Proangiogenic Effect of Angiotensin-Converting Enzyme Inhibition Is Mediated by the Bradykinin B₂ Receptor Pathway

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Abstract—Recent studies have suggested a proangiogenic effect of angiotensin-converting enzyme (ACE) inhibition. We hypothesized that such a proangiogenic effect of ACE inhibition may be mediated, in part, by bradykinin (BK) B₂-receptor pathway. This study therefore examined the neovascularization induced by ACE inhibitor treatment in B₂ receptor–deficient mice (B₂−/−) in a model of surgically induced hindlimb ischemia. After artery femoral occlusion, wild-type and B₂−/− mice were treated with or without ACE inhibitor (perindopril, 3 mg/kg/d) for 28 days. Angiogenesis was then quantitated by microangiography, capillary density measurement, and laser Doppler perfusion imaging. The protein levels of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) were determined by Western blot. In wild-type animals, vessel density and capillary number in the ischemic leg were raised by 1.8- and 1.4-fold, respectively, in mice treated with ACE inhibitor when compared with the nontreated animals (P<0.01). This corresponded to an improved ischemic/nonischemic leg perfusion ratio by 1.5-fold in ACE inhibitor–treated animals when compared with the untreated ones (0.87±0.07 versus 0.59±0.05, respectively, P<0.01). Activation of the angiogenic process was also associated with a 1.7-fold increase in tissue eNOS protein level in mice treated with ACE inhibitor (P<0.05 versus control) but not with changes in VEGF protein level. Conversely, ACE inhibition did not affect vessel density, blood flow, and eNOS protein level in ischemic hindlimb of B₂−/− mice. Therefore, proangiogenic effect of ACE inhibition is mediated by B₂-receptor signaling and was associated with upregulation of eNOS content, independently of VEGF expression. (Circ. Res. 2001;89:678-683.)

Key Words: angiogenesis ■ ischemia ■ kinins ■ angiotensin-converting enzyme ■ B₂ receptor

In ischemic diseases, both hypoxia and inflammation play a major role in the control of new vessel growth.¹ The main mechanism of hypoxia-induced angiogenesis involves the rise in hypoxia-inducible factor-1α protein, resulting in increased expression of vascular endothelial growth factor (VEGF), a specific angiogenic factor.¹ The angiogenic effect of VEGF might be mediated by activation of endothelial nitric oxide synthase (eNOS) and subsequently, by production of nitric oxide, as previously described in ischemia-induced angiogenesis.² Neovascularization appears also to be controlled by monocyte/macrophage accumulation that occurs within the ischemic area.³ The presence of these inflammatory cells is associated with local secretion of several angiogenic factors, including cytokines and growth factors.³,⁴

Numerous factors are involved in the regulation of new vessel growth from preexisting ones. Among these factors, the angiogenic role of the renin-angiotensin system remains to be defined. Angiotensin II increases vessel density in rat cremaster muscle⁵ and the chorioallantoic membrane of the chick embryo⁶ and stimulates angiogenesis in the rat subcutaneous sponge granuloma.⁷ However, angiotensin-converting enzyme (ACE) inhibition has also been shown to rise cardiac capillary length density in stroke-prone spontaneously hypertensive rats,⁸ to affect capillary density in rat limb muscle,⁹ and to promote ischemia-induced angiogenesis in ischemic rabbit hindlimb.¹⁰

ACE catalyzes the conversion of angiotensin I to angiotensin II and the breakdown of bradykinin (BK) into inactive peptides. Hence, the pharmacological effect of ACE inhibitors may be in part mediated via inhibition of angiotensin II formation but also via BK accumulation. BK is generated from the action of kallikreins on their substrate kininogen and acts by at least two BK receptor subtypes, B₁ and B₂. The B₂ receptor is constitutively expressed in various tissues and is responsible for the majority of BK effects. In contrast, B₁ has higher affinity for kinin metabolites and its expression is induced in pathological conditions. Activation of the B₂ receptor leads to the release of nitric oxide and prostacyclin, which modulate numerous biological functions.¹¹

Several lines of evidence underline the putative role of BK in the modulation of angiogenesis; hence BK activates angiogenesis of coronary venules.¹² In addition, BK, in synergy with interleukin-1, enhances the angiogenic process in the rat subcutaneous-sponge granuloma.¹³ Recently,
local delivery of tissue kallikrein gene has been shown to stimulate angiogenesis in both ischemic and normoperfused skeletal muscle through the activation of nitric oxide synthase and cyclooxygenase-2.14,15

We therefore hypothesized that the proangiogenic effect of ACE inhibition may be mediated by activation of B_2-receptor signaling. We then analyzed the angiogenic process associated with ACE inhibition in B_2 receptor–deficient mice in a model of operatively induced hindlimb ischemia. As stimulation of B_2 receptors activates the NO-cGMP pathway, we also determined the eNOS protein level in hindlimbs of ACE inhibitor–treated animals.

Materials and Methods

Experimental Protocol

Male (8-week-old) knockout mice lacking the bradykinin B_2 receptor gene (B_2^−/−) and wild-type J129SvBl6 controls (Jackson Laboratory) underwent surgery to induce unilateral hindlimb ischemia. Animals were anesthetized by isoflurane inhaling. The ligature was performed on the right femoral artery, 0.5 cm proximal to the bifurcation of the saphenous and popliteal arteries, as previously described.4 Wild-type and B_2^−/− mice (6 animals per group) were then treated with or without ACE inhibitor (perindopril, Servier, France, 3 mg/kg/d) for 28 days. This study was conducted in accordance with both institutional guidelines and those formulated by the European community for experimental animal use (L358-86/609/EEC).

Blood Pressure Measurement

To measure mean blood pressure, a polyethylene catheter was inserted into the left carotid artery of anesthetized mice. Blood pressure was then measured with a Gould transducer connected to the carotid catheter and recorded.

Quantification of Angiogenesis

Microangiography

Vessel density was evaluated by high-definition microangiography at the end of the 28-day treatment period, as previously described.4 Briefly, mice were anesthetized (isoflurane inhaling) and a longitudinal laparatomy was performed to introduce a polyethylene catheter into the abdominal aorta and to inject a contrast medium (barium sulfate, 1 g/mL). Angiography of hindlimbs was then assessed and images (3 per animal) were acquired by a digital x-ray transducer. Images were then assembled to obtain a complete view of the hindlimbs. The vessel density was expressed as a percentage of pixels per image occupied by vessels in the quantification area. Quantification zona was limited by the place of the ligature on the femoral artery, the knee, the edge of the femur, and the external limit of the leg.

Capillary Density

Microangiographic analysis was completed by assessment of capillary density, as previously described.4 Ischemic and nonischemic muscles were dissected and progressively frozen in isopentane solution cooled in liquid nitrogen. Sections (7 μm) were first incubated for 30 minutes in PBS containing 5% BSA at room temperature and then 1 hour with rabbit polyclonal antibody directed against total fibronectin (dilution 1:50) to identify capillaries (Chemicon International, Temecula, Calif). Capillaries were revealed with a fluorescent FITC anti-rabbit antibody (dilution 1:10). Capillary density was then calculated in randomly chosen fields of a definite area, using OptiLab Pro software.

Laser Doppler Perfusion Imaging

To provide functional evidence for ischemia-induced changes in vascularization, laser Doppler perfusion imaging experiments were performed, as previously described.16

Determination of VEGF and eNOS Protein Expression

Tissue samples were thawed and homogenized in 300 μL of buffer (200 mmol/L sucrose, 20 mmol/L HEPES, pH 7.4) containing protease inhibitors. Protein content was measured by the method of Bradford.17 VEGF and eNOS protein expression was then determined by Western blot in ischemic and nonischemic legs, as previously described.4

eNOS Immunostaining

Frozen tissue sections (7 μm) were incubated with rabbit polyclonal antibody directed against eNOS (dilution 1:50) to identify cells

Figure 1. A, Representative microangiography of the right ischemic and left nonischemic hindlimbs 28 days after femoral occlusion. B, Vessel density in control and B_2^−/− mice with or without ACE inhibitor (ACEI) treatment. Results are expressed as a ratio of vessel density in the ischemic leg to that of in the nonischemic one. Values are mean±SEM, n=6 per group. **P<0.01 vs controls.
producing eNOS. Immunostains were visualized by using avidin-biotin horseradish peroxidase visualization systems (Vectastain ABC kit elite, Vector Laboratories) and then analyzed in randomly chosen fields of a definite area using Histolab software.

Statistical Analysis
Results are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used to compare each parameter. Post hoc Bonferroni’s t test comparisons were then performed to identify which group differences account for the significant overall ANOVA. A value of P < 0.05 was considered significant.

Results

Physiological Data
Blood pressure was 94 ± 12 mm Hg and 84 ± 11 mm Hg in untreated and ACE inhibitor–treated wild-type mice, respectively (P > 0.05). Bradykinin B2-receptor knockout mice displayed a significant 1.3-fold increase in blood pressure when compared with untreated wild-type mice (124 ± 12 mm Hg, P < 0.05). Treatment with perindopril slightly reduced blood pressure, but this did not reach statistical significance when compared with untreated B2−/− mice (109 ± 15 mm Hg, P > 0.05).

Effect of ACE Inhibition on Vessel Density

Microangiography
In wild-type animals, the ratio of ischemic to nonischemic leg vessel density was raised by 1.8-fold in mice treated with ACE inhibitor when compared with the untreated mice (1.29 ± 0.12 versus 0.71 ± 0.05, respectively, P < 0.01). Conversely, ACE inhibition did not affect vessel density in B2−/− mice when compared with untreated B2−/− animals (0.72 ± 0.06 versus 0.56 ± 0.18, respectively; not significant versus untreated wild-type animals) (Figure 1).

Capillary Density
Microangiographic data were confirmed by capillary density analysis. In wild-type animals, the ratio of ischemic to nonischemic leg capillary density was increased 1.4-fold in mice treated with ACE inhibitor when compared with the untreated ones (0.87 ± 0.19 and 0.62 ± 0.05, respectively, P < 0.05). On the contrary, in B2−/− mice, the number of capillaries was unchanged whatever the treatment (0.59 ± 0.15 versus 0.51 ± 0.02, in B2−/− mice treated with ACE inhibitor and in untreated B2−/− mice, respectively) (Figure 2). Similar results were obtained with CD31 immunostaining to specifically reveal endothelial cells (data not shown).

Because the B2−/− mice are engineered onto the J129Sv/B6 strain, we used animals of the same background as control mice. However, other mice strains, such as C57B16, might recover differently from J129Sv/B6 after ischemia and this could bias the conclusions raised in this study. Nevertheless, in our experimental conditions (28 days of treatment) and with the surgical procedure for artery femoral ligation used in this study, we did not observe any significant differences in the recovery of angiographic score and blood flow between these two strains.

Effect of ACE Inhibition on Hindlimb Blood Flow
In wild-type animals, the ischemic/nonischemic ratio for cutaneous blood flow was increased by 1.5-fold in mice treated with ACE inhibitor when compared with the untreated mice (0.87 ± 0.07 versus 0.59 ± 0.05, respectively, P < 0.01). As for angiographic data, no changes in blood flow were observed in B2−/− mice with or without ACE inhibitor treatment (Figure 3).

Regulation of VEGF Protein Level
ACE inhibitor treatment–induced changes in vessel density and blood flow were not associated with variation in VEGF protein level. In nonischemic hindlimb, VEGF protein content was not affected whatever the treatment (Figure 4). In control mice, VEGF protein level was increased 1.6-fold in ischemic leg when compared with the nonischemic leg (P < 0.01). However, such an increase was not modulated by ACE inhibitor treatment. Indeed, in ischemic hindlimb, VEGF content was similar in control mice with or without ACE inhibitor treatment. In the same view, in ischemic
hindlimb, VEGF protein level in B_2^{-/-} mice was similar to that of control and was not affected by ACE inhibitor treatment (Figure 4).

**Regulation of eNOS Protein Level**

In contrast, changes in vessel density and blood flow were associated with variation in eNOS protein level. In nonischemic hindlimb, eNOS protein level was unchanged in both control and B_2^{-/-} mice with or without ACE inhibitor (ACEI) treatment (Figure 5). In wild-type animals, eNOS content was higher by 1.6-fold in ischemic hindlimb when compared with the nonischemic one (P<0.01). Interestingly, in ischemic leg, eNOS content was raised by 1.7-fold in mice treated with ACE inhibitor when compared with untreated animals (P<0.01). Such an effect of ACE inhibitor was not observed in B_2^{-/-} mice. Indeed, in B_2^{-/-} mice, eNOS protein level was not affected by ACE inhibitor treatment (Figure 5).

**eNOS Immunostaining**

As shown in Figure 6, eNOS immunostaining revealed that eNOS was localized within the arteriole (data not shown) and the capillary wall suggesting that endothelial cells mainly produced eNOS in the ischemic tissue. In addition, the number of capillary-producing eNOS was higher in mice treated with ACE inhibitor when compared with nontreated wild-type mice. B_2^{-/-} mice treated or not with ACE inhibitor showed similar stainings to those observed for untreated wild-type mice (data not shown).

**Figure 4.** A, Representative Western blot of VEGF protein content in the ischemic and nonischemic leg. B, VEGF protein levels in hindlimbs of control and B_2^{-/-} mice with or without ACE inhibitor (ACEI) treatment. Values are mean ± SEM, n=6 per group. Results are expressed as percentage of control in the nonischemic leg. **P<0.05 vs nonischemic leg of control mice. VEGF indicates VEGF-specific band (33 to 48 kDa). For each group, 5 μg of total protein was used.

**Figure 5.** A, Representative Western blot of eNOS protein content in the ischemic and nonischemic leg. B, eNOS protein levels in hindlimbs of control and B_2^{-/-} mice with or without ACE inhibitor (ACEI) treatment. Values are mean ± SEM, n=6 per group. Results are expressed as percentage of control in the nonischemic leg. **P<0.01 vs control mice; ††P<0.01 vs ischemic leg of control mice. eNOS indicates eNOS-specific band (140 kDa). For each group, 40 μg of total protein was used.
Discussion

The main result of this study is that ACE inhibition enhances ischemia-induced angiogenesis through the activation of the B₂-receptor pathway. This proangiogenic effect may be associated with the upregulation of eNOS expression but seems independent of the VEGF pathway.

Although evidence is accumulating on the activating role of ACE inhibition in vessel growth, little is known about the cellular signaling involved in such an angiogenic effect. Our study confirms that ACE inhibition enhanced revascularization of the ischemic hindlimb but demonstrates that such an effect was mediated by B₂-receptor activation. Indeed, ACE inhibition did not affect vessel growth in mice lacking B₂ receptor. Similarly, long-term B₂-receptor blockade prevented the ACE inhibitor–induced increase in cardiac capillary density in stroke-prone spontaneously hypertensive rats. In the same view, long-term ACE inhibitor treatment ameliorated the severity of myocardial injury in cholesterol-fed rabbits via B₂ receptor–related pathway.

In wild-type mice, the proangiogenic effect of ACE inhibition was associated with a rise in eNOS level mainly localized within the capillary wall of the ischemic leg. This ACE inhibitor–induced increase in eNOS protein content might be due to B₂ receptor activation. Indeed, ACE inhibition did not affect vessel growth in mice lacking B₂ receptor. Similarly, long-term B₂-receptor blockade prevented the ACE inhibitor–induced increase in cardiac capillary density in stroke-prone spontaneously hypertensive rats. In the same view, long-term ACE inhibitor treatment ameliorated the severity of myocardial injury in cholesterol-fed rabbits via B₂ receptor–related pathway.

In wild-type mice, the proangiogenic effect of ACE inhibition was associated with a rise in eNOS level mainly localized within the capillary wall of the ischemic leg. This ACE inhibitor–induced increase in eNOS protein content might be due to B₂ receptor activation. Indeed, the lack of B₂ receptor in B₂−/− mice resulted in the lack of ACE inhibitor effect on eNOS protein level. In the ischemic and nonischemic leg of B₂−/− mice, eNOS protein level was no different from that of wild-type animals. eNOS levels measured in the hindlimbs of B₂−/− mice might then correspond to the baseline of expression of the protein. We can speculate that other stimuli may regulate baseline eNOS levels and maintain eNOS protein at a physiological level despite the lack of B₂ receptor. In addition, the revascularization process that occurred within the ischemic leg was similar between control and B₂−/− mice, as previously described. This basal vessel growth might also reflect the basal cellular response to tissue ischemia.

Previous studies have demonstrated that binding of kinins to their receptor activates NO signal pathways. Several lines of evidence also indicate that NO is a key regulatory factor for ischemia-induced angiogenesis. Indeed, angiogenesis, in the ischemic hindlimb, was impaired in eNOS-deficient mice, whereas capillary growth was raised in rabbits receiving NO donor. Hence, ACE inhibitor–induced potentiation of endogenous BK was associated with eNOS upregulation, which, by increasing NO production, might affect neovascularization of ischemic hindlimb.

However, BK may also modulate several other cellular pathways that play an important role in the regulation of angiogenesis. Inflammation has been shown to be a key event in the angiogenic process associated with hindlimb ischemia. The B₂-receptor antagonist hampered the inflammatory process in an in vivo model of inflammation. In the same way, BK stimulated proinflammatory cytokine production by different cellular types. Taken together, these studies highlight the putative role of BK in the modulation of the inflammatory reaction and, therefore, in inflammation-induced angiogenesis. BK might also directly activate cyclooxygenase-2, which has been reported to affect the angiogenic process.

An unresolved issue is whether the potentiation of BK effects by ACE inhibitors is caused only by blocking BK enzymatic hydrolysis. B₂ receptor is a G protein–coupled receptor that is rapidly desensitized and internalized in response to high agonist concentrations. ACE inhibitors have been shown to inhibit and/or partially reverse the BK-induced internalization of the B₂ receptor and to reactivate signaling events initiated by the B₂ receptor. In fact, ACE inhibitors may augment BK effects on the B₂ receptor indirectly only when enzyme and receptor molecules are sterically closed, possibly forming a heterodimer.

Systemic hypertension developed by B₂−/− mice may also have hampered the ACE inhibition–induced angiogenesis in these mice. However, the possibility that high blood pressure
per se may impair the angiogenic process remains unclear. In fact, hypertension does not affect coronary capillary angiogenesis in hypertensive rats.

Regulation of VEGF and eNOS levels has been shown to be a major event in the angiogenic process associated with hindlimb ischemia. In addition, the angiogenic effect of VEGF might be mediated by activation of eNOS. Hence, the increase in eNOS content associated with ACE inhibitor treatment might be mediated by changes in VEGF level. Nevertheless, in the present study, ACE inhibitor treatment increases the angiogenic process without changes in the upregulation of VEGF observed in the ischemic leg. Hence, cellular events involved in activation of the angiogenic process associated with ACE inhibitor treatment were independent of the VEGF pathway. Similarly, the lack of B2 receptor did not affect the increase in VEGF content in ischemic leg suggesting that the B2-receptor pathway was not related to the VEGF pathway.

In conclusion, the present study demonstrates the proangiogenic effect of ACE inhibition in mice with operatively induced hindlimb ischemia. This proangiogenic effect was mediated by B2-receptor activation and might have involved eNOS-dependent pathways. The present work also underscores the possibility that the beneficial effects of ACE inhibition on the treatment of ischemic disease may be owing to increased vessel growth within the ischemic area.

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