Role of Bradykinin, Nitric Oxide, and Angiotensin II Type 2 Receptor in Imidapril-Induced Angiogenesis

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Abstract—The angiotensin II (Ang II)-Ang II type 1 receptor pathway is proangiogenic, whereas studies showed that some angiotensin-converting enzyme inhibitors also stimulate angiogenesis in the setting of tissue ischemia, leaving a controversy of Ang II-mediated angiogenesis. We investigated whether an angiotensin-converting enzyme inhibitor imidapril-induced angiogenesis might be mediated via the tissue bradykinin pathway. To rule out the conventional effects of Ang II on angiogenesis, we used Ang II type 1a receptor knockout (AT1aKO) mice. We examined the effects of the angiotensin-converting enzyme inhibitor imidapril on angiogenesis in a hindlimb ischemia model using AT1aKO mice. After induction of hindlimb ischemia, AT1aKO mice were treated with or without imidapril (1.0 or 0.1 mg/kg per day for 21 days). Angiogenesis was quantified by laser Doppler blood flowmetry and capillary density. Angiogenesis was reduced in AT1aKO mice compared with wild-type mice. Imidapril with either low or high doses enhanced angiogenesis in AT1aKO mice (P<0.01). Ang II type 2 receptor antagonist (PD123319; 30 mg/kg per day) and B1 receptor antagonist (DesArg9-[Leu8]-bradykinin; 50 nmol/kg per day) suppressed the imidapril-induced angiogenesis in AT1aKO mice to an extent even lower than that of nontreated AT1aKO mice. B2 receptor antagonist (Hoechst 140; 100 µg/kg/d) and NO synthase inhibitor (N'G-nitro-L-arginine methyl ester; 20 mg/kg per day) moderately attenuated the imidapril-mediated angiogenesis. RT-PCR revealed that vascular endothelial growth factor receptor 2 mRNA was reduced with PD123319, DesArg9-[Leu8]-bradykinin, or Hoechst 140, and vascular endothelial growth factor mRNA abundance was suppressed with PD123319 or DesArg9-[Leu8]-bradykinin. In conclusion, imidapril elicited angiogenesis in the setting of tissue ischemia in AT1aKO mice. This angiogenic effect might involve the Ang II-Ang II type 2 receptor pathway in addition to the bradykinin-B1 and bradykinin-B2 receptor/NO-dependent pathways. (Hypertension. 2008;51:252-258.)

Key Words: angiogenesis ■ angiotensin II ■ angiotensin-converting enzyme ■ bradykinin ■ ischemia ■ microcirculation ■ NO

The renin-angiotensin system plays essential roles in the maintenance of vascular homeostasis. Other than acting as a vasoconstrictor, angiotensin II (Ang II) modulates cardiovascular growth and remodeling. Ang II stimulates endothelial and smooth muscle cell proliferation in vitro and, thus, augments angiogenesis in vivo. We showed previously that both ischemia-induced and tumor-related angiogenesis were suppressed in Ang II type 1a receptor knockout (AT1aKO) mice compared with wild-type (WT) mice and that Ang II receptor blocker inhibited angiogenesis in WT mice. These results suggest that Ang II is proangiogenic through the AT1a receptor–mediated pathway. On the other hand, angiotensin-converting enzyme inhibitors (ACEIs), widely used as antihypertensive agents, were shown to augment cardiac capillary density in spontaneously hypertensive rats and to increase capillary density in hindlimb muscles. Fabre et al reported that an ACEI quinapril augmented angiogenesis in a rabbit model of hindlimb ischemia. Ang II is a proangiogenic molecule, whereas ACEI, which is known to reduce Ang II formation, also enhances angiogenesis in the setting of tissue ischemia. Accordingly, there is an apparent paradox regarding the role of the renin-angiotensin system in ischemia-induced angiogenesis.

Based on these controversial findings, we reasoned that there might be Ang II-independent mechanisms by which ACEI augmented ischemia-induced angiogenesis. It is well known that ACEI not only inhibits the conversion of ATI to Ang II but also blocks the breakdown of bradykinin (BK) into inactive fragments. Hence, the pharmacological efficacies of ACEI are mediated via both reduction of Ang II and accumulation of BK. Several studies have shown the roles of BK in the regulation of angiogenesis. BK exerts growth-promoting effects on coronary endothelial cells and enhanced angiogenesis in a rat subcutaneous-sponge granuloma.
model. Furthermore, local delivery of the tissue kallikrein gene has been shown to stimulate angiogenesis in ischemic skeletal muscle through the production of BK. On the other hand, upregulation of Ang II type 1 receptor and Ang II type 2 (AT2) receptor was observed in ACEI treatment, and the roles of the Ang II type 1 receptor and AT2 receptor in the angiogenesis under ACEI treatment are not fully understood.

Accordingly, we examined the effects of an ACEI imidapril on angiogenesis using a well-established mouse model of hindlimb ischemia with a special focus on the BK-mediated pathway. To rule out the influence of major vascular effects of Ang II on ischemia-induced angiogenesis, we mainly performed these experiments using AT1aKO mice. By using AT1KO mice, we would be able to observe the Ang II-Ang II receptor–independent effects of ACEI on angiogenesis. To further investigate whether the AT2 receptor plays a role in this process, we treated AT1aKO mice with imidapril and an AT2 receptor antagonist. We also examined possible roles of the 2 BK receptors (ie, BK-B1 and BK-B2 receptors) and NO using B1 and B2 receptor antagonists, as well as an NO synthase (NOS) inhibitor, in ischemia-induced angiogenesis in AT1aKO mice treated with imidapril.

Methods

Animals

Male AT1aKO (C57BL/6J strain) and WT mice (C57BL/6J) at the age of 8 to 10 weeks were used in this study. These mice were obtained as described previously. The study protocol was approved by the institutional animal care and use committee of Nagoya University School of Medicine.

Reagents

An ACEI, imidapril (Tanabe Seiyaku Co, Ltd), was administered subcutaneously with osmotic minipumps (Model 2004, Alza Corp). An AT2 receptor antagonist PD123319 (Sigma) was injected intraperitoneally everyday. A B2 receptor antagonist, DesArg9-[Leu8]-BK (DALBK; Sigma), was injected intraperitoneally everyday. A B1 receptor antagonist, Hoechst 140 (Sigma), was administered subcutaneously with osmotic minipumps. An NOS inhibitor N-nitro-l-arginine methyl ester (l-NAME; Sigma) was injected intraperitoneally everyday. All of these reagents were dissolved in normal saline.

Mouse Model of Angiogenesis

We used a mouse model of angiogenesis, in which the entire left femoral artery and vein were excised surgically. When hindlimb ischemia was induced, new blood vessels grew into the ischemic foci of the hindlimb. We prepared this model in WT and AT1aKO mice and used various pharmacological agents as described above. Mice were randomly assigned to 1 of the following experimental groups (Table S1, available online at http://hyper.ahajournals.org): (1) WT mice administered with normal saline (WT; n = 8); (2) AT1aKO mice administered with normal saline (KO; n = 10); (3) AT1aKO mice treated with high-dose imidapril (1 mg/kg per day; High Im; n = 9); (4) AT1aKO mice treated with high-dose imidapril (1 mg/kg per day) and PD123319 (30 mg/kg per day; High Im-PD123319; n = 6); (5) AT1aKO mice treated with high-dose imidapril (1 mg/kg per day) and DALBK (50 mg/kg per day; High Im-DALBK; n = 10); (6) AT1aKO mice treated with high-dose imidapril (1 mg/kg per day) and DALBK (50 ng/kg per day; High Im-DALBK; n = 10); (7) AT1aKO mice treated with high-dose imidapril (1 mg/kg per day) and l-NAME (20 mg/kg per day; High Im-l-NAME; n = 8); (8) AT1aKO mice treated with low-dose imidapril (0.1 mg/kg per day; Low Im; n = 8); (9) AT1aKO mice treated with low-dose imidapril (0.1 mg/kg per day) and PD123319 (30 mg/kg per day; Low Im-PD123319; n = 6); (10) AT1aKO mice treated with low-dose imidapril (0.1 mg/kg per day) and DALBK (50 ng/kg per day; Low Im-DALBK; n = 10); (11) AT1aKO mice treated with low-dose imidapril (0.1 mg/kg per day) and Hoechst 140 (100 µg/kg per day; Low Im-Hoe140; n = 8); and (12) AT1aKO mice treated with low-dose imidapril (0.1 mg/kg per day) and l-NAME (20 mg/kg per day; Low Im-l-NAME; n = 8).

In brief, mice were subjected to unilateral hindlimb ischemia under anesthesia with sodium pentobarbital (50 mg/kg IP). Before surgery and on postoperative days 3, 7, 14, and 21, body weight and systemic blood pressure were measured. Systemic blood pressure was determined using a tail-cuff pressure analysis system (Softron) in the conscious state. Capillary density and hindlimb blood flow were examined by the methods described below.

Laser Doppler Blood Flow Analysis

We measured hindlimb blood flow using a laser Doppler blood flowmetry (LDBF; MoorLDI, Moor Instrument), as described previously. Before and on postoperative days 0, 3, 7, 14, and 21, we performed LDBF analysis over the legs and feet. After scanning, stored images were analyzed to quantify blood flow, and mean LDBF values of the ischemic and nonischemic limbs were calculated. To avoid data variations because of ambient light and temperature, hindlimb blood flow was expressed as the ratio of the left (ischemic) to right (nonischemic) hindlimb LDBF.

Capillary Density Analysis

Capillary density was analyzed to obtain specific evidence of vascularity at the level of microcirculation. Tissue samples were obtained from the ischemic thigh adductor skeletal muscles on postoperative day 21. Frozen tissue sections with 5-µm thickness were prepared from each sample. Capillary endothelial cells were identified by immunohistochemical staining with a rat anti-mouse CD31 monoclonal antibody (PharMingen). Fifteen random microscopic fields from 2 different sections in each tissue block were examined for the presence of capillary endothelial cells, and capillary muscle fiber ratio was expressed as the ratio of the number of capillaries to the number of myofibers per high-power field (×400).

RT-PCR Analysis

Ischemic adductor muscles were obtained at 5 days after operation. Total RNA was prepared with the use of guanidium isothiocyanate-phenol chloroform solution (TRIzol reagent, Invitrogen), quantified by measuring absorption at 260/280 nm and subjected to RT-PCR analysis. Total RNA was first reverse transcribed using oligo-dT primers and RNase H reverse transcriptase (Superscript II, Invitrogen) with 1 µg of total RNA per sample. GAPDH expression was detected by RT-PCR as an internal control. PCR primers were designed for endothelial NOS (eNOS), vascular endothelial growth factor (VEGF)-A, vascular endothelial growth factor receptor (VEGFR)2, and GAPDH, as reported previously (Table S2). RT-PCR products were analyzed by 1.5% agarose gel electrophoresis.

Statistical Analysis

All of the values are expressed as mean±SEM. All of the data were subjected to 1-way ANOVA followed by Scheff’s analysis for comparison between any 2 means. P values <0.05 were considered to be statistically significant.

Results

Unilateral Hindlimb Ischemia

All of the mice survived after surgical induction of unilateral hindlimb ischemia and looked healthy during the follow-up period. Body weight did not differ among the groups throughout the experimental period (Table S3).
Effects of ACEI Imidapril on Angiogenesis in AT1aKO Mice

LDBF Analysis
Figure 1A shows representative LDBF images of hindlimb blood flow. Serial LDBF examination disclosed progressive recovery of hindlimb blood flow in WT mice after the induction of ischemia. In contrast, the LDBF ratio of AT1aKO mice remained impaired during the follow-up period, and the ratio of the ischemic:normal LDBF was persistently lower compared with WT mice (Figure 1B).

However, administration of imidapril at high dose (High Im; 1.0 mg/kg per day) significantly improved ischemia-induced angiogenesis in AT1aKO mice as assessed by the LDBF ratio, and the level was almost similar to the LDBF values observed in WT mice. Similarly, administration of imidapril at low dose (Low Im; 0.1 mg/kg per day) also improved ischemia-induced angiogenesis in AT1aKO mice, but there were no significant difference compared with AT1aKO mice at day 7.

Tissue Capillary Density
To investigate the extent of angiogenesis at the microcirculation level, we measured capillary density in a histological section harvested from the ischemic tissues. Figure 2A shows representative photomicrographs of tissue immunostained with an anti-CD31 monoclonal antibody of ischemic tissues at postoperative day 21 (×400). Brown reaction product indicates capillary endothelial cells. The ratio of ischemic:normal hindlimb LDBF decreased from 1.0 to ~0.15 after surgery in all of the groups, showing no difference in the ratio among the groups. Progressive recovery of blood flow within 21 days after surgery was observed in WT mice, whereas the blood flow ratios remained impaired in AT1aKO mice. Administration of imidapril with high- or low-dose rescued LDBF ratios.

**P < 0.01, ***P < 0.001, vs WT; ††P < 0.01, †††P < 0.001, High Im vs KO; #P < 0.05, ##P < 0.01, Low Im vs KO.

Figure 2. A, Representative photomicrographs of immunohistochemical staining with an anti-CD31 monoclonal antibody of ischemic tissues at postoperative day 21 (×400). Brown reaction product indicates capillary endothelial cells. B, Quantitative analysis revealed that the capillary density was significantly lower in AT1aKO than in WT mice. Capillary density was significantly increased in AT1aKO mice treated with imidapril compared with nontreated AT1aKO mice.

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capillary density between the high- and low-dose imidapril groups.

Effects of PD123319 on the Imidapril-Mediated Angiogenesis in AT1aKO Mice

In AT1aKO mice, the Ang II-AT2 receptor is present and may potentially affect angiogenesis even after the treatment with the ACEI imidapril. Accordingly, we tested the effects of a selective AT2 receptor antagonist, PD123319, on the imidapril-mediated angiogenesis in AT1aKO mice. PD123319 did not alter systemic blood pressure of AT1aKO mice treated with low- or high-dose imidapril (Table S3). After induction of limb ischemia, PD123319 significantly decreased the extent of angiogenesis in AT1aKO mice treated with low- or high-dose imidapril to the level even lower than that of nontreated AT1aKO mice when assessed by the ischemic/normal-limb LDBF ratio (Figure 3A and 3B) and capillary density (Figure 3C and 3D).

Effects of Hoechst 140, DALBK, and L-NAME on the Imidapril-Mediated Angiogenesis

To examine the roles of the BK-BK receptor pathway and NO formation in the proangiogenic effects of imidapril, B2 receptor antagonist Hoechst 140, B1 receptor antagonist DALBK, or NOS inhibitor L-NAME was administered with imidapril in AT1aKO mice. As shown in Figure 3A and 3B, treatment with Hoechst 140 significantly inhibited the stimulatory actions of low- and high-dose imidapril on the ischemic/normal hindlimb LDBF ratio. Similar antagonistic actions on imidapril-mediated angiogenesis were observed by L-NAME. Capillary density increase mediated by imidapril was also abolished by either Hoechst 140 or L-NAME as a similar manner observed in the LDBF ratio (Figure 3C and 3D). Interestingly, the B1 receptor antagonist DALBK administered with low- or high-dose imidapril also significantly suppressed angiogenesis to the level even lower than that of nontreated AT1aKO mice (Figure 3A through 3D).

Effects of PD123319, Hoechst 140, DALBK, and L-NAME on Imidapril-Mediated eNOS, VEGF-A, and VEGFR2 mRNA Expression

PD123319 inhibited the effects of imidapril on the expression of eNOS, VEGFR2, and VEGF mRNAs. Hoechst 140 and L-NAME inhibited the effects of imidapril on the expression of eNOS and VEGFR2 mRNA. The expression of VEGF mRNA was also suppressed by the treatment with DALBK.

Discussion

Major findings of the present study are as follows: (1) ischemia-induced angiogenesis was reduced in AT1aKO mice compared with WT mice, consistent with our previous findings; (2) an ACEI imidapril, with either low- or high-dose regimen, significantly augmented angiogenesis in ischemic hindlimb even in AT1aKO mice; (3) in AT1aKO mice, AT2 receptor antagonist PD123319 suppressed imidapril-induced angiogenesis; (4) the stimulatory effects of imidapril on angiogenesis in AT1aKO mice were attenuated by B2 receptor antagonist Hoechst 140, B1 receptor antagonist DALBK, or L-NAME treatment, and the degree of the suppression of ischemia-induced angiogenesis by DALBK was greater than that by Hoechst 140; and (5) eNOS mRNA expression was suppressed by the treatment with PD123319, Hoechst 140, and L-NAME; VEGF mRNA expression was suppressed by PD123319 and DALBK; and VEGFR2 mRNA expression was suppressed by PD123319, DALBK, Hoechst 140, and...
L-NAME and paralleled with the results of the LDBF ratio and capillary density.

Previous studies indicated that Ang II augmented angiogenesis in vitro and in vivo.\textsuperscript{2–5} We showed previously that ischemia-induced and tumor-related angiogenesis were suppressed in AT1aKO mice compared with WT animals and that Ang II type 1 receptor blocker (Ang II receptor blocker) candesartan inhibited angiogenesis in WT animals.\textsuperscript{6,7} These results support the idea that Ang II is proangiogenic through the AT1a receptor–mediated pathway. However, ACEI, which is known to reduce Ang II formation, also promoted angiogenesis in vitro and in vivo.\textsuperscript{8–10} Therefore, the role of the renin-angiotensin system in angiogenesis is still unclear, at least by experiments using the Ang II receptor blocker and ACEI. This may be because of different experimental models used in each study and different pharmacological actions between the Ang II receptor blocker and ACEI.

In the present study, capillary angiogenesis and blood flow recovery after hindlimb ischemia were impaired in AT1aKO mice compared with WT mice, consistent with our previous report.\textsuperscript{6} Imidapril treatment augmented angiogenesis in the ischemic limb in AT1aKO mice as assessed by the LDBF and capillary density. Major vascular actions of Ang II are genetically abolished in AT1aKO mice, but AT2 receptor function is still present and may potentially affect angiogenesis. In the present study, to investigate the role of AT2 receptor in the effects of ACEI, we treated AT1aKO mice with both imidapril and AT2 receptor antagonist. AT2 receptor antagonist PD123319, administered under the treatment with low- and high-dose imidapril, significantly suppressed angiogenesis to a level even lower than that of nontreated AT1aKO mice. This indicates that the AT2 receptor may also play important roles in imidapril-mediated angiogenesis, at least under the AT1a knockout condition.

To examine the role of the BK-BK receptor systems in the effects of ACEI, we used the B2 receptor antagonist Hoechst 140 and B1 receptor antagonist DALBK. The proangiogenic actions of imidapril were significantly attenuated by both Hoechst 140 and DALBK in AT1aKO mice. The B2 receptor is constitutively expressed in various tissues and is responsible for the major biological actions of BK. On the other hand, the B1 receptor is weakly expressed under physiological condition but strongly induced in response to pathological stimuli, such as inflammation, ischemia, or tissue injury.\textsuperscript{21,22} Therefore, it has been unclear as to whether angiogenic actions of ACEI are mediated by B1 receptor, B2 receptor, or both in ischemic tissues. Interestingly, the B1 receptor antagonist more remarkably inhibited imidapril-induced angiogenesis than did the B2 receptor antagonist in AT1aKO mice in the present study. The B1 receptor antagonist reduced LDBF to a level even lower than that of nontreated AT1aKO mice. Previous studies indicated that both B2 and B1 receptors were important for ischemia-induced angiogenesis.\textsuperscript{23–26} However, relative involvement of the 2 receptors remained unclear, because experimental models used varied, and the extent of tissue inflammation and/or injury might have been different in these previous studies as in our current study. Because B1 receptor expression is increased in the inflammatory tissues, contribution of the BK-B1 pathway may become greater in the ischemia-induced angiogenesis than in nonischemic angiogenesis. In fact, Emanueli et al.\textsuperscript{26} demonstrated that the BK-B1 receptor plays an essential role in the ischemia-induced angiogenesis in the mouse hindlimb tissues. These previous reports, as well as our current findings, suggest that the BK-B1 receptor pathway, in addition to the BK-B2 receptor pathway, is important for ischemia-induced angiogenesis by ACEI treatment.

Another interesting finding is that the NOS inhibitor L-NAME attenuated the imidapril-mediated angiogenesis in AT1aKO mice. We demonstrated previously that eNOS-derived NO in physiological concentration is an important regulatory molecule for ischemia-induced angiogenesis,\textsuperscript{15} and activation of the NO signaling pathway is also 1 of the

\begin{figure}[h]
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\caption{A, mRNAs of eNOS, VEGF, and VEGFR2 in ischemic limb tissues were determined by RT-PCR at postoperative day 5 (n=5 in each group). B through D, Quantitative evaluation of eNOS, VEGF, and VEGFR2 mRNA levels was expressed as a percentage of GAPDH mRNA. \textsuperscript{*}P<0.05, \textsuperscript{**}P<0.01 vs High Im group.}
\end{figure}
main mechanisms of BK-mediated angiogenesis.\textsuperscript{23–25} Therefore, our results suggest that the BK-B1/B2 receptor and the NO system are involved in the imidapril-induced angiogenesis in AT1aKO mice.

Furthermore, several studies suggested possible links between the AT2 receptors and BK receptors.\textsuperscript{27–30} In the present study, PD123319 suppressed the mRNA expressions of eNOS, VEGFR2, and VEGF. DALBK suppressed the mRNA expressions of VEGFR2 and VEGF. These indicated that the effect of the B1 receptor on imidapril-induced angiogenesis may be mediated by VEGF, not by the eNOS pathway, and the AT2 receptor might be also involved in this process. In addition, Hoechst 140 and L-NAME inhibited the expression of VEGFR2 and eNOS. Transactivation of VEGFR2 by BK and subsequent eNOS activation in endothelial cells were reported previously.\textsuperscript{31,32} These previous studies, together with our present findings, suggest that the BK/VEGFR2/eNOS and AT2/VEGFR2/eNOS pathways were also activated by imidapril treatment. Furthermore, a link between the AT2 receptor and B2 receptor might be present. These molecular cascades and interactions may be additional mechanisms by which the ACEI imidapril stimulated angiogenesis via AT2 receptor-, BK-B1-, and BK-B2-eNOS-dependent manners (Figure 5).

Our present findings have several important clinical implications. Clinical studies have demonstrated that ACEI is beneficial for suppressing major cardiovascular events post-myocardial infarction.\textsuperscript{33,34} The mechanisms have been largely attributed to the suppression of the development of heart failure because of myocardial remodeling. Our present findings further suggest that treatment with an ACEI, such as imidapril, would be beneficial in patients with acute limb ischemia and/or myocardial infarction by facilitating early postischemic angiogenesis (ie, collateral formation) and subsequent tissue healing. These are previously underestimated new mechanisms for the beneficial actions of ACEI in patients with postmyocardial infarction. Further clinical studies are warranted to examine the effects of ACEI on angiogenesis in patients with acute myocardial or peripheral tissue ischemia.

Perspectives

An ACEI, imidapril, induced angiogenesis in ischemic tissues via the BK-B1- and B2-receptor/NO-dependent and AT2 receptor-dependent pathways, which suggests that ACEI would be beneficial in patients with acute limb ischemia and/or myocardial infarction by facilitating early postischemic angiogenesis (ie, collateral formation) and subsequent tissue healing.

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Disclosures

None.

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