Endothelin Antagonism Prevents Diabetic Retinopathy in NOD Mice: A Potential Role of the Angiogenic Factor Adrenomedullin

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Altered activity of retinal endothelin-1 (ET-1) and nitric oxide may play a causal role in the hemodynamic and histopathological changes of diabetic retinopathy. This study evaluated the therapeutic potential of long-term selective blockade of the ET-1_A receptor (ETRA) to prevent the development of retinopathy in a genetic mouse model of nonobese type 1 diabetes (NOD). Mice with NOD that received subcutaneous implantation of insulin pellets and wild-type control mice were treated for 4 months with the selective ETRA antagonist LU208075 (30mg/kg/day) via drinking water. At the end of the study, blood glucose levels were evaluated, and animals were anesthetized and perfused intracardially with FITC-labeled dextran. Retinas were removed and either fixed in formalin for confocal microscope evaluation of retinal vascular filling or transferred to RNALater for quantitative reverse transcriptase–polymerase chain reaction to evaluate expression of NOS-3, NOS-1, ET-1, ETRA, ETRB, and the angiogenic factor adrenomedullin. Compared with wild-type controls, expression of ET-1, ETRA, ETRB, and adrenomedullin in mice with NOD were markedly upregulated in the retinas of nontreated mice (cycle time values relative to GAPDH [ΔCt], 14.8 vs. 13.7, 18.57 vs. 17.5, 10.76 vs. 9.9, and 11.7 vs. 9.1, respectively). Mean integral fluorescence intensity (MIFI) of retinal vascular filling was reduced from normal values of 24 to 12.5 in nontreated animals. LU208075 treatment normalized the upregulated expression of ET-1 and adrenomedullin, as well as the deficit in MIFI, but did not affect the increased ETRA and ETRB expression or the elevated plasma glucose levels found in nontreated animals. NOS isoform expression was essentially unchanged. ETRA antagonists may provide a novel therapeutic strategy to slow or prevent progression of retinal microvascular damage and proliferation in patients for whom there is clear evidence of activation of the ET-1 system. Exp Biol Med 231:1101–1105, 2006

Key words: endothelin-1; diabetes; retinopathy; adrenomedullin

Introduction

Diabetic retinopathy is a complex phenomenon characterized by increased microvascular permeability and damage, abnormal blood flow autoregulation, disordered angiogenesis, and increased adhesive properties of the endothelium (1–5). This can lead to capillary occlusion (6), microvascular degeneration, abnormal neovascularization, and eventually irreversible blindness. Diverse mutually interactive pathogenic mediators are simultaneously activated. Among these, endothelin-1 (ET-1), angiotensin II (AII), vascular endothelial growth factor (VEGF), adrenomedullin (ADM), prostaglandins (PG), and nitric oxide (NO) are thought to play a particularly important role (7–13). In the eye, blood flow autoregulation occurs through nonnervous mechanisms intrinsic to the retina that involve contractile pericytes possessing ET-1 receptors. ET-1 causes vasoconstriction of retinal microvessels and is a mitogenic stimulus for retinal pericytes. In contrast, NO and ADM may normally function to counterbalance effects of vasoconstrictor peptides but under pathological conditions could also contribute to disordered angiogenesis. These data suggest that imbalances in ET-1 and vasodilator/angiogenic mediators may play an important role in contributing to the hemodynamic and histopathological abnormalities associated with diabetic retinopathy.

The aims of the present study were to evaluate the role that dysregulation of vasoconstrictor and vasodilatory mediators, particularly ET-1, NO, and ADM, plays in retinal damage and to examine the potential of long-term selective blockade of ETRA to attenuate or prevent these pathological changes.

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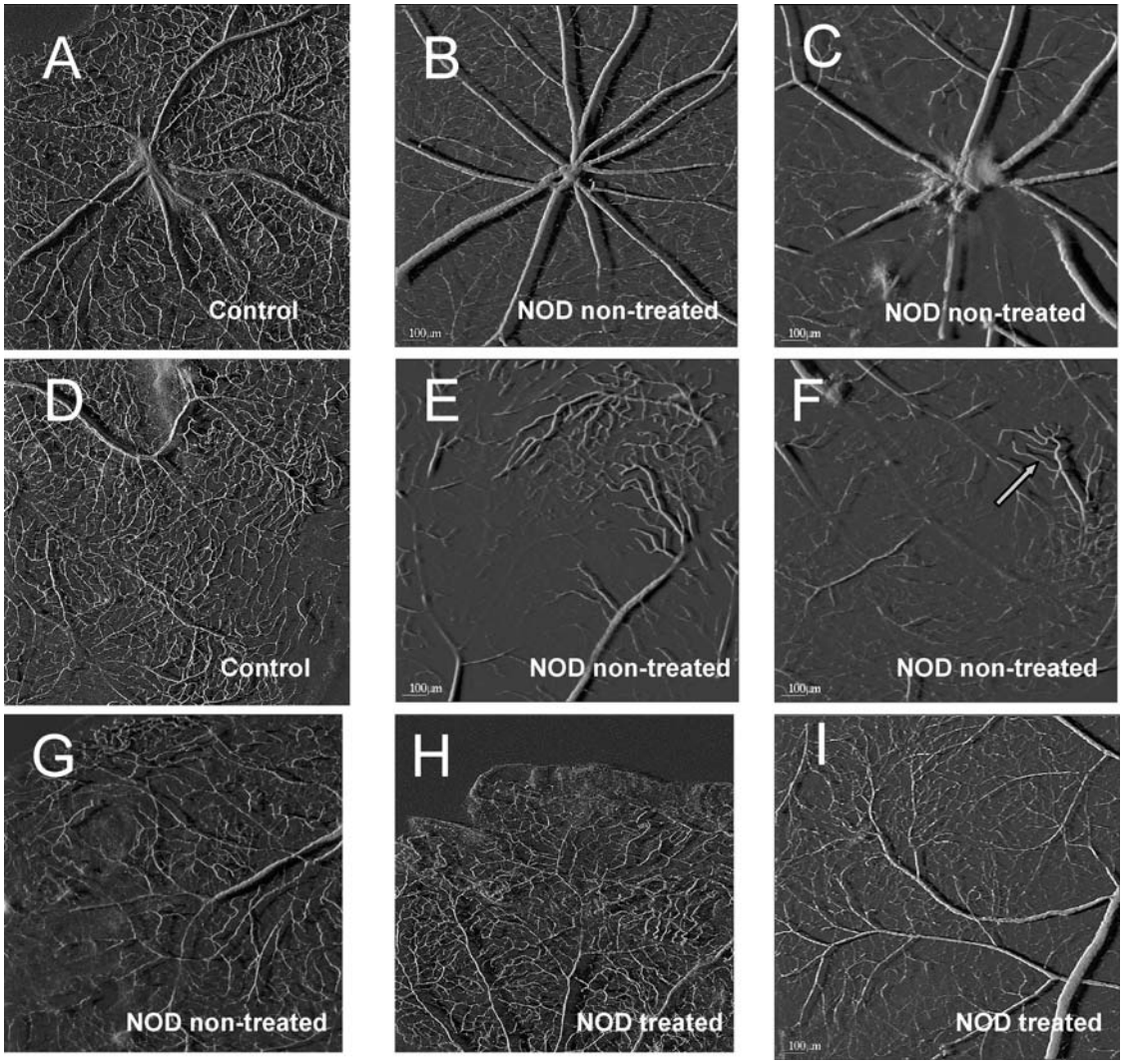

Figure 1. Composite fluorescent confocal microscope images showing the extent of vascular filling in control (A and D) and NOD retinas. Note the poorer definition of microvessels in the nontreated NOD retinas (B and G). In central regions of the retina where the main arterial system enters the eye, there is also evidence of vasoconstriction or degeneration of more major vessels (B and C). Other areas show vessel denudation with evidence of disordered focal proliferation of new vessels (E). In some cases, abnormal focal vascular proliferation appears to protrude from the retinal surface into the chamber of the eye (F, arrow). In contrast, treatment of NOD animals for 4 months with the selective ETRA antagonist LU 208075 prevented the microvascular abnormalities and uneven perfusion of the retinal microvessels, which now appear to be similar to those of controls (H and I vs. A and D).

Materials and Methods

The genetic nonobese diabetic (NOD) mouse, an insulin-dependent model closely analogous to human type-I diabetes was used. Experimental subjects consisted of the following four groups, each of which included six animals: two groups of female, 8-week-old NOD mice and two groups of age-matched wild-type controls. One NOD group and one control group received no treatment, whereas the other NOD and control groups received the selective endothelin receptor A (ETRA) antagonist LU 208075 (Knoll, Ludwigshaven, Germany) via drinking water for 4 months. NOD mice were implanted with slow-release insulin pellets to control the otherwise severe hyperglycemia. Plasma glucose levels were monitored every 3 days, and animals on average required another pellet implantation every 7–10 days. During this period, plasma glucose levels in NOD mice varied between 120 and 250 mg/dl, compared with normal levels of approximately 100 mg/dl in control mice. Separate groups of animals were used to evaluate vascular integrity and molecular biology characteristics. After the treatment period, animals were anesthetized with a triple anesthesia mix (Dormisol, Climasol, Fentanyl [Graeub AG, Bern, Switzerland]) and perfused through the heart with high–molecular weight FITC-dextran (Sigma; average molecular weight, 2,000 kDa) in saline. Eyes were removed and fixed in formol-buffered saline, and retinas were whole mounted in glycerol for fluorescence confocal microscopy or were transferred to RNAlater for quantitative reverse tran-

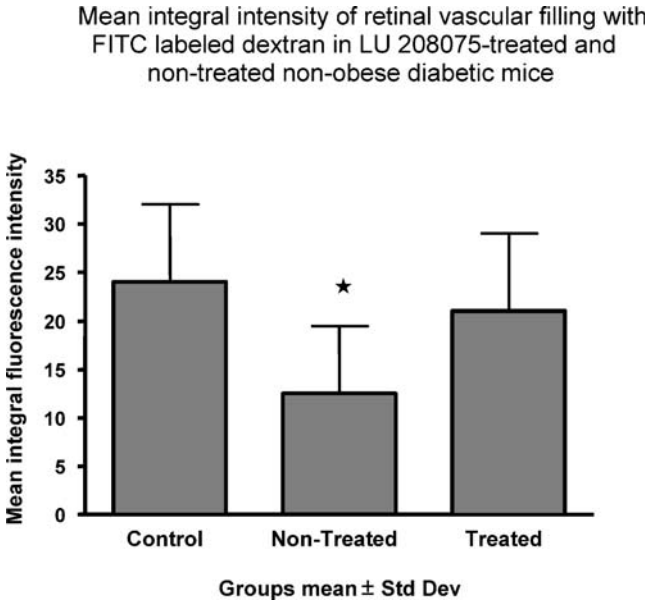

Figure 2. Quantification of the extent of vascular perfusion in retinas from control, NOD, and NOD animals treated with LU 208075. The approximate 50% reduction in vascular filling is almost completely prevented by ETRA blockade ($n = 6$). *$P < 0.001$.

scriptase–polymerase chain reaction (RT-PCR) with Taq-Man technology. For confocal assessment of retinal vascular filling, a series of sequential photographs were taken at different depths throughout the thickness of the retina (approximately 600 μm). A total of 30–36 separate images for a given area were obtained, which were then integrated using machine software into a single 3-D–like image. Quantification was performed using the mean integral intensity of the fluorescence signal of all the photographic planes.

Results

Compared with retinas from wild-type controls (Fig. 1A and D), there was clear evidence of regional microvascular degeneration and/or lack of perfusion in retinas from nontreated NOD mice (Fig. 1B and G). In some animals, even central major vessels were affected (Fig. 1C), whereas in more peripheral regions, this was accompanied by focal areas of disorganized vascular proliferation (Fig. 1E), with some vessels appearing to protrude from the retinal surface into the lumen of the eye (Fig. 1F). In contrast, NOD-treated animals showed near normal vascularization and vascular filling with no evidence of abnormal vascular proliferation (Fig. 1H and I). Quantitatively, vascular filling was reduced by approximately 50% in nontreated NOD animals, compared with wild-type controls. This was prevented by long-term ETRA blockade (Fig. 2).

Quantitative RT-PCR showed a marked increase (2–4-fold, on average) in expression of ET-1, ETRA, and ETRB (Fig. 3). This was accompanied by a similar marked increase of 4–5-fold in the expression of ADM (Fig. 4). Increased expression of ET-1 and ADM, but not of ETRA or ETRB, was prevented by long-term blockade of ETRA. Apart from a tendency for NOS3 expression to increase in NOD-treated animals after long-term receipt of LU 208075, no other changes were observed in NOS1 or NOS3 expression in any of the other groups (data not shown). Fasting plasma glucose levels after long-term treatment did not differ between the treated and nontreated groups (Fig. 4).

Discussion

The data show that retinopathy in the NOD mouse is associated with upregulation of ET-1 mRNA, ETRA mRNA, ETRB mRNA, and marked increases in expression of the multifunctional vasorelaxant angiogenic peptide ADM. Histologically, there was a loss of retinal microvessels and/or reduced perfusion of the retina, resulting in hypoxia with evidence of disordered focal proliferation of new vessels. These changes were prevented by long-term administration of the ETRA antagonist LU 208075 in association with an attenuation of the overexpression of ADM and ET-1. Treatment was effective after onset of diabetes and occurred independently of differential changes in the degree of hyperglycemia in treated and nontreated animals.

The involvement of ADM in the retinopathy process is a relatively new concept and as such its function under these conditions still remains unclear. ADM levels have recently been shown to be increased in the vitreous humor of diabetic patients with retinopathy, which suggests that the associated increases observed in the present study are not simply a phenomenon of this particular model (14, 15). Normally, ADM is primarily considered to have beneficial effects on vascular function, with relaxatory, antioxidative, and antiproliferative effects on vascular smooth muscle and endothelium (16). As such, it could be argued that the increased expression seen in nontreated NOD animals may represent a counterregulatory response to enhanced activation of the ET-1 system in an attempt to reset the imbalance of vasoconstrictor and vasodilatory factors.

It remains unclear, therefore, why, despite elevated levels of ADM expression, retinas from untreated NOD animals still show extensive damage and why the beneficial response to ETRA blockade is accompanied by a decrease, rather than an increase, in ADM transcription. Recent new aspects of ADM regulation and pharmacologic data may shed some light on these apparent anomalies. ADM transcription is under the control of hypoxia-inducible-factor (HIF), the level of which may be increased under conditions of retinal underperfusion (17, 18). In the face of marked activation of the ET-1 system, elevated ADM levels may be insufficient to overcome the prolonged potent local vasoconstricting effect of ET-1. Under these conditions, additional properties of ADM, including its effects on angiogenesis, may play a detrimental role in contributing to the abnormal focal vascular proliferation (19, 20). In addition, an associated phenomenon may also be relevant.

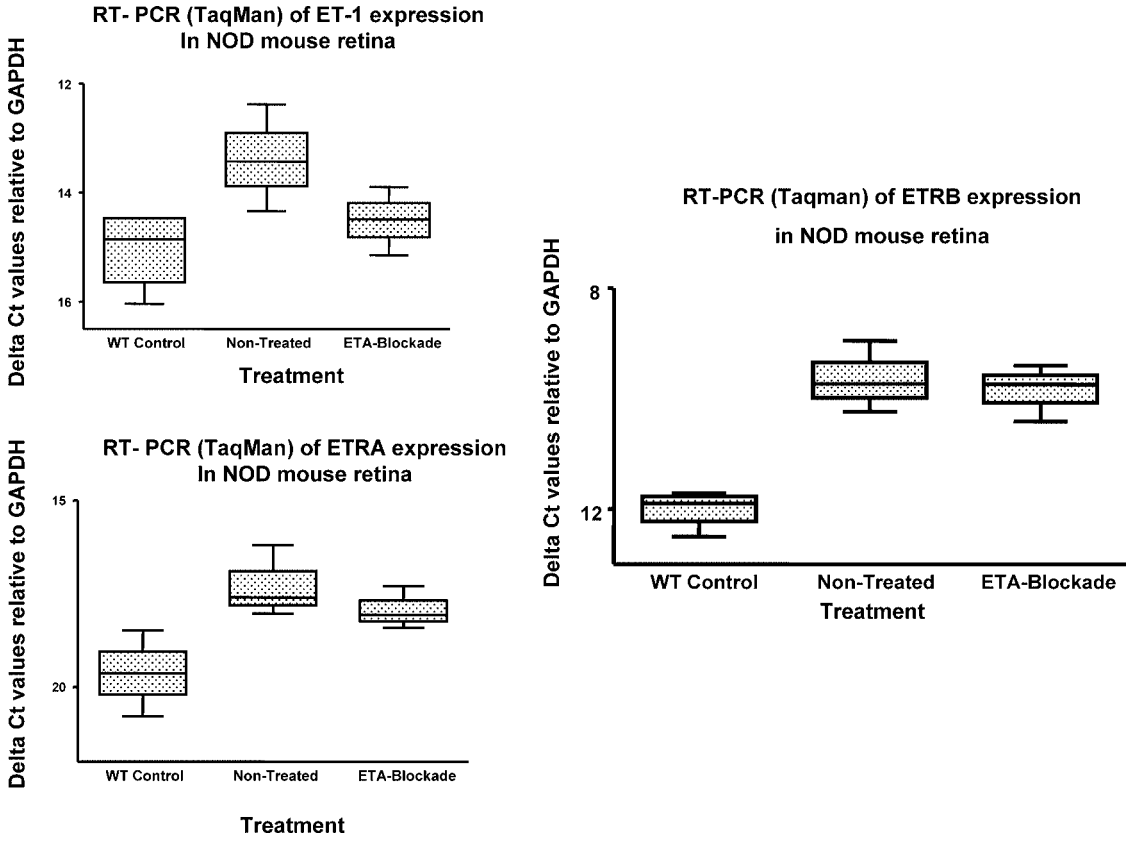

Figure 3. Real-time PCR of retinal ET-1, ETRA, and ETRB expression. A decrease of one cycle represents a doubling of target mRNA. The upregulation of ET-1 seen in nontreated NOD animals was attenuated by ETRA blockade. In contrast, the marked upregulation of ETRA and ETRB expression was unaffected by ETRA blockade.

A marked upregulation and increased activity of matrix metalloprotease-2 (MMP2) has been shown to occur in human retinopathy (21). Recent studies show that, in the presence of MMP2, ADM can be converted to an 11–21-peptide fragment, which acts as a potent vasoconstrictor that may contribute to the vasoconstriction leading to a vicious circle that promotes further vascular underperfusion (22).

Blockade of ETRA by reducing retinal ischemia and decreasing HIF-mediated ADM formation and concomitant MMP2 activation may explain some of the apparent

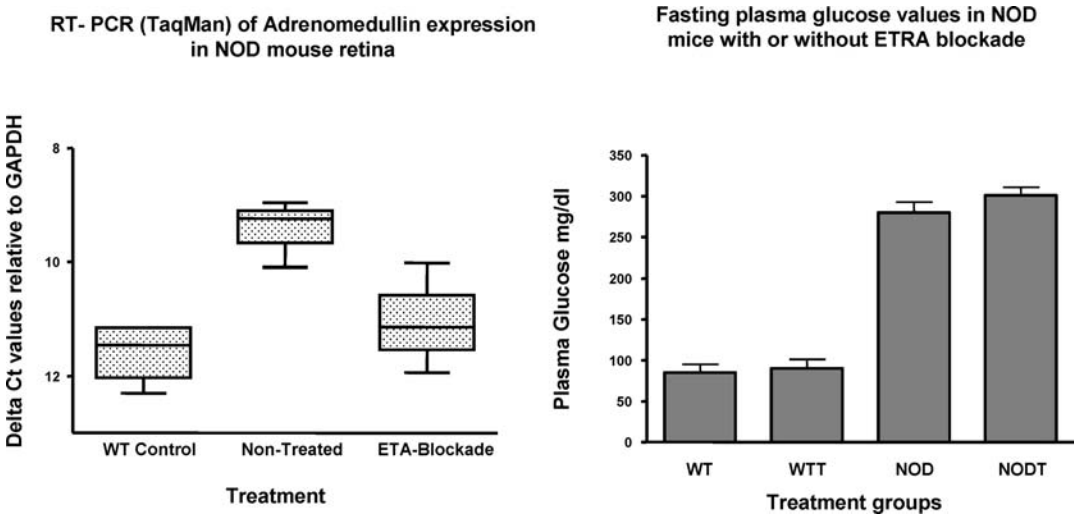

Figure 4. Real-time PCR of retinal expression of the vasoactive and angiogenic peptide adrenomedullin showing a 4–5-fold increase that was attenuated by ETRA blockade. There were no differences in fasting plasma glucose values in wild-type (WT), NOD-treated (T), and nontreated animals (NOD) between groups.

anomalies in the experimental findings. Although these suggestions remain speculative at present, further studies are clearly needed to clarify the role of the ADM system under these conditions. Taken together, these data suggest that upregulation of retinal ADM may represent a newly associated factor influencing the course of diabetes-related hemodynamic and proliferative retinal vascular disease. Furthermore, long-term treatment with selective ETRA antagonists may provide a novel therapeutic strategy to slow or prevent the progression of retinal microvascular damage and proliferation in patients for whom there is clear evidence of activation of the ET-1 system.

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