Endothelin Expression in Ocular Tissues of Diabetic and Insulin-Treated Rats

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Purpose. The endothelins are potent vasoactive peptides that are widely distributed in ocular tissues. There is evidence linking the endothelins to vascular dysfunction in diabetic microangiopathy. Thus, the synthesis and distribution of endothelin-1 (ET-1) and endothelin-3 (ET-3) were studied in the retinas of diabetic and nondiabetic animals.

Methods. Levels of ET-1 and ET-3 were determined by radioimmunoassay in ocular tissues of normal rats, and in rats with streptozotocin-induced diabetes of 6 and 12 weeks' duration, insulin-treated and untreated. In a separate cohort of similarly treated animals, retinal vascular trypsin digest preparations were immunostained, using antibodies raised against ET-1 and ET-3.

Results. Ocular ET-1 levels were elevated twofold in diabetic animals that received insulin treatment for 7 days when compared with levels in normal rats. Insulin treatment for 10 days before death caused a fourfold elevation of ET-1 in ocular tissues. Endothelin-1 was also increased in 12-week-old diabetic animals and in those maintained on insulin throughout their period of diabetes. Immunofluorescence to anti-ET-1 within the capillary bed and veins of the retina in diabetic insulin-treated animals was elevated when compared with digests from normal litter-matched control animals. Ocular tissue ET-3 levels were unaffected by diabetes.

Conclusions. Overall ocular and retinal tissue levels of ET-1 were selectively elevated by diabetes and insulin treatment, suggesting that the endothelins may be involved in the pathogenesis of diabetic retinal microangiopathy. Invest Ophthalmol Vis Sci. 1997;38:2144-2151.

The endothelins are potent vasoactive and neural peptides. There are three well-characterized isoforms of these 21 amino acid peptides (ET-1, ET-2, ET-3) of which ET-1 and ET-3 are the most widely distributed in mammalian tissues.1-4 Ocular tissues are a particularly rich source of both peptides, and ET-1 and ET-3 gene transcription has been demonstrated in vascular and extravascular sites in retina, uveal tract, and optic nerve, 5 indicating important physiological roles for these peptides in the control of vascular tone, regulation of aqueous flow, and neural modulation in retina and optic nerve. The endothelins act through specific receptors, which are transmembrane G-proteins; and the two major subtypes ET\textsubscript{A} and ET\textsubscript{B} have been described in ocular tissues and its associated vasculature.6,7 Endothelin-1 is consistently found in human plasma (0.15 to 1.5 pmol/l) and ET-3 levels have been variously reported as ranging from undetectable to extremely low.8 Both endothelins are considered to be paracrine hormones, exerting their effects locally at the site of synthesis.9 The endothelins have been implicated in the pathophysiology of a number of vascular diseases, including hypertension.10 Evidence in vitro suggests that elevated levels of glucose11 and insulin,12 individually, and perhaps synergistically, cause an enhanced synthesis of ET-1. Endothelin-1 is a potent mitogen in vascular smooth muscle,13,14 prompting speculation that increased secretion of this peptide is responsible for the link between diabetes and hypertensive atherosclerotic disease.

Markedly elevated plasma ET-1 levels, which did not correlate with blood pressure, retinopathy or duration of diabetic disease, have been demonstrated in
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Figure 1. Graph showing (A) immunoreactive endothelin-1 and (B) immunoreactive endothelin-3 in ocular tissue extracts, with each point representing the mean values ± SEM from 15 animals per group (5 within each category, with a = normal, b = diabetic, c = insulin-treated diabetic). Animals within each group were matched, belonging to the same litter and having been rendered diabetic at the same time. Insulin treatment was given for 7 days before death (group 1), 10 days before death (group 2), or from induction of diabetes (group 3). Significant differences in immunoreactive endothelin-1 levels were found between 2a versus 2c (P = 0.009) and 2b versus 2c (P = 0.0088). Immunoreactive endothelin-1 was also significantly elevated in insulin-treated animals in group 3 (c) when compared with that in its matched control animals (a; P = 0.008). Endothelin-3 levels were not significantly different in normal, untreated diabetic, or insulin-treated diabetic animals within each group.

Figure 2. Radioimmunoassay of endothelin-1 and endothelin-3 in normal rat ocular tissue extracts subjected to high-performance liquid chromatography. Immunoreactive endothelin-1 and immunoreactive endothelin-3 peaked in fractions 37 and 35, respectively, corresponding to peak elution times of standard synthetic endothelin-1 and endothelin-3 of 37.8 minutes and 35.7 minutes, respectively.

Vascular angiopathy may yet be proposed if tissue levels of these peptides are elevated. In the retina, a characteristic feature of diabetes is capillary closure followed by ischemia. Furthermore, clinical and experimental evidence indicates compromised autoregulation and altered blood flow patterns in the retinal and uveal circulation. To help clarify the putative role of the endothelins in functional and microangiopathic changes seen in diabetes, we studied ocular tissue endothelin levels in normal rats, in rats with streptozotocin-induced diabetes (STZ-diabetic), and in insulin-treated rats with streptozotocin-induced diabetes.

Materials and Methods

Animals

All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Wistar rats were maintained on standard laboratory food and water ad libitum under temperature- and light-controlled conditions. In any one group, two thirds of the animals (weighing approximately 250 g) were rendered diabetic by a single injection of 50 mg/kg of streptozotocin (STZ), and blood glucose was measured in samples obtained from the tail vein. Animals in which blood sugar levels were consistently in excess of 10 mmol/1 were diagnosed diabetic patients.

Although ET-1 exerts most of its vasoconstrictor effects through ET \(_{A}\) receptors, which are preferentially expressed in the vascular smooth muscle on the abluminal side of the blood vessel, the endothelium itself contains significant numbers of ET \(_{B}\) receptors that mediate vasodilation. Also, a role for the endothelins in the pathogenesis of microvascular angiopathy may yet be proposed if tissue levels of these peptides are elevated. In the retina, a characteristic feature of diabetes is capillary closure followed by ischemia. Furthermore, clinical and experimental evidence indicates compromised autoregulation and altered blood flow patterns in the retinal and uveal circulation. To help clarify the putative role of the endothelins in functional and microangiopathic changes seen in diabetes, we studied ocular tissue endothelin levels in normal rats, in rats with streptozotocin-induced diabetes (STZ-diabetic), and in insulin-treated rats with streptozotocin-induced diabetes.
as diabetic, and half were treated with daily insulin injections.

Groups of animals (15 per group) were used, each consisting of three categories comprising a minimum of 5 each of matched control, untreated diabetic, and insulin-treated diabetic rats (for 7 days or 10 days before death). In a separate experiment, animals that had been rendered STZ-diabetic for 12 weeks were treated with insulin daily from diagnosis until death. Each insulin-treated animal received 4 U insulin (Rapitard, Novo Nardisk, Crawley Sussex, UK) per day.

The mean blood glucose value in normal control rats at death was 8 ± 0.8 mmol/l, compared with values of 39.1 ± 4.9 mmol/l in untreated diabetic rats and 4.1 ± 0.9 mmol/l in insulin-treated diabetic rats.

**Tissue Preparation**

After removal of the crystalline lens through an incision made at the equator, the remaining ocular tissue was weighed. Pairs of eyes from each animal were combined, homogenized, and extracted into acidified ethanol (100%) for 48 hours at 4°C on a rotary shaker, as previously described. The samples were dried under a stream of N2 and were reconstituted in 50 mmol phosphate buffer (pH 7.4) containing 0.1% bovine serum albumin (BSA) and 0.1% Triton X-100 at the time of assay or were aliquoted and stored.

**High-Performance Liquid Chromatography**

Standard synthetic ET-1 and ET-3 were purchased from Peninsula Laboratories (Merseyside, UK). Each peptide (500 ng) was dissolved in 10 μl of distilled water and subjected to reverse-phase high-performance liquid chromatography (HPLC; System Gold Beckman Instruments, San Paolo, CA) using a C-18 300A column (Waters, UK) at a flow rate of 1 ml/min. A linear gradient of acetonitrile (0% to 50%) containing 0.05% trifluoroacetic acid was developed in a 45-minute period and was used to elute the peptides. One-milliliter fractions were collected, freeze-dried, and reconstituted in assay buffer before examination by radioimmunoassay. To confirm the specificity of the radioimmunoassay, ethanol-extracted samples from one group of rats were reconstituted in 25 μl of water containing 0.05% trifluoroacetic acid and were subjected to HPLC under the same conditions used to elute standard synthetic endothelins.

**Iodination of Peptides**

The peptides ET-1 and ET-3 were iodinated, using iodo-gen (Pierce Warriner, UK). Briefly, 10 to 15 μg of the peptides were incubated with iodo-gen and Na125I (Amersham, UK) in 50 mmol phosphate buffer for 20 minutes at room temperature. The reaction mix was loaded onto a C-18 μbondapak column and purified by reverse-phase HPLC (Waters). The specific activities of the radiolabeled peptides were calculated by the method of self-displacement.

**Radioimmunoassay**

For ET-1, the assay volume was 300 μl, and the intra- and interassay coefficients of variation were 12% and 17%, respectively (n = 5). The IC50 value was 7.3 fmol/tube. The sensitivity of the assay allowed the detection of 0.76 ± 0.3 fmol/tube from zero standard with 95% confidence. Cross-reactivity was 100%, 15%, and 7% with ET-1, ET-2 and ET-3, respectively. The antibody to ET-1 did not cross-react with substance P, angioten-
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FIGURE 4. Bar graph showing (A) immunoreactive endothelin-1 and (B) immunoreactive endothelin-3 in ocular (open bar), renal (hatched bar), and intestinal (solid bar) tissue in matched control animals, in untreated diabetic animals (6 weeks since induction), and in insulin-treated diabetic animals (after 10 days' treatment). Marked increases in immunoreactive endothelin-1 are seen in ocular and renal tissues after 10 days of insulin treatment. Immunoreactive endothelin-3 levels are not significantly different in intestinal or renal tissue.

sin II, or other vasoactive peptides. Nonspecific binding was always less than 3%.

For ET-3, the intra- and interassay coefficients of variation were 5% and 21%, respectively (n = 7). The IC_{50} value was 23 fmol/tube. The detection limit of the assay was 1.43 ± 0.2 fmol/tube from zero standard with 95% confidence. Cross-reactivity was 100%, with ET-3, and less than 5% with ET-1 and ET-2. The antibody to ET-3 did not cross-react with other vasoactive peptides. Nonspecific binding was less than 5%.

In Vitro Glycation Experiments

Synthetic ET-1 and ET-3 were incubated in 0, 5, 15, and 25 mM glucose in distilled water for 24, 72, and 240 hours. The peptides were then subjected to reverse-phase HPLC and the fractions collected and subjected to radioimmunoassay, as previously described.

Endothelin-1 and Endothelin-3 in Other Tissues

To establish whether changes in ET levels were organ-specific, two other tissues were examined. The kidney and the small intestine were chosen, because both tissues show considerable levels of ET-1-like immunoreactivity.21,22 Also, the vasculature of the kidney is affected by diabetes and suffers pathologic changes similar to those that occur in the retina. Animals that had been diabetic for 6 weeks and then subjected to insulin treatment for 10 days were chosen for this experiment. At death, a segment of jejunum approxi-

mately 2.5 cm long, the left kidney, and both eyes of each rat were removed. Tissues were weighed; extracted in acid−ethanol, as previously described; spun down; and dried under a stream of N₂.

Immunodetection of Endothelins in Retinal Vascular Digests

For this experiment, animals were assigned to one of three groups consisting of controls, 6-week STZ-diabetic untreated, and STZ-diabetic insulin-treated rats for 10 days before death (n = 6, each group). After death, the eyes were enucleated and sectioned in half under a dissecting microscope. The retinas were carefully dissected from the posterior calotte, fixed for 12 hours in 4% buffered formalin, washed in distilled water, and incubated in a 3% trypsin solution at 37°C for 2 to 4 hours. The neural tissue was gently tapped away and the vascular tree mounted on a silane-coated glass slide. After permeabilization in Tris-buffered saline (TBS) containing 0.5% bovine serum albumin and 0.01% TX-100, the trypsin digests were exposed to a polyclonal anti-ET-1 or anti-ET-3 (final dilution 1:1000) per the manufacturers recommendation (Cambridge Research Biochemicals, MA) in 20 mM TBS, pH 7.2, overnight at 4°C. Control digests were exposed to antibody preabsorbed with 10 nM ET-1. The slides were sequentially exposed to a biotinylated goat anti-rabbit antibody, followed by streptavidin−fluorescein isothiocyanate (FITC) conju-
RESULTS

Ocular Endothelin-1- and Endothelin-3-Like Immunoreactivity

The results reported are from a minimum of 15 animals per group (5 control, 5 untreated diabetic, and 5 insulin-treated diabetic), with each sample assayed in duplicate in two separate radioimmunoassays. The results are graphically summarized in Figure 1. Endothelin-1-like immunoreactivity (irET-1) in whole eye extracts of normal rats was 36 ± 18 fmol/g wet weight. Profiles obtained in HPLC of ocular extracts from normal rats showed that irET-1 was present in fractions 33 through to 39 and peaked at fraction 37 (Fig. 2). This profile was identical to that of standard synthetic ET-1 eluted under the same conditions (data not shown but published previously).19 No immunoreac-

Statistical Methods

The results were analyzed using analysis of variance to test for significant differences between populations. Specific group differences were tested using the Mann–Whitney test for independent samples. Because multiple comparisons were carried out within groups, only $P$ values less than 0.01 were considered significant.
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FIGURE 7. (A) Trypsin digest of retina from a rat with 6 weeks' induced diabetes, treated with insulin for 10 days before death. There is intense immunoreactivity to endothelin-1 in the retinal artery (RA), capillary bed (arrows) and vein (V). (B) High-power micrograph of digest shown in A. The venous and capillary endothelia show strong, positive immunoreactivity to anti-endothelin-1. Magnification, ×100.

Activity was present in any of the other fractions. In rats with 6 weeks' induced diabetes, irET-1 was not significantly different from that in control animals (Fig. 1A). In ocular tissues of animals that had been maintained with insulin for 7 days before death, irET-1 was twofold higher; but the difference did not reach significance (P = 0.09). By contrast, treatment with insulin for 10 days before death resulted in a twofold elevation in irET-1, which was highly significant when compared with that in the matched control (P = 0.009) or in untreated diabetic (P = 0.0088) animals within that particular group.

In rats with 12 weeks' STZ-induced diabetes, irET-1 in ocular tissue extracts was elevated at 57 ± 36 fmol/g wet weight but was not significantly different from that of matched controls at 28 ± 6.9 fmol/g wet weight. Animals maintained continuously on insulin treatment from induction of diabetes, showed irET-1 (93 ± 14 fmol/g wet weight) significantly increased over that seen in controls (P = 0.0088). High-performance liquid chromatography profiles of ocular tissue extracts from diabetic and diabetic insulin-treated rats, showed a much broader peak of irET-1, which was found in fractions 30 through 40 peaking at fractions 34 and 35 (Fig. 3A).

As previously reported, ET-3 like-immunoreactivity (irET-3) was higher than irET-1 in ocular tissues of normal rats. In rats with 6 weeks' and 4 months' induced diabetes, with or without insulin treatment, ocular tissue ET-3 levels were not significantly different from control rats within the same group (Fig. 1B). High-performance liquid chromatography profiles showed that irET-3 peaked at 35 minutes in normal rat ocular extracts (Fig. 2) and was identical to that seen when standard synthetic ET-3 was eluted under the same conditions. Samples from diabetic and insulin-treated diabetic rats showed that similar to ET-1, ET-3 eluted earlier than its standard synthetic counterpart (Fig. 3B).

In Vitro Glycation Experiments

The earlier appearance of the ET-1 and ET-3 peaks revealed in HPLC in untreated and insulin-treated diabetic animals suggested increases in hydrophilicity. Incubations of the peptides with glucose for 24 and 72 hours did not alter the peak elution times of ET-1 or of ET-3 in any of the concentrations used. Incubation of either peptide for 240 hours in 15 and 25 mM glucose resulted in a left shift of the peak, eluting approximately 2 minutes earlier in each case.

Tissue Endothelin Levels in Renal and Intestinal Samples

Extracts of normal kidney tissue contained 7.9 ± 3 fmol/g of ET-1, which was increased twofold and fivefold in untreated and insulin-treated diabetic animals, respectively (Fig. 4A). In the small intestine of control rats, irET-1 levels were 13 ± 7 fmol/g wet weight and were not significantly different from that in untreated diabetic rats or in their insulin-treated counterparts (Fig. 4A).

Endothelin-3-like immunoreactivity was low in duodenal tissue (1.9 fmol/g wet weight) and in kidney tissue (0.5 fmol/g wet weight) and was unchanged after 6 weeks of diabetes and 10 days of insulin treatment (Fig. 4A).

Immunodetection of the Endothelins in Retinal Vascular Digests

Immunofluorescence to ET-1 was present in the normal rat retinal vasculature and was most marked in the large arteries and arterioles, with gradually decreasing fluorescence within the capillaries and negligible in the venules and veins (Fig. 5A). In digests from diabetic animals increased fluorescence was seen in the
capillary and venous endothelia (Fig. 6). In diabetic animals treated with insulin for 10 days, a marked increase in fluorescence was seen within the capillary and venous endothelium (Figs. 7A, 7B). Normal and diabetic retina-derived digests showed no immunostaining after preincubation or coincubation of the antibody with 10 nM ET-1 (Figs. 5B, 6B) confirming the absence of significant nonspecific binding of antibody. Digests from normal, untreated diabetic, and insulin-treated diabetic animals exposed to anti-ET-3 antibody showed no positive immunostaining within the retinal vasculature.

DISCUSSION

Microangiopathy is a major complication of diabetes mellitus, and in experimental animal models, increased circulating levels of such vasoconstrictors as ET-1 and prostanoids in the plasma of hypertensive streptozotocin diabetes. Also, high concentrations of circulating ET-1, through interaction with the ETB receptor mediators that mediate vasodilation and that are expressed in the endothelium, could further aggravate increases in blood flow.

Results in a recent study have suggested that ET-1 gene expression is reduced in the kidney in the STZ-diabetic rat and that this effect is reversed by the treatment of such animals with insulin. Our results only partially support these observations, because in the current results, tissue ET-1 levels were not reduced by hyperglycemia but were elevated after treatment with insulin. Our results support the observations of Yamauchi et al who detected increased ET-1 synthesis after preincubation or coincubation of the antibody with 10 nM ET-1 (Figs. 5B, 6B) confirming the absence of significant nonspecific binding of antibody. Digests from normal, untreated diabetic, and insulin-treated diabetic animals exposed to anti-ET-3 antibody showed no positive immunostaining within the retinal vasculature.

The current work implicates the vasculature as a major source of the abnormal increases in peptide; increased irET-1 was seen within the retinal vasculature in trypsin-digest preparations. The absence of immunostaining in preabsorbed control vascular digests from matched diabetic animals confirmed that increased protein glycation occurring as a consequence of diabetes could not have been responsible for the increased immunofluorescence in the experimental digests.

The possibility that the increase in ET-1 documented by the current data may simply reflect decreased degradation of the peptide was considered. Hyperglycemia per se results in increased nonenzymatic glycation of proteins. Endothelin-1 and ET-3 contain lysine residues that are potential sites for glycation, and the decreased retention time of these peptides after exposure to either 15 or 25 mM glucose for periods in excess of 120 hours suggests that significant glycation may have occurred. Such a change can cause alteration in charge, blocking of critical amino groups, and loss of cellular recognition and hydrogen-bonding capacity. Furthermore, glycation may also affect posttranslational processing and degradation of the peptides.

Further studies are clearly required to understand the mechanisms involved in the control of synthesis of ET-1 and associated peptides in hyperglycemia and in diabetes.

Key Words
endothelin-1, endothelin-3, insulin, radioimmunoassay, streptozotocin diabetes

References

6. de Juan JA, Moya FJ, de Llaca G, Cruz F, Fernandez-
Endothelin Expression in Diabetic Ocular Tissues