dr. M. Clozel) and was given by daily oral gavage (100 mg/kg of body weight/day) [24]. The animals were given rat chow and water ad libitum. They were monitored daily with respect to body weight, urine glucose, urine sugar and ketones. Diabetic animals received small daily doses (0.1–3.0 U) of ultra lente insulin (Novo Nordisk, Princeton, N.J. USA). Before killing the animals they were anaesthetized with methoxyflurane inhalation (Mallinckrodt Veterinary Inc. Mundelein, Ill., USA) and laser Doppler analysis of retinal blood flow of the right eye was done. Following death, the retina of the right eye of each animal was snap frozen in liquid nitrogen. The left eyes were fixed in 10% buffered formalin for histological analysis. Blood was collected and analysed for glucose (Sure step, Lifescan, Burnby, BC, Canada) and glycated haemoglobin levels (Glycotest , Pierce, Rockford, Ill., USA). All investigations were done in a masked fashion.

Blood flow analysis. Following anaesthesia, the central retinal vasculature was localized with a 8L5 8.0MHZ probe for colour Doppler sonography (Acuson Sequoia 2.5, Mountainview, Calif., USA) and Doppler waveforms were examined [25]. Several trial recordings were carried out to optimize the sensitivity at an acceptable noise level. The colour Doppler showed blood flow towards the transducer in red, and blood flow away from the transducer in blue. Colour images were obtained in real time and Doppler spectral analyses were done. From the recordings of the arterial blood flow, using peak systolic velocity (SV) and end diastolic velocity (DV), resistivity indices (RI) were calculated as (SV-DV)/SV (Fig. 1). At least three measurements from each recording were carried out and the mean of the three readings was taken as the representative. An increase in the RI in the central retinal artey is indicative of increased resistance in the distal vascular bed, i.e, vasoconstriction at the precapillary or at the capillary level in the retina [25].

RNA isolation. TRIZOL reagent (Canadian Life Technologies Inc. Burlington, ON, Canada) was used to isolate RNA from the retina according to the previously described methodologies [10]. Quantitation of RNA was done by determining the absorbance at 260 nm and 280 nm.

First strand cDNA synthesis. First strand cDNA synthesis was carried out using Superscript-II system (Canadian Life Technologies Inc.) according to the previously described methodologies [10]. The resulting products were stored at –20 °C.

Polymerase Chain Reaction (PCR). The amplification was carried out using the following of our previously described methodologies with some modifications [10].

For ET-1, primer 1 (sense, 5'- GCTCCTGCTCCTCCTTGATG-3') and primer 2 (antisense, 5'- CTCGCTCTATGTAAGTCATGG-3') with a predicted product size of 499 bp and for ET-3, primer 1 (sense, 5'-GCACTTGCTTCACTTATAAGG-3') and primer 2 (antisense 5'-ACAGAAGCAAGAAGCATCAGTTG-3') with a predicted product size of 383 bp were used [26]. For ET_A primer 1 (antisense, 5'-TTCGTCATGGTACCCTTCGA-3') and primer 2 (sense 5'-GATACTCGTTCCATTCATGG-3') with a predicted product size of 546 bp and for ET_B primer 1 (antisense 5'-TTCACCTCAGCAGGATTCTG-3') and primer 2 (5'-AGGTGTGGAAAGTTAGAACG-3') with a predicted product size of 475 bp were used [26].

Reactions were carried out in 30-µl volumes containing 1.5 mmol/l $MgCl_2$ and 4 µl of the RT product and following the cycling parameters as described previously [10]. The linearity of the PCR reaction was established by analysing PCR products with variable amounts of template and variable cycle numbers. It has been previously shown that in this reaction the PCR amplification is log-linear up to 40 cycles [26, 27, 28]. In this study, we used 30 cycles of amplification for ET-1 and ET-3, and 35 cycles for ET_A and ET_B. Simultaneously a housekeeping gene (β-actin) was amplified in a separate set of tubes using the same RT-product, 5'-TGGTGGTATGGGTCAGAAGG-3' and 5'-ATCCTGTCAGCGATGCCTGGG –3' primer sets using the same cycling parameters with a predicted product size of 813 bp [29]. The amplification products were

Table 1. The clinical data of animal groups after 1 month and 6 months of follow-up

	1 month			6 months		
	Control ($n = 9$)	Diabetic ($n = 8$)	Diab + Bosentan ($n = 6$)	Control ($n = 7$)	Diabetic ($n = 8$)	Diab + Bosentan ($n = 8$)
Body weight (g)	399.41 ± 10.66	347.88 ± 11.04[1]	326.83 ± 12.83[1]	629.8 ± 25.51	455.4 ± 11.48[1]	434.8 ± 10.49[1]
Blood glucose (mmol/l)	5.73 ± 0.22	22.64 ± 1.15[1]	21.28 ± 1.47[1]	5.84 ± 0.24	24.7 ± 1.19[1]	23.54 ± 1.40[1]
Glycated haemoglobin (%)	5.45 ± 0.43	10.72 ± 1.06[1]	10.62 ± 0.89[1]	4.2 ± 0.2	10.44 ± 0.22[1]	10.73 ± 0.36[1]

Data are presented as means ± standard error (SEM).
n, number of animals in the group; [1] $p < 0.05$ or less compared with the control group

analysed on a 2.5% agarose gel under ultraviolet light following electrophoresis and ethidium bromide staining.

Southern hybridization. The specificity of the amplifications were confirmed by Southern blot of the PCR products using biotinylated specific internal oligoprobes (ET-1: 5'-CAAAGACCACAGACCAAGGG-3', ET-3: 5'-CCTGCACAGCCTGGAAATGC-3' ET_A : 5'-CGAGGTCATGAGGCTTTTGG-3' and ET_B: 5'-TGCAGACCTTTCCGCAAGCACG-3', β-actin5'-CTGACCCTGAAGTACCCCATT-3') using previously described methodologies [10, 26–28]. The detection was carried out using a NBT/BCIP system (Sure blot, Oncor, Gaithersburg, Md., USA).

Quantitation. Quantitation was carried out by serial dilution slot-blot hybridization and densitometry of the products from the upstream (following 30 cycles for ET-1 and ET-3, and 35 cycles for ET_A and ET_B) of amplification. Hybridizations were carried out using the biotinylated oligoprobes as described above. The blots were analysed by a Hewlett-Packard 4 C scanner and using Mocha software (Jandel Scientific, San Rafael, Calif., USA). The densitometric values were measured as arbitrary units per ug of total RNA and the ratio of β-actin (which did not vary among various groups) to the specific gene(s) was obtained [10].

Immunoctyochemistry. Five μm thick sections on positively charged slides were used for immunocytochemistry using polyclonal anti-rabbit ET-1 and ET-3 antibodies (Peninsula Laboratories, Belmont, Calif., USA) and a streptavidin-biotin-peroxidase technique (Vector Laboratories, Burlingame, Calif., USA), as previously described by us [7]. The specificity of these antibodies has already been established [7, 8]. Negative controls included non-immune rabbit serum and antibodies preabsorbed with an excess of purified peptides (Peninsula Laboratories).

ET receptor localization. The receptor autoradiography was carried out using paraffin embedded retinal tissues as described previously [30]. Briefly, the sections were deparaffinized and preincubated in PBS containing 0.1 mmol/l phenylmethyl-sulphonyl-fluoride for 30 min. The sections were then incubated for 2 h in 0.12 mmol/l ^{125}I-ET-1 (NEN Dupont, Mississanga, Canada) in the presence or absence of cold ET-1. The slides were dipped into Kodak NTB-3 emulsion, dried and exposed for 5 days in the dark. The autoradiograms were developed in Kodak D19 developer and counterstained with haematoxylin and eosin.

Statistical analysis. The data are expressed as means ± SEM and were analysed by Student's *t* test. A *p* value of less than 0.05 was accepted as being significant.

Results

Diabetic animals with or without bosentan treatment showed reduced body weight gain, hyperglycaemia, glycosuria and increased glycated haemoglobin levels (Table 1). After one month of diabetes the RI, measured by colour Doppler sonography, was significantly ($p < 0.05$) increased in the diabetic animals compared with the control animals (Fig. 1). This increased RI was corrected by treatment with bosentan. After 6 months of diabetes this alteration of RI was not verifiable in the diabetic animals. The data further show an age-related increase in the RI in the normal rats (Fig. 1).

Both ET peptides and their receptors were present in the retina of diabetic and non-diabetic rats. Analysis of RNA by semi-quantitative RT-PCR from the linear phase of amplification showed that, after 1 month of diabetes retinal mRNA for ET-1, ET-3 and ET_A were significantly ($p < 0.03$ or less) raised (Fig. 2). In addition after 6 months of follow-up, ET_B mRNA was also significantly ($p < 0.007$) raised (Fig. 3). As expected bosentan treatment did not have any effects on any of these mRNA levels (Fig. 2, 3).

Immunocytochemical analysis showed that, similar to our previously published results [7], both ET-1 and ET-3 were distributed in the retinal vasculature (endothelial cells), ganglion cells, Müller cells, bipolar cells, photoreceptor outer segment, and retinal pigment epithelium. Both after 1 and 6 months of follow-up an increased ET-1 and ET-3 immunoreactivity was present in the retina of diabetic animals with or without bosentan treatment (data not shown).

Autoradiographic receptor localization as shown by the presence of silver grains showed ^{125}I-ET-1 binding in all layers of the retina. The localization was stronger on the ganglion cell layers and on the outer and inner nuclear layers. Preincubation with cold ET-1 caused a reduction of the binding to the level of background. In diabetes the binding was increased (Fig. 4). Retinas from the animals treated with bosentan showed a reduction of the ^{125}I-ET-1 binding.

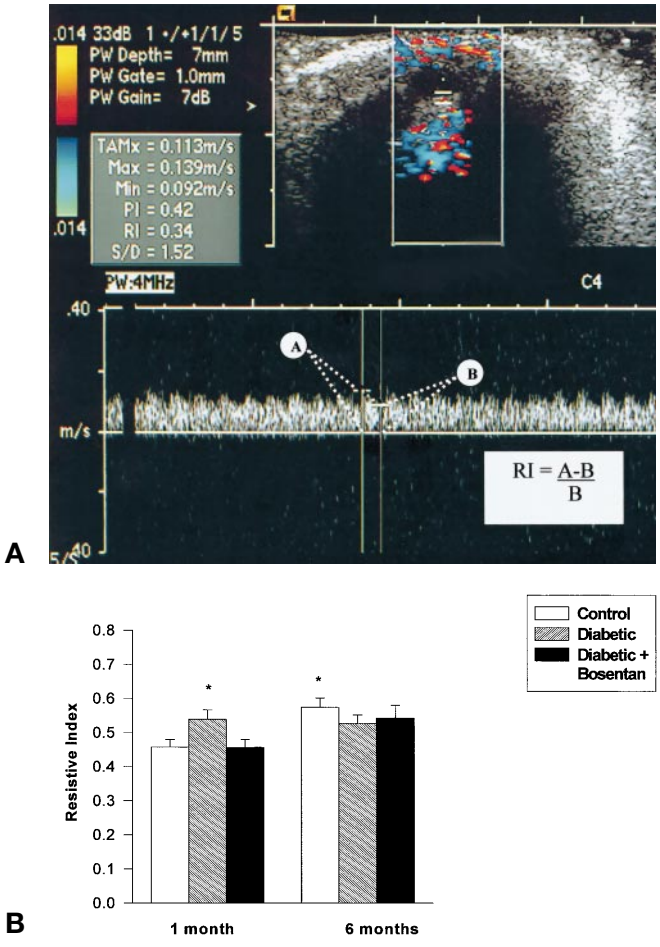

Fig. 1 A, B. **A** Representative colour Doppler sonography from a rat eye showing localization of central retinal artery and vein; the lower pannel shows spectral analysis. Method of resistivity index (RI) measurement is shown in the inset (A = peak systolic velocity, B = end diastolic velocity). **B** RI from diabetic, bosentan-treated diabetic and non-diabetic control animals after 1 month and after 6 month of follow-up. * = significantly ($p < 0.05$) different from 1 month non-diabetic controls

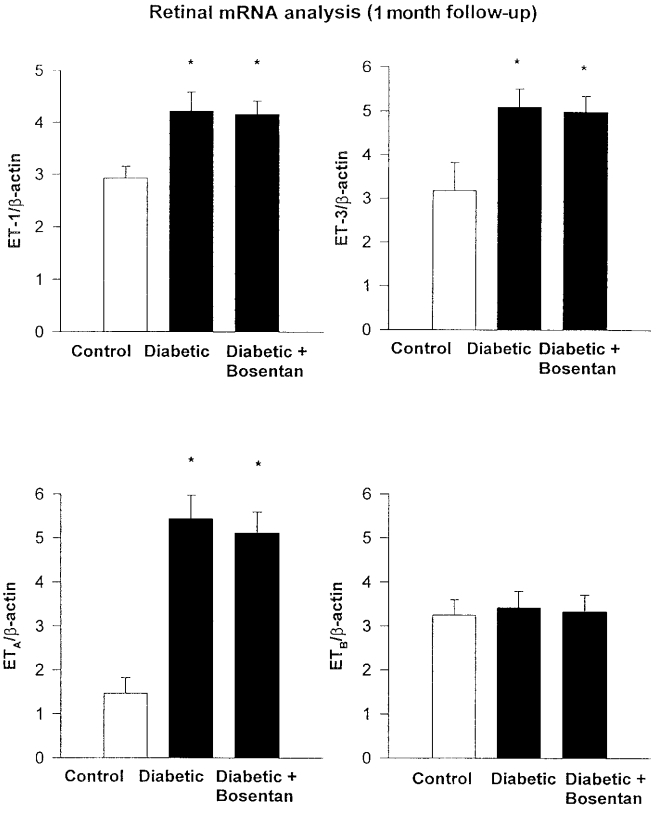

Fig. 2. Semiquantitative mRNA analysis by RT-PCR from the retina of diabetic, bosentan-treated diabetic and non-diabetic control animals after 1 month of follow-up. The data are expressed as the ratio of housekeeping gene β-actin, which did not alter in various groups * = significantly ($p < 0.03$ or less) different from the controls

Discussion

In this study we have shown that retinal vasoconstriction in early diabetes was associated with ET-1, ET-3 and ET_A mRNA up regulation, increased immunoreactive ET-1 and ET-3 and raised ET receptor concentrations. Diabetes-induced retinal vasoconstriction was prevented by treatment with ET receptor antagonist bosentan. Vasoconstriction was not present after 6 months of diabetes. At this stage ET_B receptor mRNA was also upregulated. The RT-PCR based method used in this study allowed us to measure the mRNA expression of multiple genes from a small amount of RNA. This method has previously been used to quantitate gene expression in the retina by us and other investigators [10, 29]. In keeping with the present data, others and we have previously established that several cell types in all segments of the eye showed immunoreactivity for ET-1 and ET-3 with overall increased immunoreactivity in diabetes [7–9]. Furthermore, ET receptors have previously been shown in the vascular and neuronal components of the retina [9, 31].

This study has provided direct evidence linking the ET system to the pathogenesis of vascular dysfunction in the retina in experimental diabetes. We used colour Doppler sonography to detect retinal blood flow in the rats in diabetes. This sensitive, non-invasive technique provided the opportunity to do a real-time recording in the blood vessels of choice. This technique, although it has been used in human diabetic subjects, to our knowledge has not been used previously in diabetic animals [32–34]. The present data showing increased RI in early diabetes suggest a vasoconstriction which was not verifiable in long-term follow-up. This is in keeping with previous studies by other investigators suggesting a vasoconstriction in several organs in early diabetes [14, 15, 19]. On the other hand, some investigators have shown an increased blood flow in multiple organs in diabetes [11]. It is to be pointed out that the RI measured in

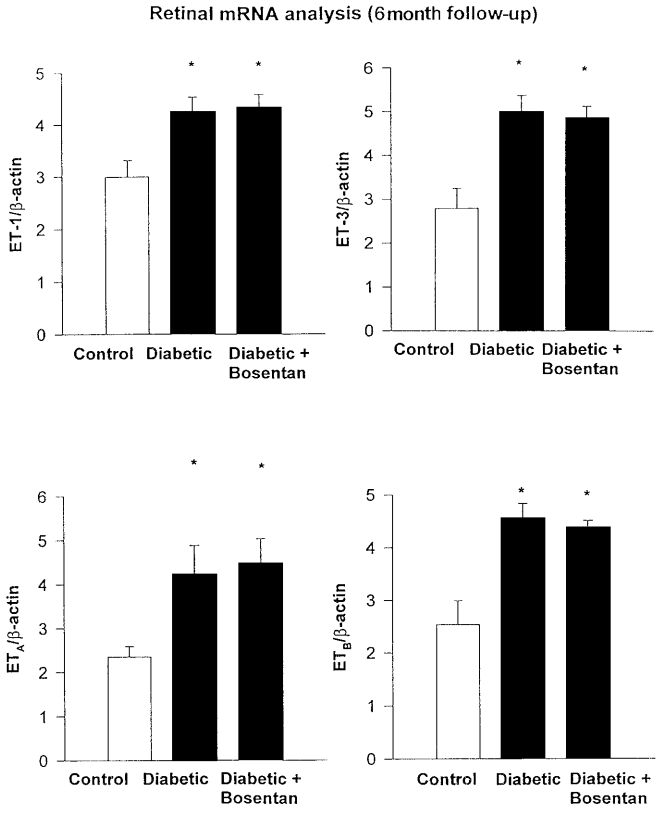

Fig. 3. Semiquantitative mRNA analysis by RT-PCR from the retina of diabetic, bosentan-treated diabetic and non-diabetic control animals after 6 month of follow-up. The data are expressed as the ratio of housekeeping gene β-actin, which did not alter in various groups * = significantly ($p < 0.04$ or less) different from the controls

this study does not indicate actual blood flow [25, 32, 33]. It is possible for the blood flow to increase or decrease depending on the grades of vasoconstriction within a physiologic range. The pattern of blood flow in the central retinal arteries, however, is like that of large arteries with low end diastolic pressure. As there was no local stenosis in any of our animals, increased RI was indicative of distal vasoconstriction, i.e, at the precapillary or at the capillary level [25]. This could have led to a reduction of blood flow in the retinal capillary bed, although the exact volume of blood flow reduction was not known [25, 32–34]. In human diabetes using a similar method, it has been shown that diabetic patients with or without retinopathy compared with non-diabetic subjects have a higher RI [32]. There is much discrepancy between various studies in human and animal diabetes regarding alterations in blood flow as apparent from an in-depth review published recently [35]. Nevertheless our study suggests the important part played by the ET system in the generation of this functional abnormality in diabetes. In the peripheral nerve ET-receptor blockade has been shown to improve diabetes-induced reduced nerve conduction velocity and blood flow abnormalities [20].

The differential expression of ET_A and ET_B mRNA after 1 and 6 months of follow-up suggests that the components of the ET system might change over time leading to a wide range of effects. Among the ET receptors, ET_A binds with ten times higher affinity with ET-1 compared with ET-3. ET_B, however, binds with both ET-1 and ET-3 with equal affinity [1–6]. ET_A is predominantly responsible for mediating vasoconstricting effects of ET-1 by way of phospholipase C, inositol trisphosphate formation and increase in intracellular Ca^{++} [2–5]. On the other hand, it has been shown that binding of ET-3 with ET_B receptors might cause vasodilatation mediated by an in-

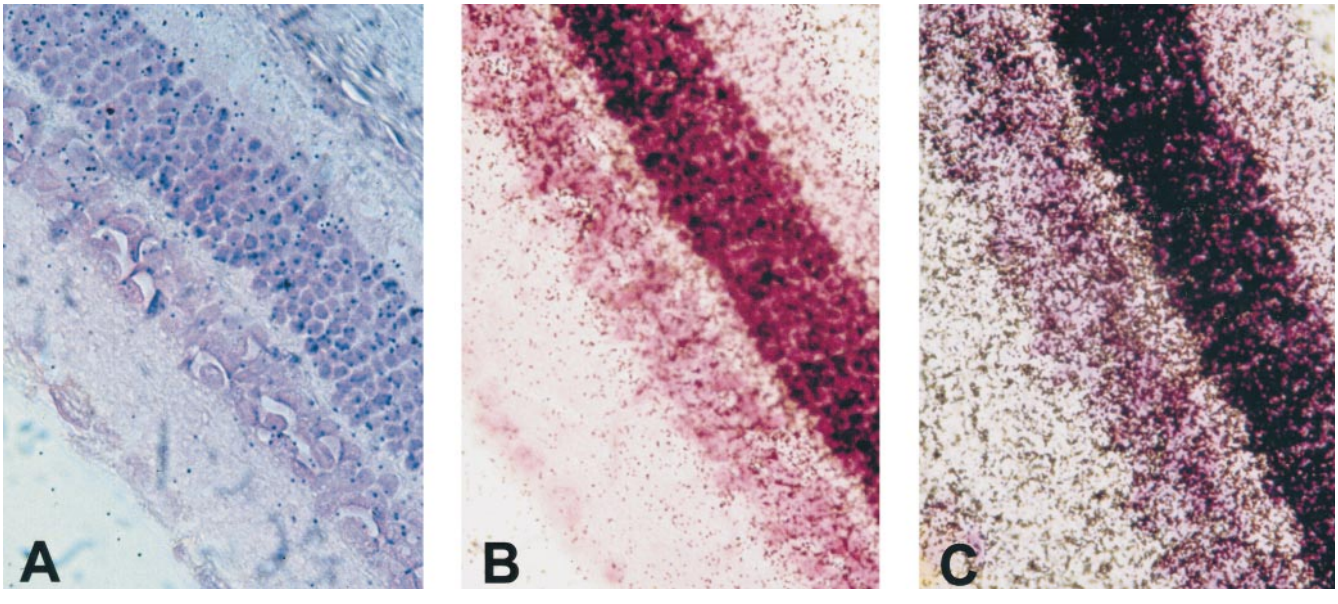

Fig. 4. ET-receptor localization in the retina of **B**) non-diabetic controls and **C**) diabetic rats. Note the higher grain density in **C**. The signals could be abolished to the background level by incubation with cold ET-1 (**A**)

crease in NO and prostaglandin production and activation of K^+ channels [2–5]. It is possible, that in this study increased ET_B production after 6 months might have a balancing effect on ET-1-induced vasoconstriction. Note that after 6 months of diabetes, retinal RI in the non-diabetic animals was significantly ($p < 0.05$) higher than that in 1 month non-diabetic animals. This change probably reflects the effect of normal ageing processes. In human diabetic subjects time-dependent alteration of ET-1 has been shown in the cutaneous microvessels in which, although ET-1 was increased in short-term diabetes, it was decreased after long-term follow-up [36].

Apart from vasoconstriction, an altered ET system might have various effects in the retina in diabetes. In the retina, ETs probably play important parts in neurotransmission and neuromodulation [1–9]. Several modulators of ETs, such as protein kinase C (PKC), Ca^{++} and nitric oxide (NO) are altered in diabetes [11–15, 37]. Diabetes-induced PKC activation could be responsible for ET up regulation in the retina [1–6, 11, 14, 15, 37]. Other potent vasoactive substances such as NO and prostacyclin interact with ET which in turn possesses a positive feedback regulatory action on NO-synthesis [1–6]. ET-1 and ET-3 have been shown to up regulate VEGF production in endothelial cells [21, 22]. On the other hand VEGF increases ET-1 mRNA and protein expression in the endothelial cells [21, 22].

It is, however, not clear what impact altered expression of ETs and their receptors possibly have on the development of diabetic retinal lesions in the long-term. It has been postulated that loss of contractile function of pericytes in diabetes might be related to changes in ET-1 [38]. The long-term consequences of an augmented ET system possibly further contribute to long-term nuclear signalling and involve cellular changes secondary to altered gene expression [1–6, 39]. Increased extracellular matrix protein synthesis and subsequent structural changes such as basement membrane thickening are hallmarks of diabetic retinal microangiopathy [11, 12, 15]. It has been shown that diabetes-induced up regulation of glomerular α1(I), α1(III), α1(IV) collagen, laminin B1 and B2, tumour necrosis factor α, platelet derived growth factor, transforming growth factor β, and basic fibroblast growth factor genes can be completely blocked by treatment with an ET_A receptor antagonist [23]. It is possible that, similar to kidneys in diabetic rats, ETs influence gene expression of extracellular matrix proteins in the retina leading to the development of structural changes [1–6, 23, 39].

Acknowledgements. This work was supported by grants from the Canadian Diabetes Association in honour of F. Langlie and from the faculty of Medicine and Dentistry, University of Western Ontario.

References

1. Vanhoutte PM (1994) A matter of life and breath. Nature 368: 693–694
2. Simonson MS (1993) Endothelins: Multifunctional peptides in the kidney. Physiol Rev 73: 375–411
3. Rubanyi GM, Polokof MA (1994) Endothelins: Molecular biology, biochemistry, pharmacology, physiology and pathophysiology. Pharmacol Rev 46: 326–414
4. Huggins JP, Pelton JT, Miller RC (1993) The structure and specificity of endothelin receptors: Their importance in physiology and medicare. Pharmacol Ther 59: 55–123
5. Levin ER (1995) Endothelins. New Engl J Med 333: 356–363
6. Levin ER (1996) Endothelins as cardiovascular peptides. Am J Nephrol 16: 246
7. Chakrabarti S, Sima AAF (1997) Endothelin-1 and Endothelin-3 like immunoreactivity in the eyes of diabetic and non-diabetic BB/W rats. Diabetes Res Clin Prac 37: 109–120
8. Chakravarthy U, Douglas AJ, Bailie R, McKibben B, Archer DB (1994) Immunoreactive endothelin distribution in ocular tissue. Invest Ophthalmol Vis Sci 35: 2448–2454
9. MacCumber MW, Ros CA, Glaser BM, Snyder SH (1989) Endothelin: Visualization of mRNAs by in situ hybridization provides evidence for local action. Proc Natl Acad Sci USA 86: 7285–7289
10. Chakrabarti S, Gan XT, Merry A, Karmazyn M, Sima AAF (1998) Augmented retinal endothelin-1, endothelin –3, $Endothelin_A$ and $Endothelin_B$ gene expression in chronic diabetes. Curr Eye Res 17: 301–307
11. Williamson JR, Chang K, Frargos M et al. (1993) Perspective in diabetes: Hyperglycemic pseudohypoxia and diabetic complications. Diabetes 42: 801–813
12. Brownlee M, Cerami A, Vlassara H (1988) Advanced products of non-enzymatic glycosylation and the pathogenesis of diabetic vascular disease. Diabetes Metab Rev 4: 323–337
13. Greene DA, Lattimer SA, Sima AAF (1987) Sorbitol myoinositol and sodium potassium ATPase in the pathogenesis of diabetic complication. N Engl J Med 316: 559–606
14. Koya D, King GL (1998) Protein kinase C activation and the development of diabetic complications. Diabetes 47: 859–866
15. King GL, Brownlee M (1996) The cellular and molecular mechanisms of diabetic complications. Endocrinol Metab Clin North Am 25: 255–269
16. Gupta S, Ruderman S, Cragoe EJ, Susman I (1991) Endothelin stimulates Na + -K + -ATPase activity by a protein kinase C-dependent pathway is rabbit aorta. Am J Physiol 261:H38–H45
17. Awazu M, Parker RE, Harvie BR, Ichikawa I, Kon V (1991) Down regulation of endothelin-1 receptor prevented by protein kinase C inhibitor in streptozotocin diabetic rats. J Cardiovasc Pharmacol 17 [suppl 7] S500–S502
18. Kohner M, Patel V, Rassam SMB (1995) Role of blood flow and impaired autoregulation in the pathogenesis of diabetic retinopathy. Diabetes 44: 607
19. Bursell SE, Clermont AC, Osen B, King GL (1995) The in vivo effect of endothelin retinal circulation in non-diabetic and diabetic rats. Invest Ophthalmol Vis Sci 36: 596–607
20. Cameron NE, Dines KC, Cotter MA (1994) The potential contribution of endothelin-1 to neurovascular abnormalities in streptozotocin-diabetic rats. Diabetologia 37: 1209–1215
21. Pedram A, Razandi M, Hu RM, Levin ER (1997) Vasoactive peptides modulate vascular endothelial cell growth

factor production and endothelial cell proliferation and invasion. J Biol Chem 27: 17097–17103
22. Matsuura A, Yamochi W, Hirata K, Kawashina S, Yokoyana M (1997) Stimulatory interaction between vascular endothelial growth factor and endothelin-1 on each gene expression. Biochem Biophys Res Commun 235: 713–716
23. Nakamura T, Ebihara I, Fukui M, Tomuno Y, Koide H (1995) Effect of a specific endothelin receptor A antagonist on mRNA levels for extracellular matrix components and growth factors in diabetic glomeruli. Diabetes 44: 895–899
24. Clozel M, Gray GA, Kalina B et al. (1994) Pharmacological characterization of Bosentan, an orally active non-peptide endothelin receptor antagonist. J Pharmacol Exp Ther 270: 228–235
25. Zwiebel WJ (1992) Spectrum analysis in doppler vascular diagnosis. In: Zwiebel WJ (ed) Introduction to vascular ultrasonography 3rd edn. Saunders, Philadelphia, pp 46–65
26. Wang X-K, Douglas S, Louden C, Vickery-Clark LM, Feuerstein GZ, Ohlstein EH (1996) Expression of endothelin-1, endothelin-3, endothelin converting enzyme-1, and endothelin-A and endothelin-B receptor mRNA after angioplasty induced neointimal formation in the rat. Circ Res 78: 322–338
27. Yang T, Terada Y, Nonoguchi H, Ujiie K, Tomita K, Marumo F (1993) Effect of hyperasmolality on production and mRNA expression of ET-1 in inner medullary collecting ducts. Am J Physiol 264: F684–F689
28. Tarada Y, Tomita K, Nonoguchi H et al. (1993) Expression of endothelin-3 mRNA along rat nephron segment using polymerase chain reaction. Kidney 44: 1273–1280
29. Roy S, Lorenzi M (1996) Early biosynthetic changes in the diabetic like retinopathy of galactose fed rats. Diabetologia 39: 735–738
30. Chakrabarti S, Ghahary A, Murphy LJ, Sima AAF (1991) Insulin like growth factor I expression is not increased in the retina of diabetic BB/W rats. Diab Res Clin Pract 14: 91–98
31. MacCumber MW, D'Anna SA (1994) Endothelin receptor–binding subtypes in the human retina and choroid. Arch Ophthalmol 112: 1231–1235
32. Arai T, Numata K, Tanaka K et al. (1998) Ocular arterial flow dynamics in patients with diabetes mellitus. J Ultrasound Med 17: 675–681
33. Guthoff RF, Berger RW, Winkler P, Helmke K, Chumbley LC (1991) Doppler sonography of the ophthalmic and central retinal vessels. Arch Ophthalmol 109: 532–537
34. Lieb WE (1993) Color Doppler ultrasonography of the eye and orbit. Curr Opin Ophthalmol 4: 68–82
35. Schmetterer L, Wolzt M (1999) Ocular blood flow and associated functional deviations in diabetic retinopathy. Diabetologia 42: 387–405
36. Properzi G, Terenghi G, Gu XH et al. (1995) Human microvs. Early increase precedes a depletion of endothelin–but not of von Willebrand factor in cutaneous microvessels of diabetic patients. A quantitative immunohistochemical study. J Pathol :175: 243–252
37. Ishii H, Jirousek MR, Koya D et al. (1996) Ameloriation of vascular dysfunction in diabetic rats by an oral PKC β inhibitor. Science 272: 728–731
38. De La Rubia G, Oliver FJ, Inoguchi, King GL (1992) Induction of resistance to endothelin-1's biochemical actions by elevated glucose levels in retinal pericytes. Diabetes 41: 1533–1539
39. Simonson MS, Jones JM, Dunn MJ (1992) Differential regulation of fos and jun gene expression and AP-1 Cis element activity by endothelin isopeptides: Possible implications for mitogenic signalling by endothelin. J Biol Chem 267: 8643–8649