Dual Effect of Angiotensin-Converting Enzyme Inhibition on Angiogenesis in Type 1 Diabetic Mice

Téni G. Ebrahimian, Radia Tamarat, Michel Clergue, Micheline Duriez, Bernard I. Levy, Jean-Sébastien Silvestre

Objective—We analyzed the beneficial therapeutic effect of angiotensin converting enzyme inhibitor (ACEI) on both retinal and hind limb neovascularization in diabetic mice.

Methods and Results—Diabetic mice (streptozotocin, 40 mg/kg) were treated with or without ACEI (Perindopril, 3 mg/kg per day) or AT1 receptor blocker (Candesartan, 20 mg/kg) for 4 months. Hind limb ischemia was then induced by right femoral artery ligation for 1 additional month. In the ischemic leg, angiographic score, capillary density, and foot perfusion were increased by 2.7, 2.0-fold, and 1.6-fold, respectively, in ACEI-treated diabetic mice compared with untreated diabetic animals (P<0.01). ACEI also raised vascular endothelial growth factor (VEGF) protein level by 1.4-fold in ischemic diabetic leg. This ACEI pro-angiogenic effect was totally blunted in diabetic bradykinin B2 receptor-deficient animals, suggesting that it was mediated by the bradykinin pathway. In the diabetic retina, angiotensinogen and ACE mRNA levels were increased by 2.8-fold and 4.1-fold, respectively (P<0.01 versus nondiabetic mice), highlighting a local activation of renin-angiotensin system. Diabetes also raised VEGF protein level by 1.5-fold (P<0.05 versus nondiabetic mice). Treatments with ACEI and AT1 receptor blocker hampered diabetes-induced VEGF upregulation and retinal neovascularization.

Conclusion—ACE inhibition improved neovascularization in the diabetic ischemic leg through activation of bradykinin signaling, whereas it reduced vessel growth in the diabetic retina through inhibition of overacting Ang II pathway.

Key Words: angiotensin-converting enzyme ■ diabetes ■ angiogenesis ■ ischemia ■ retina

The morbidity and mortality of diabetes are related to the development of both macrovascular and microvascular complications.1 Macrovascular diseases include accelerated atherosclerosis affecting arteries that supply the heart, brain, and limb and are characterized by poor outcomes after vascular occlusion. Hence, the impaired collateral vessel development after thrombotic vessel obstruction probably contributes to the severe course of limb ischemia in diabetic patients. Current explanations for the abrogate neovascularization in diabetic subjects have involved alterations in vascular endothelial growth factor (VEGF) signaling and in inflammation-related pathway.2,3 Alternatively, the pro-angiogenic effect of bone marrow-derived progenitor cells is affected in diabetes and may account for the reduction in the postnatal neovascularization in this setting.4–6 Increased formation of advanced glycation end products by affecting the proteolytic process has also been shown to decrease the postischemic revascularization reaction in diabetes.7 The development of angiogenic strategies designed to increase such native neovascularization process is of major therapeutic importance for the treatment of diabetes-induced macrovascular disease.

All forms of diabetes are also characterized by the development of diabetes-specific microvascular pathology in the retina, a major common cause of blindness.1 The factors that stimulate retinal blood vessel growth have not been fully defined but there is accumulating evidence that angiotensin II (Ang II), the effector peptide of the renin-angiotensin system (RAS), may be involved in a number of retinal vascular disorders, including retinopathy of prematurity and proliferative diabetic retinopathy.8–10 Besides its well-known hemodynamic action, Ang II has been shown to promote cell proliferation and blood vessel growth.11–13 Ang II acts by binding to its 2 isoform receptors, AT1 and AT2. The known pro-angiogenic actions of Ang II are thought to be mediated via the AT1 receptor, in part, through activation of VEGF-related pathway.12,13 With respect to the retina, Ang II increases VEGF and VEGF receptor type 2 expression in retinal endothelial cells, and RAS blockade attenuates VEGF upregulation in experimental model of retinopathy.10,14,15 Taken together, these studies provide a rationale for the use of agents that interrupt RAS in the treatment of retinal microvascular pathology. In this view, RAS blockade prevents...
retinal neovascularization in different models of retinopathy.\textsuperscript{9,10,15} A therapeutic role for RAS blockade has also been suggested in the EUCLID study, in which the angiotensinconverting enzyme (ACE) inhibitor lisinopril slowed the progression of proliferative diabetic retinopathy.\textsuperscript{16}

In contrast, ACE inhibition promotes ischemia-induced neovascularization in ischemic rabbit and rodent hind limbs.\textsuperscript{17–19} ACE catalyzes the conversion of angiotensin I to Ang II and the breakdown of bradykinin into inactive peptides. Hence, the pharmacological effect of ACE inhibitors may be in part mediated via inhibition of Ang II formation and also via bradykinin accumulation. We therefore hypothesized that ACE inhibition may correct both macrovascular and microvascular diseases in diabetes: (1) ACE inhibition, by activating the local bradykinin proangiogenic pathway, may improve posts ischemic neovascularization in the hind limb; and (2) ACE inhibition, by reducing local Ang II formation, may abrogate blood vessel growth in the retina. We then analyzed the beneficial therapeutic effect of ACE inhibitor on both retinal and hind limb neovascularization in diabetic mice with surgically induced hind limb ischemia.

**Materials and Methods**

**Experimental Protocol**

This study was conducted in accordance with institutional guidelines and those formulated by the European community for experimental animal use (L358-86/609ECC). To induce diabetes, mice were injected intraperitoneally with 40 mg/kg streptozotocin in 0.05 mol/L Na citrate, pH 4.5, daily for 5 days. Three days after the fifth injection, blood glucose levels were measured. If serum glucose was <9 mmol/L, mice were additionally injected twice per week at the same dosage. Glycemia was tested every week to ensure serum glucose levels >10 mmol/L. Mice with glucose levels <10 mmol/L were excluded from the study, as previously described.\textsuperscript{4,5} On first confirmation of hyperglycemia, J129Sv/B6 wild-type mice and J129Sv/B6 mice deficient for the bradykinin B\textsubscript{2} receptor gene (10-week-old: Jackson Laboratory, Bar Harbor, Me) were treated with ACE inhibitor (perindopril; Servier, France; 3 mg/kg per day in the drinking water) or AT1 receptor blocker (Candesartan; Astra, Sweden; 20 mg/kg per d). These treatments continued throughout the study. After 4 months, mice were anesthetized by isoflurane inhalation and unilateral hind limb ischemia was induced by ligation on the right femoral artery for 1 additional month.\textsuperscript{18,19} Body weight and glucose plasma level were recorded weekly throughout the study.

**Quantification of Neovascularization in the Hind Limb**

At time of euthanization, vessel density was evaluated by 3 different methods, as previously described:\textsuperscript{18,19} (1) high-definition microangiography using Barium sulfate (1 g/mL) injected in the abdominal aorta, followed by image acquisition with a digital X-ray transducer and computerized quantification of vessel density expressed as a percentage of pixels per image occupied by vessels in the quantification area; (2) assessment of capillary densities by immunostaining with a rabbit polyclonal antibody directed against total fibronectin (dilution 1/50; Chemicon International) and morphometric quantification using Histolab software (Microvisions); and (3) laser Doppler perfusion imaging to assess in vivo tissue perfusion in the legs.

**Quantification of Neovascularization in the Retina**

At time of euthanization, a contrast medium (black ink) was also injected through a catheter introduced into the carotid. Mice retina were gently dissected free, cut at the optic disk after enucleation, and those formulated by the European community for experimental animal use (L358-86/609ECC). To induce diabetes, mice were injected intraperitoneally with 40 mg/kg streptozotocin in 0.05 mol/L Na citrate, pH 4.5, daily for 5 days. Three days after the fifth injection, blood glucose levels were measured. If serum glucose was <9 mmol/L, mice were additionally injected twice per week at the same dosage. Glycemia was tested every week to ensure serum glucose levels >10 mmol/L. Mice with glucose levels <10 mmol/L were excluded from the study, as previously described.\textsuperscript{4,5} On first confirmation of hyperglycemia, J129Sv/B6 wild-type mice and J129Sv/B6 mice deficient for the bradykinin B\textsubscript{2} receptor gene (10-week-old: Jackson Laboratory, Bar Harbor, Me) were treated with ACE inhibitor (perindopril; Servier, France; 3 mg/kg per day in the drinking water) or AT1 receptor blocker (Candesartan; Astra, Sweden; 20 mg/kg per d). These treatments continued throughout the study. After 4 months, mice were anesthetized by isoflurane inhalation and unilateral hind limb ischemia was induced by ligation on the right femoral artery for 1 additional month.\textsuperscript{18,19} Body weight and glucose plasma level were recorded weekly throughout the study.

**Physiological Data**

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>Glycemia (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont</td>
<td>35.2±1.1</td>
</tr>
<tr>
<td>Cont + ACE</td>
<td>36.1±2.2</td>
</tr>
<tr>
<td>SPTZ</td>
<td>31.2±1.3*</td>
</tr>
<tr>
<td>SPTZ + ACE</td>
<td>30.3±1.2*</td>
</tr>
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</tr>
<tr>
<td>SPTZ-B2K0</td>
<td>28.9±4.4*</td>
</tr>
<tr>
<td>SPTZ + Cand</td>
<td>30.4±1.4*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. n=6 per group. P<0.05, †P<0.01 vs control group.

**Determination of VEGF Protein Expression**

VEGF protein expression was determined by Western blot in ischemic and nons ischemic legs and in the retina, as previously described.\textsuperscript{18,19}

**Statistical Analysis**

Results are expressed as mean±SEM. One-way analysis of variance ANOVA was used to compare each parameter. Post hoc Bonferroni t test comparisons were then performed to identify which group differences account for the significant overall ANOVA. P<0.05 was considered significant.

**Results**

**Physiological Data**

Body weight was reduced by 12% in treated and untreated diabetic mice when compared with control mice. There was a comparable hyperglycemia in all diabetic groups (P<0.001, when compared with controls) (Table).

**Effect of ACE Inhibition on Posts ischemic Neovascularization in Hind Limb: Microangiography**

As expected, blood vessel growth was impaired in diabetic mice compared with control animals (P<0.05) (Figure I, available online at http://atvb.ahajournals.org; Figure 1). Angiographic score showed significant improvement in ischemic/nons ischemic leg ratio of 1.7-fold in control mice receiving ACE inhibitor when compared with untreated controls (P<0.01). Similarly, ACE inhibition improved vessel density by 2.7-fold in ACE inhibitor-treated diabetic mice in reference to untreated diabetic animals (P<0.001).

**Capillary Density**

Microangiographic data were confirmed by capillary density analysis (Figure I and Figure 1). The ischemic/nons ischemic leg capillary number ratio was reduced by 1.4-fold in diabetic
mice when compared with control \((P<0.05)\). ACE inhibitor administration improved this ratio by 1.5-fold in reference to untreated animals \((P<0.01)\). Similarly, capillary number ratio was increased by 1.6-fold in ACE inhibitor-treated diabetic mice compared with untreated diabetic animals \((P<0.01)\).

**Laser Doppler Perfusion Imaging**

Microangiographic and capillary density measurements were associated with changes in blood foot perfusion (Figure I and Figure 1). The ischemic/nonischemic ratio for cutaneous blood flow recovery was impaired by 1.4-fold in diabetic mice compared with controls \((P<0.05)\) (Figure I and Figure 1). ACE inhibition enhanced by 1.5-fold cutaneous foot perfusion in reference to untreated controls \((P<0.01)\). In the same view, ACE inhibitor administration improved by 2-fold blood perfusion recovery in treated diabetic mice compared with untreated diabetic animals \((P<0.01)\).

**Molecular Mechanisms Associated With ACE Inhibition-Induced Increase in the Neovascularization Process in Ischemic Hind Limb**

The pharmacological effect of ACE inhibitors may be in part mediated via inhibition of Ang II formation but also via bradykinin accumulation.

**Involvement of RAS-Related Pathway**

We first analyzed the effect of diabetes on RAS components mRNA level in ischemic hind limb (Figure 2A). Angiotensinogen, ACE, and AT1 receptor mRNA contents were unaffected in the ischemic leg of diabetic mice compared with control animals, suggesting that RAS-related pathway was not activated in this setting. In this view, AT1 receptor blockade did not affect the impaired-neovascularization reaction in the ischemic leg of diabetic animals (Figure 1).

**Involvement of Bradykinin-Related Pathway**

We determined the effect of diabetes on kallikrein–kinin component mRNA level in ischemic hind limb (Figure 2B). Bradykinin type 1 \((B1R)\) and type 2 \((B2R)\) and kallikrein mRNA contents were unaffected in the ischemic leg of diabetic mice compared with control animals. We also analyzed the role of bradykinin signaling in ACE inhibition-induced pro-angiogenic effect. We evidenced that the effect of ACE inhibitor on the neovascularization process in the ischemic diabetic leg was fully blunted in B2R-deficient diabetic mice \((P<0.01)\) versus ACE inhibitor-treated diabetic mice; Figure 1). In addition, VEGF protein level was enhanced by 1.4-fold in ACE inhibitor-treated diabetic mice in reference to untreated diabetic animals \((P<0.001)\) and reached the level observed in treated and untreated control animals (Figure 2C). This increase was also abrogated in diabetic mice deficient in B2R. Overall, these results evidence that ACE inhibitor pro-angiogenic effect in the ischemic leg of diabetic animals was mediated, at least in part, by the bradykinin-related pathway.

**Effect of ACE Inhibition on Neovascularization in the Retina**

Angiographic score showed significant improvement in vessel density in the retina of diabetic mice in reference to control animals \((P<0.01)\) (Figure 3). ACE inhibition hampered the diabetes-induced increase in vessel density \((P<0.001)\) versus untreated diabetic mice).  

**Molecular Mechanisms Associated With ACE Inhibition-Induced Decrease in the Neovascularization Process in the Retina: Involvement of RAS-Related Pathway**

We first analyzed the effect of diabetes on RAS components mRNA level in the retina (Figure 4A). Angiotensinogen and
ACE mRNA levels were strongly increased in the diabetic retina by 2.8- and 4.1-fold, respectively, in reference to control animals \( (P < 0.001) \), suggesting that RAS-related pathway was activated in this setting. Interestingly, AT1 receptor blockade totally hampered the neovascularization reaction observed in the retina of diabetic animals \( \text{Figure 3} \), demonstrating that RAS blockade prevented the retinal disorders observed in diabetic mice. Finally, VEGF protein level was raised in the retina of diabetic mice, and this upregulation was blocked by both ACE inhibition and AT1 receptor blockade \( \text{Figure 4C} \).

**Involvement of Bradykinin-Related Pathway**

Diabetes did not affect B1R, B2R, and kallikrein mRNA contents \( \text{Figure 4B} \). We next analyzed the role of bradykinin signaling in ACE inhibition-induced anti-angiogenic effect. The effect of ACE inhibitor on the neovascularization process in the retina of diabetic mice was still observed in B2R-deficient diabetic animals \( \text{Figure 3} \). In addition, the angiographic score was unaffected in the retina of diabetic mice deleted in B2 receptor. Taken together, these results suggest that the bradykinin-related pathway was not involved in the ACE inhibition-induced prevention of microvascular disease in the retina of diabetic animals.

**Discussion**

The present study demonstrates that the local environment may influence final ACEI biological responses and identifies ACE inhibition as a novel therapeutic strategy for the treatment of macrovascular and microvascular diseases in setting of diabetes.

One of the most common complication of diabetes is peripheral vascular disease. Therapeutic strategies designed to augment native collateral vessel blood flow represent a novel means of achieving perfusion of ischemic tissue. Recent studies have established the feasibility of using angiogenic growth factors or progenitor cells to enhance neovascularization in patients with limb or myocardial ischemia.\(^2^2\) One potential alternative strategy may be the use of drugs with pro-angiogenic activity, available in an oral...
Similarly, chronic B2 receptor blockade prevents the ACE inhibition promotes the neovascularization reaction may also mediate, at least in part, the bradykinin pro-angiogenic effect.26,27

Diabetes is also associated with alterations to the retinal vasculature. Identification of RAS components including angiotensinogen, ACE, renin, and Ang II receptors expression within the eyes of humans and other species has provided evidence for a local RAS.8–10,15,16 In addition, overactivity of RAS has been shown to accompany diabetic ocular microvascular complications. Overexpression of renin in Ren-2 diabetic rats is associated with both retina and iris neovascularization.9 An angiogenic role for Ang II in the eye is also provided by in vitro studies on bovine retinal endothelial cells and by reports showing that application of Ang II to the rabbit cornea induces an angiogenic response.14 We confirm and extend these previous studies because we evidenced that angiotensinogen and ACE mRNA levels are upregulated in the retina of diabetic mice. We also demonstrate that ACE inhibition and AT1 receptor antagonist hamper vessel growth in the diabetic retina, suggesting that RAS blockade prevents diabetes-induced neovascularization in the retina. VEGF upregulation in the retina of diabetic mice is also reduced by ACE inhibition. ACE inhibitor anti-angiogenic effect is therefore likely mediated by reduction in VEGF signaling. Overall, these results highlight the potential for RAS blockade as an important anti-angiogenic strategy for the attenuation of diabetic retinopathy.

Interpretation of our findings must involve a consideration of hypertension. The changes in vessel density observed in both retina and ischemic legs of diabetic mice treated with ACE inhibitor may be related to reduction in systemic blood pressure. However, the possibility that high blood pressure per se may affect the angiogenic process remains unclear. In fact, hypertension has been shown to decrease capillary density in hind limbs but not in heart of hypertensive rats.28,29 In addition, endothelial cell proliferation was observed in retina of diabetic Ren-2 rats but not in that of spontaneously hypertensive diabetic rats, despite equivalent blood pressure suggesting a local role of RAS in the retina independently of changes in systemic blood pressure.9

In conclusion, ACE inhibition improves neovascularization in the diabetic ischemic leg through activation of bradykinin signaling, whereas it reduces vessel growth in the diabetic retina through inhibition of overacting Ang II pathway. The present report highlights the concept that the local environment may influence final ACEI biological responses and provides a rationale for the use of ACE inhibitor in the prevention of macrovascular and microvascular diseases in diabetes.

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