Background—Endothelial cells (ECs) represent the critical cellular element responsible for postnatal angiogenesis. Because ACE inhibitors may favorably affect endothelial function, we investigated the hypothesis that administration of the ACE inhibitor quinaprilat could enhance angiogenesis in vivo.

Methods and Results—Ten days after resection of 1 femoral artery, New Zealand White (NZW) rabbits were randomly assigned to receive recombinant human vascular endothelial growth factor (rhVEGF) administered as a single intra-arterial injection (n = 6), quinaprilat (n = 8) or captopril (n = 7) administered as a daily subcutaneous injection, or no treatment (controls, n = 6). Angiogenesis was monitored in vivo by measurement of blood pressure, vasoreactivity, and resistance in ischemic versus normal limbs at day 10 (D10) and D40; angiographic studies to identify sites of neovascularization were performed at D10 and D40, and morphometric analysis of capillary density in the ischemic limb was performed at necropsy (D40). Both functional and morphological outcomes documented augmented angiogenesis in quinaprilat-treated rabbits similar to that observed for rhVEGF and superior to that observed with either captopril or no drug (controls). Residual ACE activity was equivalent for the captopril and quinaprilat groups in plasma (42.54 ± 0.03% versus 41.53 ± 0.02%, P = NS) but not in tissue, where quinaprilat lowered ACE activity significantly (P < 0.01) compared with captopril (13% versus 61%).

Conclusions—ACE inhibition with quinaprilat promotes angiogenesis in a rabbit model of hindlimb ischemia. Thus, nonsulfhydryl ACE inhibitors with high tissue affinity may be potentially useful for therapeutic angiogenesis in ischemic tissues. Moreover, previous evidence that ACE inhibition benefits patients with myocardial ischemia may be due in part to augmented collateral development. (Circulation. 1999;99:3043-3049.)

Key Words: angiogenesis ■ angiotensin ■ growth substances ■ ischemia

Angiotensin-converting enzyme is a dipeptidyl carboxypeptidase that converts inactive angiotensin I to angiotensin II (Ang II) and inactivates bradykinin. Whereas circulating ACE may act on circulating angiotensin I derived from the action of renal renin on angiotensinogen, tissue-bound ACE may also act on circulating or local angiotensin I. Indeed, <10% of ACE found in the body is freely circulating; the remaining 90% is found locally as tissue-bound ACE.1 Endothelial cells (ECs) constitute an important source of ACE in the vessel wall,2,4 where circulating or locally produced angiotensin I serves as available substrate for local production of Ang II.5

ACE inhibition targeting the Ang II–mediated inactivation of bradykinin may increase local NO production, and clinical application of drugs intended to inhibit ACE activity has been shown to exert a favorable effect on endothelial dysfunction.6

Previous data regarding the role of the renin-angiotensin system in angiogenesis are controversial. Ang II has been shown to augment microvessel density in rat cremaster muscle7 and promote angiogenesis of precapillary and post-capillary vessels in the chorioallantoic membrane of the chick embryo.8 Although administration of captopril was shown to block neovascularization in the rat cornea9 and microvascular development in hypertensive and normotensive rats,10 other ACE inhibitors have been reported to increase capillary density in rat limb muscle,11 sciatic nerve,12 and coronary microvasculature.13–15

The present study was carried out to evaluate the effects of ACE inhibition on neovascularization of ischemic tissue in vivo. We investigated 2 ACE inhibitors, quinaprilat and captopril, both of which may achieve equivalent levels of ACE inhibition in plasma but 1 of which, quinaprilat, inhibits ACE activity more efficiently at the tissue level. Vascular endothelial growth factor (VEGF), an EC-specific mitogen shown to promote angiogenesis in the same animal model, was used as a positive control. Both functional and morphological outcomes documented evidence for augmented angiogenesis in quinaprilat-treated rabbits similar to that observed for VEGF and superior to that observed with either captopril or no drug (controls).
**Methods**

**Animal Model**

New Zealand White rabbits (3.8 to 4.4 kg, n=42, Pine Acre Rabbitry, Norton, Mass) undergoing surgery to create unilateral hindlimb ischemia were used as previously described. All protocols were approved by St Elizabeth’s Institutional Animal Care and Use Committee.

**Study Design**

Animals with limb ischemia were randomly divided into 4 groups. Rabbits in the captopril group (n=7) and the quinaprilat group (n=8) received a single daily subcutaneous (SC) injection of captopril (25 mg) or quinaprilat (2 mg), beginning on day 10 (D10) postsurgery and continuing through D35. The recombinant human (rh)VEGF group, as a positive control group, consisted of 6 rabbits receiving 500 μg rhVEGF intra-arterially at D10. The ischemic control group consisted of 6 rabbits receiving no treatment. All 4 groups were investigated at D10 and D40 postsurgery, at which time lower-limb blood pressure, vasomotor reactivity, and angiographic score were evaluated. All animals from each group were euthanized on postoperative D40 and studied at necropsy.

An additional group of 15 rabbits underwent ischemic hindlimb surgery and either were not treated (n=3) or were treated from D10 through D35 with captopril (n=7) or quinaprilat (n=5). All 15 of these animals were euthanized at D35, at which time sections of hindlimb muscle were harvested to measure tissue ACE activity.

**Measurement of Plasma Levels of ACE Activity**

**Plasma ACE Activity**

Plasma ACE activity in blood samples obtained from the central ear vein was determined by spectrophotometry using the synthetic tripeptide FAPGG (Sigma). ACE catalyzes hydrolysis of FAPGG to furylacryloylphenylalanine and glycerylglycine, which results in a decrease in absorbance at a wavelength of 340 nm. ACE activity in the sample was determined by comparing the sample reaction rate to that obtained with an ACE calibrator.

In the 2 groups treated with either quinaprilat or captopril and studied from D10 to D40, ACE activity was measured in plasma at D10 for baseline values, at D14 to assess the efficacy and equivalence of treatments, and at D40 to document the absence of a residual effect after 5 washout days. Efficiency of each regimen was calculated as the percentage of the D10 plasma activity according to the following ratio: D14 plasma activity/D10 plasma activity.

**Tissue ACE Activity**

To measure tissue ACE activity, muscles from the additional group were harvested, weighed, and homogenized in PBS. The solution obtained was used as a substrate for the ACE determination kit (Sigma), and the result, divided by sample weight, is expressed as international units per liter per gram of tissue (U · L⁻¹ · g⁻¹).

**Lower-Limb Blood Pressure Ratio**

Blood pressure was measured in both hindlimbs on D10 and D40 as previously described. Blood pressure ratio (BPR) was defined for each rabbit as systolic pressure of the ischemic limb/systolic pressure of the normal limb.

**Vasomotor Reactivity and Vascular Resistance**

After measurement of lower-limb blood pressure, a 3F end-hole infusion catheter (Tracker-18, Target Therapeutics) was inserted into the left common carotid artery and advanced to the abdominal aorta. A 0.018-in Doppler guidewire (Cardiometrics) was advanced through the 3F infusion to the proximal segment of the internal iliac artery supplying the ischemic limb. The Doppler wire records a real-time, spectral analysis of the Doppler signal, from which the average peak velocity (the temporal average of the instantaneous peak velocity wave form) was calculated and displayed on line. A second catheter (Tracker-18) was introduced into the left common carotid artery through the same cutdown and advanced to the origin of the common iliac artery of the ischemic limb by use of a separate 0.018-in guidewire (Hi-Torque Floppy II, Advanced Cardiovascular Systems) under fluoroscopic guidance. This catheter was used for infusion of vasoactive drugs, for measurement of intra-arterial blood pressure via connection to a pressure transducer, and for selective angiography of the ischemic limb (see below).

The vasodilator acetylcholine chloride, sodium nitroprusside, and serotonin creatine sulfate were administered intra-arterially over 2 minutes via a constant-infusion pump (1 mL/min). Each was administered at a dose of 1.5 μg · min⁻¹ · kg⁻¹ on D10 and on D40. A lag time of 5 minutes between each drug sufficed for return to basal flow values.

**Calculation of Vascular Reactivity**

Vascular reactivity was assessed after infusion of vasodilators into the internal iliac artery of the ischemic limb. Blood flow was calculated as described previously. Angiography was performed immediately after drug administration and intra-arterial blood pressure recording with 1 mL of contrast medium (RenoCal-76, Squibb).

Calculated blood flow ratios (BFRs) are expressed as blood flow after vasodilator injection/basal blood flow, measured just before injection.

**Calculation of Vascular Resistance**

Vascular resistance was calculated according to the formula $R = P_R/Q_D$, where $R$ is vascular resistance at internal iliac artery, $P_R$ is blood pressure at the common iliac artery, and $Q_D$ is Doppler-derived flow at internal iliac artery. Calculated resistance ratio is expressed as resistance after vasodilator injection/basal resistance, measured just before injection.

**Angiographic Score**

Angiograms of the thigh were performed on postoperative D10 and D40, and a quantitative angiographic score was derived as previously described.

**Capillary Density**

Microscopic angiogenesis was assessed by measuring the number of capillaries in light-microscopic sections taken from the ischemic hindlimb at D40. Tissue sections snap-frozen in liquid nitrogen were stained for alkaline phosphatase to detect capillary ECs as previously described. Capillaries in 21 randomly selected fields were counted under a ×10 objective to determine capillary density.

**Drugs**

rhVEGF protein (165-amino-acid isoform of VEGF-A) was generously provided by Dr Bruce Keyt (Genentech). Quinaprilat (CI 928; Acupril ampoules, 1 mg/mL), the active diacid of quinapril, was generously provided by Parke-Davis, and captopril (Capoten, 12.5-mg tablets) by Bristol-Meyers Squibb. In a preliminary experiment, we determined the dose of each drug required to induce the same level of plasma ACE inhibition. A 50% to 60% inhibition was achieved in ischemic rabbits with 2 mg of quinaprilat (~2 mL) or 25 mg of captopril, both after SC administration. For the latter, tablets were dissolved daily in saline, and the resulting suspension was injected.

**Statistical Analysis**

All results are expressed as mean±SEM. Multiple comparison among 4 groups was performed by ANOVA. When a significant difference was detected, a protected $t$ test was used for comparisons between 2 means. A value of $P<0.05$ was interpreted to denote statistical significance.

**Results**

**Plasma ACE Activity**

Plasma measurements of ACE activity for captopril and quinaprilat were similar at baseline (D10, Table). Plasma...
ACE activity was again identical at D14 (42.54 ± 0.03% versus 41.53 ± 0.02%, respectively, P = 0.78). These measurements, obtained 24 hours after the previous drug injection and immediately before the subsequent injection, thus denote a level of ACE inhibition >50% for either agent. Five days after the injection of ACE inhibitors was discontinued, ie, at D40, plasma measurement of ACE activity was restored to nearly normal for both the captopril (92.87 ± 0.04%) and quinaprilat (80.71 ± 0.08%) (Figure 1).

Tissue ACE Activity
Mean tissue activity in thigh muscles of 15 additional rabbits was significantly different among the 3 groups (P < 0.01 by ANOVA): 12.65 ± 2.60 IU/g in controls, 7.65 ± 1.69 IU/g for the captopril group (P = 0.14 versus controls), and 1.69 ± 0.70 IU/g for quinaprilat-treated rabbits (P < 0.05 versus controls). These measurements correspond to a residual tissue activity level of 13% for quinaprilat versus 61% for captopril of that measured in nontreated (control) tissues (Figure 1).

Lower-Limb BPR
Quinaprilat, like rhVEGF, resulted in superior recovery of ischemic/normal BPR compared with either captopril or controls. At D40, BPR for quinaprilat was 0.86 ± 0.03 compared with 0.45 ± 0.04 for controls (P < 0.01). BPR for VEGF (0.74 ± 0.03) was superior to that of controls (P < 0.01). There was no statistically significant difference between values recorded for captopril (0.47 ± 0.05) versus controls (Figure 2). No statistically significant difference was found between rhVEGF and quinaprilat or between captopril and controls.

Vasomotor Reactivity and Vascular Resistance
Mean BFR (stimulated flow/basal flow) calculated for the 27 rabbits disclosed moderate dilation induced by acetylcholine or nitroprusside (1.64 ± 0.01 and 1.69 ± 0.01, respectively). Serotonin induced dramatic reduction in blood flow, equivalent among the 4 groups (mean BFR = 0.37 ± 0.04), reflecting intense vasoconstriction.

At D40 (Figure 3), the response to acetylcholine was improved in each group, but the magnitude of this improvement was greatest for quinaprilat and VEGF: control and captopril groups showed a moderate increase in BFR (1.74 ± 0.09 and 1.93 ± 0.07, respectively), whereas quinaprilat and rhVEGF elicited more profound vasodilation (3.06 ± 0.25 and 2.72 ± 0.22, respectively). Comparison with controls disclosed a statistically significant difference for both quinaprilat and rhVEGF (P < 0.01 for each) but not for captopril (P = 0.09). Nitroprusside likewise resulted in significantly greater vasodilation for quinaprilat (2.94 ± 0.24, P < 0.05) and rhVEGF (2.66 ± 0.35, P < 0.05) but not for captopril (2.05 ± 0.11, P = NS) compared with controls (1.81 ± 0.09). BFR in response to serotonin at D40 remained diminished for control and captopril (0.63 ± 0.09 and 0.62 ± 0.05, respectively) but was substantially increased with quinaprilat and rhVEGF (1.17 ± 0.08 and 1.19 ± 0.09, P < 0.01 versus control for both).

Vascular resistance at D10 was comparable among the 4 groups after acetylcholine, nitroprusside, or serotonin (Table). At D40, however, vascular resistance measured in the internal iliac artery (Figure 4) varied significantly among the 4 groups (P < 0.01 by ANOVA): resistance remained significantly greater for controls and captopril versus rhVEGF and quinaprilat in response to either acetylcholine (0.57 ± 0.04 and 0.51 ± 0.03 versus 0.32 ± 0.03 and 0.35 ± 0.03; P < 0.01 versus control for both quinaprilat and rhVEGF) or nitroprusside (0.42 ± 0.02 and 0.38 ± 0.03 versus 0.28 ± 0.02 and 0.29 ± 0.04; P < 0.01 for quinaprilat and P < 0.05 for rh-

### Reference Data for 4 Rabbit Groups

<table>
<thead>
<tr>
<th></th>
<th>Plasma ACE Activity, U/L</th>
<th>Body Weight, kg</th>
<th>SBP in Healthy Limb, mm Hg</th>
<th>Resting Blood Flow, mL/min</th>
<th>Resting Vascular Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (n=6)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>D10</td>
<td>4.15 ± 0.07</td>
<td>96.9 ± 2.4</td>
<td>15.38 ± 0.64</td>
<td>4.21 ± 0.11</td>
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<tr>
<td>D40</td>
<td>4.02 ± 0.18</td>
<td>124.5 ± 2.5</td>
<td>19.19 ± 0.31</td>
<td>3.44 ± 0.05</td>
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<tr>
<td>rhVEGF group (n=7)</td>
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<tr>
<td>D10</td>
<td>4.08 ± 0.06</td>
<td>85.3 ± 1.8</td>
<td>14.78 ± 0.48</td>
<td>3.95 ± 0.11</td>
<td></td>
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<tr>
<td>D40</td>
<td>4.01 ± 0.08</td>
<td>92.2 ± 2.7</td>
<td>14.32 ± 0.77</td>
<td>4.46 ± 0.21</td>
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<td>Captopril group (n=7)</td>
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<tr>
<td>D10</td>
<td>55.94 ± 0.75</td>
<td>4.17 ± 0.13</td>
<td>89.7 ± 2.2</td>
<td>15.03 ± 0.38</td>
<td>3.69 ± 0.10</td>
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<tr>
<td>D14</td>
<td>23.92 ± 0.60</td>
<td>4.39 ± 0.13</td>
<td>126.0 ± 3.6</td>
<td>16.04 ± 0.19</td>
<td>3.93 ± 0.07</td>
</tr>
<tr>
<td>D40</td>
<td>51.80 ± 0.80</td>
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<td>Quinaprilat group (n=8)</td>
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<tr>
<td>D10</td>
<td>59.50 ± 1.38</td>
<td>4.19 ± 0.10</td>
<td>99.5 ± 1.3</td>
<td>14.37 ± 0.32</td>
<td>4.63 ± 0.19</td>
</tr>
<tr>
<td>D14</td>
<td>24.32 ± 0.46</td>
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<td></td>
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</tr>
<tr>
<td>D40</td>
<td>46.40 ± 0.98</td>
<td>4.54 ± 0.20</td>
<td>117.1 ± 1.7</td>
<td>13.87 ± 0.48</td>
<td>5.47 ± 0.18</td>
</tr>
</tbody>
</table>

The body weight and basal hemodynamic data at D10 and D40 are reported for reference groups (control and rhVEGF) and ACE inhibitor groups (captopril and quinaprilat). The ACE activity is also displayed at D10, D14, and D40 for ACE inhibitor–treated groups. ANOVA testing disclosed no significant difference in body weight, systolic blood pressure (SBP) in healthy hindlimb, resting blood flow, and resting vascular resistance among the 4 groups at D10 and D40. Similarly, there was no statistically significant difference in plasma ACE activity between captopril and quinaprilat groups at D10 (basal value), D14 (after 4 days of treatment), and D40 (5 days after discontinuation).
VEGF). Finally, the severe increase in resistance induced by serotonin at D10 was reversed only by quinaprilat and rhVEGF (0.94±0.06 and 0.77±0.04, *P*<0.05 versus control for both); increased resistance persisted for both controls and captopril (2.15±0.59 and 1.88±0.20).

**Angiographic Assessment of Hindlimb Collateral Circulation**

The angiographic score (Figure 5) measured at D40 was significantly higher for both quinaprilat (51.19±0.02%, *P*<0.01) and rhVEGF (50.88±0.04%, *P*<0.01) than for control (33.66±0.02%) and captopril (35.03±0.02%, *P*=NS).

**Capillary Density**

Morphometric light microscopic analysis of the medial thigh muscles from the ischemic limbs on D40 demonstrated a prominent increase in capillary density in animals receiving quinaprilat and rhVEGF (Figure 5). Capillary density with quinaprilat (214.0±6.4/mm²) and rhVEGF (228.8±9.6/mm², *P*<0.01) was significantly greater than in controls (140.5±4.3/mm²) or with captopril (147.2±11.9 mm²).

**Discussion**

The results of the present study establish that ACE inhibition with quinaprilat promotes angiogenesis in the rabbit ischemic hindlimb to the same extent as rhVEGF. This outcome,
however, is not generic for ACE inhibitors, because captopril, another ACE inhibitor, failed to augment angiogenesis. Improvement in the functional indices recorded 5 days after discontinuation of ACE inhibition suggests that these findings were not simply the result of transient, pharmacologically mediated improvement in endothelium-dependent blood flow but instead reflect persistent modification of the hindlimb vasculature. Augmented endothelium-independent flow was also achieved with nitroprusside, consistent with the notion that quinaprilat resulted in a more capacious hindlimb circulation; similar results have been described previously in response to rhVEGF.17

The differing consequences of ACE inhibition noted for quinaprilat versus captopril in this study may be the result of 2 factors. First, these 2 agents do not achieve the same level of tissue ACE inhibition because of differences in tissue affinity. To avoid variation in bioavailability, the dose of each drug was selected to induce an equivalent level of ACE inhibition in plasma. At these doses, however, quinaprilat inhibited ACE activity at the tissue level more efficiently than captopril. The higher tissue inhibition achieved with quinaprilat was previously assessed by both a radioinhibitor-binding assay and autoradiography.19 The evidence in the present study that angiogenesis is more sensitive to tissue than plasma ACE inhibition is consistent with the demonstration that tissue ACE inhibition was more efficient than plasma inhibition for reducing neointima formation after vascular injury.20

Second, captopril but not quinaprilat has a sulfhydryl group that has been implicated in a variety of effects,21,22 including inhibition of neovascularization in the rat cornea. This inhibition could not be shown to result from reduced enzymatic ACE activity but instead appeared to result from captopril inhibition of Zn2+–dependent metalloproteinase activity that ECs require to respond to an angiogenic stimulus.9

The mechanisms responsible for augmented angiogenesis in response to ACE inhibitors remain to be elucidated. There is insufficient evidence to support a direct effect of the renin-angiotensin system on endogenous angiogenic cytokines; in fact, Ang II has previously been reported to induce a concentration- and time-dependent increase in VEGF expression by vascular smooth muscle cells23 as well as ECs.24 We analyzed VEGF gene expression at the protein level in rabbit muscles 5 days after initiation of quinaprilat treatment by Western blot analysis and were unable to show a difference versus controls (data not shown). It is likely that hypoxia in the ischemic limb25,26 is sufficiently potent to upregulate VEGF expression independently of Ang II levels.

In contrast, published data do suggest the possibility of an indirect effect of ACE inhibition, mediated by augmented EC levels of nitric oxide (NO), as a potentially important contributing mechanism. Recent reports have implicated NO as a critical regulatory molecule for angiogenesis. We observed, for example, that angiogenesis is impaired in eNOS−/− mice and that administration of supplemental L-arginine to rabbits with hindlimb ischemia significantly upregulated hindlimb neovascularization.27 Indeed, VEGF directly upregulates EC production of NO.28 and data from other laboratories suggest that NO may be crucial for VEGF-induced angiogenesis.29,30
ACE inhibition has previously been shown to promote NO accumulation in coronary microvessels, and certain salutary effects of ACE inhibitors on vasomotor reactivity have been linked to augmented NO release. Moreover, NO may completely abrogate the cascade cascade activated by Ang II and shown by Dimmel et al. to induce apoptosis of human umbilical vein ECs.

At least 2 possible mechanisms may account for increased NO in response to ACE inhibition. First, endothelial ACE degrades bradykinin; ACE inhibition blocks this effect, potentiating the accumulation of bradykinin, a potent activator of the L-arginine–NO pathway, which has been shown to promote growth of ECs from postcapillary venules via B1 receptors. The observations that intracerebral augmentation of NO with low doses of ramipril or perindopril and cardiac capillary length density induced by ACE inhibition are both inhibited by the B1-receptor antagonist icatibant constitute inferential evidence that bradykinin may mediate angiogenesis in response to ACE inhibitors.

Second, Ang II can stimulate NADH oxidase present in the vascular wall, increasing the generation of superoxide anions capable of degrading NO. Reduced levels of superoxide anions in conjunction with a reduction in Ang II after ACE inhibition would thus be expected to further augment NO bioavailability.

The demonstration that inhibition of tissue ACE activity by quinapril promotes angiogenesis has potential clinical implications. In contrast to certain growth factors, including VEGF, currently under clinical investigation for therapeutic angiogenesis, ACE inhibitors, including quinapril, are available in an oral formulation that could facilitate treatment of patients with lower-extremity or myocardial ischemia. Alternatively, previous demonstrations of synergy between 2 angiogenic growth factors—VEGF plus quinaprilat—might well yield a synergistic angiogenic response. Finally, the finding that ACE inhibition may promote angiogenesis raises the possibility that the favorable effects of ACE inhibition on the treatment of patients with ischemic heart disease may be due in part to augmentation of coronary collateral circulation.

Acknowledgments

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