Retinal Expression of Vascular Endothelial Growth Factor Is Mediated by Angiotensin Type 1 and Type 2 Receptors

Xiaoli Zhang, Markus Lassila, Mark E. Cooper, Zemin Cao

Abstract—Angiotensin II is a known stimulus for the expression of vascular endothelial growth factor (VEGF). This action of angiotensin II is mediated by the angiotensin type 1 (AT1) receptor. However, the role of the angiotensin type 2 (AT2) receptor subtype in inducing VEGF expression has been controversial. The aim of the present study was to assess the effects of AT2 receptor blockade on VEGF expression in the retina, initially in experimental diabetic rats induced by injection of streptozotocin. The AT1 receptor antagonist, valsartan, or the AT2 receptor antagonists, PD123319, were administered to diabetic rats for 4 weeks. Increased gene and protein expressions of VEGF, as assessed by real-time reverse transcription-polymerase chain reaction and immunostaining, respectively, were observed in the retina in diabetic rats. Treatment with either valsartan or PD123319 attenuated retinal VEGF expression. To further explore the link between angiotensin receptor subtypes and VEGF expression, valsartan, or PD123319 were administered to rats that were infused with angiotensin II for 2 weeks. VEGF expression was also increased in the retina from angiotensin II infused rats, and this was attenuated by valsartan and PD123319. These findings suggest that VEGF expression is modulated by AT1 and AT2 receptors, thereby implicating angiotensin II receptor subtypes in retinal diseases such as diabetic retinopathy. (Hypertension. 2004;43:276-281.)

Key Words: angiotensin receptors • vascular endothelial growth factor • diabetes

Vascular endothelial growth factor (VEGF) is a potent growth factor with multiple cellular actions. Increased VEGF expression is found in the retina in experimental diabetes,1 and inhibition of VEGF is associated with suppression of retinal neovascularization.2 Therefore, VEGF is implicated in the pathogenesis of diabetic retinopathy.1,3,4 Although hypoxia is considered the major stimulus for VEGF expression in a site such as the retina, angiotensin II, the major effector molecule of the renin–angiotensin system (RAS), is also a known stimulus for VEGF expression.5 Inhibition of the production of angiotensin II with angiotensin-converting enzyme (ACE) inhibition is associated with suppression of VEGF expression in experimental diabetes.6 All components of the RAS, including the angiotensin receptor subtypes, the type 1 (AT1) and type 2 (AT2) receptors, have been demonstrated to be present in the retina.7–11 Previous studies have shown that the increase in VEGF expression by angiotensin II stimulation was mediated by the AT1 receptor.12 However, the role of the AT2 receptor in mediating VEGF expression remains controversial. The findings from an in vitro study in cultured bovine pericytes suggested no role for this receptor in VEGF induction.5 However, administration of the AT2 receptor antagonist was associated with not only less vascular proliferation but also reduced retinal VEGF expression in a rat model of retinopathy of prematurity.13 Therefore, the aim of the present study was to assess the effect of AT2 receptor antagonism on VEGF expression in two different models, experimental diabetes and angiotensin II infusion.

Methods

Protocol 1: Experimental Diabetes

Eight-week-old male Sprague-Dawley (SD) rats (body weight 230 to 280 g) were purchased from the Animal Resource Centre, Perth, Australia. SD rats were injected intravenously with streptozotocin at a dose of 55 mg/kg in citrate buffer (pH 4.5) to induce diabetes after an overnight fast (n=24). Control rats received an intravenous injection of citrate buffer alone (n=8). Diabetic rats were further randomly allocated to no treatment or treatment with either an AT1 receptor antagonist, valsartan (Novartis Pharma AG, Basel, Switzerland), or an AT2 receptor antagonist (n=8 per group). The AT1 receptor antagonist, valsartan (Novartis Pharma AG, Basel, Switzerland), was administered at a dose of 30 mg/kg per day by once-daily oral gavage. The AT2 receptor antagonist, PD123319 (Parke-Davis, Ann Arbor, Mich), was administered at a dose of 5 mg/kg per day delivered by subcutaneous osmotic minipump (Alzet model 2004, Alza Corporation, Palo Alto, Calif). For insertion of minipumps, animals were anesthetized with ethrane (Abbott Australasia, Kurnal, Australia) and the skin at the mid scapular region was cut open. The minipumps were then implanted subcutaneously into the mid scapular region.14 Drug therapy was started within 24 hours after induction of diabetes and lasted for a period of 4 weeks. The doses of valsartan and PD123319 were chosen based on our previous studies that demonstrated blockade of AT1 receptor binding by valsartan and inhibition of AT2 receptor binding by PD123319 at the

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Hypertension is available at http://www.hypertensionaha.org

Received October 14, 2003; first decision November 11, 2003; revision accepted December 9, 2003.
From The JDRF Danielle Alberti Memorial Centre for Diabetes Complications, Baker Heart Research Institute, Melbourne, Australia.
Correspondence to Dr Zemin Cao, The JDRF Danielle Alberti Memorial Centre for Diabetes Complications, Baker Medical Research Institute, P.O. Box 6492, St Kilda Road, Central Melbourne 8008, Victoria, Australia. E-mail zemin.cao@baker.edu.au

DOI: 10.1161/01.HYP.0000113628.94574.0f
aforementioned doses. At the doses described, neither inhibition of valsartan on AT2 receptor binding nor inhibition of AT1 receptor binding by PD123319 was detected using in vitro autoradiographic techniques.

Protocol 2: Angiotensin II Infusion

Eight-week-old male SD rats were anesthetized with enflurane, then an Alzet osmotic minipump (Model 2002) filled with vehicle (0.15 mol/L NaCl, 1 mmol/L acetic acid, n=8) or human angiotensin II at a concentration of 7 mg/mL was subcutaneously implanted in the mid scapular region as described. Based on the pumping rate of the minipumps (0.5 μL/h), angiotensin II was administered at a dose of 58.3 ng/min. Rats infused with angiotensin II were randomly allocated to angiotensin II infusion alone or treatment with either valsartan or PD123319 (n=8 per group). Administration of valsartan and PD123319 is described above. Separate Alzet minipumps were infused with valsartan or PD123319 (n/H11005 58.3 ng/min. Rats infused with angiotensin II were randomly assigned a value of 1.

The protocols for animal experimentation and the handling of animals were approved by the animal ethic committee of our institute and were in accordance with the principles established by the National Health and Medical Research Council of Australia.

Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed conscious animals. Blood samples were collected from the tail veins of conscious diabetic rats before the animals were euthanized for measurement of glycated hemoglobin (HbA1c). HbA1c was measured by a high-performance liquid chromatography method (Biorad, Richmond, Calif). The animals were euthanized by intravenous injection of pentobarbitone sodium at a dose of 60 mg/kg body weight (Boehringer Ingelheim, Artarmon, Australia). One eye from each rat was removed and fixed in 10% formalin and then processed to paraffin for subsequent immunostaining with VEGF. Another eye was enucleated immediately and the retina isolated by blunt dissection using a dissecting microscope. Retinas were then frozen in liquid nitrogen and stored at −80°C for subsequent reverse transcription-polymerase chain reaction (RT-PCR) studies.

RT-PCR

Three micrograms of total RNA extracted from each retina was used to synthesize cDNA with the Superscript First Strand synthesis system for RT-PCR (Life Technologies BRL, Grand Island, NY). VEGF gene expression was analyzed as real-time RT-PCR performed with the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700; Perkin-Elmer, PE Biosystems, Foster City, Calif). To control for variation in the amount of DNA available for PCR in the different samples, gene expression of the target sequence was normalized in relation to the expression of an endogenous control, 18S ribosomal RNA (rRNA) (18S rRNA TaqMan Control Reagent kit; ABI Prism 7700). Primers and TaqMan probe for VEGF and the endogenous reference 18S rRNA were constructed with the help of Primer Express (ABI Prism 7700). For amplification of the VEGF cDNA, the forward primer was 5′-GGGAGGCTGCTGCAATG3′, and the reverse primer was 5′-TGAAGGAGCAGTCTGTGT-3′. The probe specific for VEGF was 5′-FAM-5′-TGGCCACGTCCGAGAAGACGT-3′-TAMRA. The amplification was performed with the following time course: 50°C for 2 minutes and 95°C for 10 minutes and 50 cycles of 94°C for 20 s and 60°C for 1 minute. Each sample was tested in triplicate. Results were expressed relative to control retina values, which were arbitrarily assigned a value of 1.

Immunohistochemistry

Four-micron-thick sections were cut, dehydrated, and subsequently used for immunohistochemistry. In brief, after dewaxing, sections were treated in a microwave oven at low power for 10 minutes in 10 mmol/L sodium citrate buffer (pH 6.0). Endogenous peroxidase was inactivated using 3% hydrogen peroxide in methanol for 20 minutes. The sections were then incubated in protein-blocking agent

TABLE 1. Metabolic and Hemodynamic Parameters in Protocol 1

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>BW, g</th>
<th>SBP, mm Hg</th>
<th>HbA1C, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>365±13</td>
<td>128±5</td>
<td>3.8±0.2</td>
</tr>
<tr>
<td>Diabetic</td>
<td>8</td>
<td>281±6*</td>
<td>145±5*</td>
<td>12.8±0.4*</td>
</tr>
<tr>
<td>Diabetic+ valsartan</td>
<td>8</td>
<td>282±7*</td>
<td>130±7†</td>
<td>12.9±0.5*</td>
</tr>
<tr>
<td>Diabetic+PD123319</td>
<td>8</td>
<td>276±7†</td>
<td>140±3</td>
<td>14.3±0.4*</td>
</tr>
</tbody>
</table>

The data are shown as mean ± SEM. BW indicates body weight; SBP, systolic blood pressure; HbA1C, glycated hemoglobin.

*P<0.05 versus control. †P<0.05 vs diabetic.

Figure 1. VEGF gene and protein expression in protocol 1. VEGF mRNA levels (A) were expressed as relative to control retina, which were arbitrarily assigned a value of 1. The VEGF immunostaining (B) was assessed using the following grade scales: 0, not detected; 1, weak staining; 2, moderate staining; 3, strong staining; 4, very strong staining. They were graded under a light microscope at a magnification of ×200. This analysis was performed in a masked and randomized fashion on for 30 minutes followed by incubation with a monoclonal mouse antibody to VEGF (Santa Cruz Biotechnology, Santa Cruz, Calif) overnight at 4°C. Biotinylated horse anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, Calif) was used as the secondary antibody. Sections were then incubated with horseradish peroxidase-conjugated streptavidin. Peroxidase conjugates were localized by 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, Mo) as a chromogen. Sections were counterstained with hematoxylin.

Retinal VEGF immunostaining from both protocols was performed in the same run and then was quantitated using the Imaging Analysis System (AIS; Imaging Research, St. Catherines, Canada) attached to a video camera and computer. Each slide was graded using the following scale: 0, not detected; 1, weak staining; 2, moderate staining; 3, strong staining; 4, very strong staining. They were graded under a light microscope at a magnification of ×200. This analysis was performed in a masked and randomized fashion on
3 retinal sections from each animal and the mean value was determined.

Statistics
Data were analyzed by ANOVA using Statview SE (Brainpower, Calabasas, Calif) on a Macintosh iMac Computer (Cupertino, Calif). Comparisons of group means were performed by Fisher least significant difference method. Data are shown as mean ± SEM. A P<0.05 was viewed as statistically significant.

Results

Protocol 1: Experimental Diabetes

Metabolic and Hemodynamic Parameters
Diabetic rats had reduced body weight and increased HbA1c levels compared with control rats (Table 1). Treatment with valsartan or PD123319 did not significantly attenuate these metabolic parameters. Systolic blood pressure (SBP) in diabetes was mildly increased when compared with control rats (Table 1). Treatment with valsartan, but not PD123319, was associated with reduced systolic blood pressure.

VEGF Gene Expression
VEGF gene expression was increased approximately 3-fold in the retina of diabetic rats when compared with control animals (Figure 1A). Treatment with either valsartan or PD123319 attenuated VEGF gene expression to a similar level to that observed in the control rats (Figure 1A).

VEGF Immunostaining
VEGF protein expression was detected in the cornea, iris, retina, the choroid–retinal pigment epithelial complex, ciliary body, the walls of blood vessels, and ocular muscles (data not shown). In the retina of control rats, VEGF staining was predominantly in the inner limiting membrane, ganglion cell layer, and inner nuclear layer (Figure 2A). Weak positive staining for VEGF was also found in the outer limiting membrane and retinal pigment epithelium (Figure 2A). Retinal VEGF protein expression was increased significantly in diabetic rats when compared with control animals (Figure 1B and 2B). VEGF immunostaining was localized to the same intraretinal sites in diabetic rats as in control animals (Figure 1B and 2B). The increased VEGF immunostaining in the
retina of diabetic rats was attenuated by treatment with either valsartan or PD123319. There was no significant difference in VEGF immunostaining in the retina of diabetic animals treated with either valsartan or PD123319. The levels were similar to that seen in retinae from control rats (Figure 1B, 2C and D).

Protocol 2: Angiotensin II Infusion

**Metabolic and Hemodynamic Parameters**
The body weight of rats receiving angiotensin II infusion was less than in the rats treated with vehicle at the end of the experiment (Table 2). This effect of angiotensin II on body weight was prevented by co-administration of valsartan II but not PD123319 (Table 2). Angiotensin II infusion was associated with a significant elevation of SBP compared with control rats (Table 2). Treatment with valsartan was associated with prevention of the increase in SBP induced by angiotensin II infusion. By contrast, the AT2 receptor antagonist, PD123319, did not significantly influence SBP in angiotensin II-infused rats.

**VEGF Gene Expression**
VEGF gene expression in the retina in angiotensin II-infused rats was increased approximately 2-fold when compared with control rats (Figure 3A). Treatment with either valsartan or PD123319 was associated with reduced VEGF gene expression. VEGF gene expression in animals treated with either valsartan or PD123319 was similar to that seen in control rats (Figure 3A).

**VEGF Immunostaining**
Retinal VEGF immunostaining was increased in angiotensin II-infused animals when compared with control animals. Both valsartan and PD123319 treatment were associated with attenuated VEGF immunostaining (Figure 3B and Figure 4). No changes in distribution of VEGF immunostaining was observed in any of the treatment groups with VEGF detected specifically in the inner limiting membrane, the ganglion cell layer, and the inner nuclear layer (Figure 4).

**Discussion**
The present study has demonstrated that administration of an AT2 receptor antagonist is associated with attenuation of VEGF expression in the retina of experimental diabetes. To further confirm that the AT2 receptor may play a role in retinal VEGF expression, an AT2 receptor antagonist was administered to angiotensin II-infused rats. Increased VEGF expression was also documented in the retina of angiotensin II-infused animals. The AT2 receptor antagonist abrogated the increased in VEGF expression in response to angiotensin II infusion. The effects of the AT2 receptor antagonist on VEGF expression in the retina observed in the present studies are similar to those seen with an AT1 receptor antagonist, valsartan. These findings suggest that AT1 and AT2 receptors modulate retinal expression of VEGF.

Previous studies have described VEGF gene expression in the retina. In the present study, we have localized VEGF protein expression to the ganglion cell layer, Müller cells, the inner nuclear layer, and the retinal pigment epithelium. Increased gene and protein expression of VEGF was observed in the retinas of diabetic animals. These findings are consistent with previous reports, albeit using different approaches to assess expression of this growth factor. VEGF is a major regulator of endothelial cell proliferation, angiogenesis, and vascular permeability. Increased VEGF production in ocular tissues has been observed at an early stage of diabetes, before the appearance of detectable retinal changes in diabetic animals.

There is now considerable evidence for a direct relationship between the RAS and VEGF. All components of the RAS including angiotensinogen, renin, ACE, angiotensin II, and AT1 and AT2 receptors have been demonstrated to be present in ocular tissues of normal rabbit eyes. In cultured bovine retinal pericytes, angiotensin II induced a significant increase in VEGF mRNA levels in a time- and dose-dependent manner. In the present study, we demonstrated increased gene and protein expression of VEGF in the retina after long-term angiotensin II infusion. In addition, our

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**TABLE 2. Metabolic and Hemodynamic Parameters in Protocol 2**

<table>
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<th>SBP, mm Hg</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>8</td>
<td>340±6</td>
<td>124±3</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>8</td>
<td>267±8*</td>
<td>186±5*</td>
</tr>
<tr>
<td>Angiotensin II + valsartan</td>
<td>8</td>
<td>312±8†</td>
<td>130±3†</td>
</tr>
<tr>
<td>Angiotensin II + PD123319</td>
<td>8</td>
<td>264±8*</td>
<td>204±3*</td>
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</tbody>
</table>

The data are shown as mean ± SEM. *P<0.05 vs control or vehicle.
†P<0.05 vs angiotensin II.
Previous studies have demonstrated that ACE inhibition reduces retinal VEGF expression. Furthermore, angiotensin II had a growth-promoting effect on cultured endothelial cells, and this effect was inhibited by a VEGF neutralizing antibody. These findings suggest that angiotensin II stimulates VEGF expression, and this may be implicated in the pathogenesis of retinal disease.

In the present study, the AT1 receptor antagonist, valsartan, reduced retinal VEGF expression in diabetic and angiotensin II-infused rats. These findings are consistent with the previous reports in which AT1 receptor antagonists significantly diminished retinal VEGF gene expression. These findings followed initial in vitro studies in bovine retinal pericytes, which demonstrated increased VEGF expression in response to angiotensin II via the AT1 receptor subtype. The present study extends these findings by confirming that these changes in retinal VEGF gene expression ultimately translate to effects on VEGF protein expression.

The present study demonstrated that the AT2 receptor antagonist reduces retinal VEGF expression in diabetic and angiotensin II-infused animals. This is consistent with the effects of the AT2 receptor antagonist on retinal VEGF expression in the model of retinopathy of prematurity. In that study, increased retinal VEGF expression and angiogenesis were observed in the rat model of retinopathy of prematurity. Administration of the AT2 receptor antagonist was associated with reduced VEGF expression and retinal angiogenesis. These findings from in vivo studies are opposite to a previous in vitro study in cultured bovine pericytes that showed no effects of AT2 receptor blockade on VEGF expression in response to angiotensin II stimulation. One must be cautious in extrapolating these in vitro findings in bovine pericytes to the in vivo context. Indeed, the status of the AT2 receptor in cultured bovine pericytes is unclear and needs to be clarified. Expression of the AT2 receptor in the rat retina has been demonstrated using autoradiography.

Figure 4. VEGF staining in vehicle infusion (A), angiotensin II infusion (B), angiotensin II infusion with valsartan (C), or PD123319 (D). Sections were counterstained with hematoxylin. Original ×200. ILM indicates inner limiting membrane; GNL, ganglion cell layer; INL, inner nuclear layer; OLM, outer limiting membrane; RPE, retinal pigment epithelium.

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and RT-PCR techniques. Furthermore, using immunohistochemical techniques, the AT2 receptor has been localized to blood vessels, the inner limiting membrane, and in the inner nuclear layer in rat retina.

It has previously been considered that the AT1 and AT2 receptors may exert opposite effects in terms of cellular proliferation, apoptosis, and blood pressure regulation, and that only the AT1 receptor subtype is important in mediating the actions of angiotensin II. However, there is increasing evidence to suggest that the AT2 receptor plays an important role in modulating cellular proliferation and matrix protein accumulation in the kidney, as well as in mediating antiproliferative effects in the mesenteric arterial tree. Our findings show that the AT2 receptor antagonist PD123319 reduces the retinal expression of VEGF in diabetic animals, similar to effects seen with the AT1 receptor antagonist, valsartan. These findings highlight the importance of angiotensin receptor subtypes in retinal angiogenesis and suggest that blockade of the AT2 receptor as well as the AT1 receptor may confer end organ protection in various retinal diseases.

Acknowledgments

This study was supported by the Juvenile Diabetes Research Foundation (JDRF) and the National Health and Medical Research Council of Australia (NH&MRC). Dr Xiaoli Zhang was supported by the Chinese Scholarship Council. Dr Markus Lassila was supported by grants from the Finnish Academy, Einar and Karin Stroem’s Foundation, The Helsingin Sanomat Centennial Foundation, and Paavo Nurmi Foundation. Dr Zemin Cao is a recipient of an advanced postdoctoral fellowship from the JDRF. We thank Novartis Pharma AG for providing valsartan and Parke-Davis for provision of PD123319.

References


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Hypertension. 2004;43:276-281; originally published online January 12, 2004;
doi: 10.1161/01.HYP.0000113628.94574.0f
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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