Antiangiogenic Effect of Angiotensin II Type 2 Receptor in Ischemia-Induced Angiogenesis in Mice Hindlimb

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Abstract—This study examined the potential role of angiotensin type 2 (AT2) receptor on angiogenesis in a model of surgically induced hindlimb ischemia. Ischemia was produced by femoral artery ligation in both wild-type and AT2 gene–deleted mice (Agtr2−/Y). After 28 days, angiogenesis was quantitated by microangiography, capillary density measurement, and laser Doppler perfusion imaging. Protein levels of vascular endothelial growth factor (VEGF), endothelial nitric oxide synthase (eNOS), Bax, and Bcl-2 were determined by Western blot analysis in hindlimbs. The AT2 mRNA level (assessed by semiquantitative RT-PCR) was increased in the ischemic hindlimb of wild-type mice. Angiographic vessel density and laser Doppler perfusion data showed significant improvement in ischemic/nonischemic leg ratio, 1.9- and 1.7-fold, respectively, in Agtr2−/Y mice compared with controls. In ischemic leg of Agtr2−/Y mice, revascularization was associated with an increase in the antiapoptotic protein content, Bcl-2 (211% of basal), and a decrease (60% of basal) in the number of cell death, determined by TUNEL method. Angiotensin II treatment (0.3 mg/kg per day) raised angiogenic score, blood perfusion, and both VEGF and eNOS protein content in ischemic leg of wild-type control but did not modulate the enhanced angiogenic response observed in untreated Agtr2−/Y mice. Finally, immunohistochemistry analysis revealed that VEGF was mainly localized to myocyte, whereas eNOS-positive staining was mainly observed in the capillary of ischemic leg of both wild-type and AT2-deficient mice. This study demonstrates for the first time that the AT2 receptor subtype may negatively modulate ischemia-induced angiogenesis through an activation of the apoptotic process. (Circ Res. 2002;90:1072-1079.)

Key Words: angiogenesis ■ ischemia ■ angiotensin II ■ AT2 receptor ■ apoptosis

Angiogenesis is the development of new vessels from preexisting blood vessels. This tightly controlled process is associated with pathological conditions such as tumor growth, diabetic retinopathy, and ischemic diseases. Hence, understanding mechanisms of angiogenesis is of major therapeutic importance. In ischemic diseases, both hypoxia and inflammation play a major role in the control of new vessels growth. 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. The growth-stimulating 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. Neovascularization appears to be also controlled by endothelial cell apoptosis. Growth factors, which are essential for angiogenesis, not only stimulate endothelial cell proliferation but concomitantly inhibit endothelial cell apoptosis. Hence, VEGF and basic fibroblast growth factor, 2 key factors involved in the angiogenic reaction, markedly inhibit endothelial cell apoptosis through the activation of the survival promoting phosphatidylinositol 3-kinase/Akt pathway and upregulation of the antiapoptotic protein Bcl-2. The execution phase of apoptosis is controlled by the Bcl-2 family members, which contain both inhibitors (Bcl-2) and inducers (Bax) of apoptosis. The balance between anti- and proapoptotic Bcl-2 family members is critical to determine if a cell undergoes apoptosis.

Numerous factors modulate vessel growth. Among these factors, angiotensin II (Ang II), the main effector peptide of the renin-angiotensin system, appears to be implicated in the regulation of the angiogenic process. Ang II stimulates endothelial and smooth muscle cell proliferation in vitro, increases vessel density in rat cremaster muscle in vivo angiogenesis in the rat subcutaneous sponge granuloma. The biological effects of Ang II are mainly exerted through 2 isoform receptors, AT1 and AT2. It is generally accepted that most of the well-known Ang II functions in the cardiovascular system are attributable to AT1, Ang II–induced activation of endothelial and smooth muscle cell growth is...
mediated by the AT1 receptor. Similarly, in the rat subcutaneous sponge granuloma, the Ang II angiogenic response is hampered by a selective AT1 receptor antagonist. Little information is available regarding the physiological roles of AT2 and its signal-transduction pathway. Several lines of evidence suggest that the AT2 receptor might mediate opposite effects to those related to AT1 receptor activation. AT2 receptor activation has been shown to suppress the Ang II–induced stimulation of endothelial cell proliferation, whereas AT2 receptor blockade enhanced the Ang II angiogenic effect in the rat subcutaneous sponge granuloma. Interestingly, one important emerging function of the AT2 receptor concerns its proapoptotic role. AT2 promotes apoptosis in a wide variety of cell types and in vascular smooth muscle cells of spontaneously hypertensive rats in vivo. These findings suggest a putative antiangiogenic effect of AT2. However, number of recent reports indicate that AT2 in cardiovascular tissues may be growth-promoting and share at least in part a common signaling pathways with AT1. Hence, AT2 mediates cardiac hypertrophy resulting from pressure overload and aortic hypertension induced by Ang II treatment. One of the important reasons for the disparity may be due to different experimental conditions. In particular, AT2 expression is known to be unstable or even absent in cultured cells.

To eliminate these variabilities, we used AT2-deficient (Agtr2/Y) mice and evaluated the hypothesis that endogenous AT2 could modulate ischemia-induced angiogenesis. To analyze the mechanism of the AT2 action, we evaluated the role of the VEGF/eNOS-related pathway and the role of the apoptotic reaction in the revascularization process associated with the genetic deletion of AT2.

Materials and Methods

Mouse Model of Ischemic Hindlimb

Male congenic C57BL/6J Agtr2/Y mice produced by 8 backcrosses to C57BL/6J, and their littermate wild-type controls underwent surgery to induce unilateral hindlimb ischemia, as previously described. Animals were anesthetized by isoflurane inhalation. A ligature was placed on the right femoral artery, 0.5 cm proximal to the bifurcation of a saphenous and popliteal arteries. Wild-type and AT2-deficient mice (5 animals per group) were then treated with or without Ang II (0.3 mg/kg per day) by using an osmotic minipump (Model 2004, Alza Corp) for 28 days. Animals were supplied by T. Inagami. 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).

Quantification of Angiogenesis

Microangiography

Vessel density was evaluated by high-definition microangiography at the end of the treatment period, as previously described. Briefly, animals were anesthetized (isoflurane inhalation) and a contrast medium (barium sulfate, 1 g/mL) was infused through a catheter introduced into the abdominal aorta. Images acquired by a digital x-ray transducer were assembled in order to obtain a complete view of the hindlimbs. The angiographic score was expressed as a percentage of pixels per image occupied by vessels in the quantification area. Quantification zone was delineated by the place of the ligature on the femoral artery, the knee, the edge of the femur, and the external limit of the leg.
chemiluminescent signal was detected, as previously described. The proteins were then stained with Ponceau Red (Sigma) for 10 minutes. Quantifications were performed by densitometric analysis after scanning using the Bio-Rad gel Doc 1000. Results are expressed as a ratio of quantification of the specific band to quantification of the transferred total protein bands stained with Ponceau Red.

In Situ Detection of Apoptotic Cell Death
In situ detection of apoptotic cells, using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) method of fragmented DNA was performed on cryostat sections, as previously described. Negative controls for TUNEL labeling were obtained after omission of the enzyme TdT.

Immunostaining
Frozen tissue sections (7 µm) were incubated with rabbit polyclonal antibody directed against either AT₁ (Santa-Cruz, dilution 1:25), VEGF (Santa-Cruz, dilution 1:25), eNOS (Santa-Cruz, dilution 1:50), and Bcl-2 (Santa-Cruz, dilution 1:25) to identify cells producing such factors. 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

Changes in AT₁ and AT₂ Receptor Level
We first verified whether ischemia-induced angiogenesis was associated with a rise in AT₁ mRNA level in the ischemic tissue. In wild-type mice, a marked increase in AT₁ mRNA level was detected in the ischemic legs compared with the nonischemic ones (169±13% versus 100±15%, respectively; *P<0.01; Figure 1).

We also assessed AT₁ receptor variation in AT₂-deficient mice. After 28 days of ischemia, in control animals, no significant changes were observed in AT₁ mRNA and protein levels between ischemic and nonischemic legs (data not shown). In the ischemic leg, we observed a 1.4-fold increase in AT₁ mRNA level in AT₂-deficient mice when compared with control animals (138±10% versus 102±9%, respectively; *P<0.05). Similarly, AT₁ protein level tended to be increased in AT₂-deficient mice when compared with control animals. Nevertheless, this raise did not reach statistical significance (131±21% versus 108±15%; *P>0.05). Finally, we observed by using immunohistochemistry a positive AT₁ staining in skeletal myocyte (Figure 7) and vessels (data not shown) in ischemic leg of both wild-type and AT₂-deficient mice.

Antiangiogenic Effect of AT₂ Receptor Subtype

Microangiography
The ischemic/nonischemic leg angiographic score ratio was increased by 1.9-fold in AT₂-deleted mice when compared with wild-type control (P<0.05). Ang II treatment raised the score by 1.7-fold in reference to that of untreated wild-type control (P<0.05). Conversely, administration of Ang II did not affect the activated angiogenic reaction observed in ischemic hindlimb of AT₂-deleted mice (Figure 2). No significant changes were observed in the nonischemic hindlimb (data not shown).

Capillary Density
Microangiographic data were confirmed by capillary density analysis. The ischemic/nonischemic leg capillary number ratio was enhanced by 1.7-fold in AT₂-deleted mice when compared with wild-type control (1.03±0.07 versus 0.61±0.05, respectively; *P<0.05). Ang II administration increased by 1.8-fold the ischemic/nonischemic leg capillary number ratio in reference to untreated control (1.10±0.09; *P<0.05). On the contrary, capillary number ratio was similar between Ang II-treated and untreated AT₂-deleted mice (1.11±0.08 versus 1.03±0.07; *P>0.05). No significant changes were observed in the nonischemic hindlimb (data not shown). Similar results were obtained with CD31 immunostaining, a specific staining of endothelial cells (data not shown).

Laser Doppler Perfusion Imaging
Microangiographic and capillary density measurements were associated with changes in blood perfusion. Hindlimb blood
flow recovery occurred in both wild-type and AT2-deficient mice treated or not with Ang II. However, in AT2-deficient mice, a greater increase in blood flow was evident by day 28 compared with control animals (1.7-fold; \( P < 0.05 \); Figure 3). Similarly, at day 28, a 1.7-fold increase was induced by Ang II treatment in the ischemic/nonischemic leg ratio in control mice (\( P < 0.01 \) versus untreated control mice). In contrast, Ang II treatment did not affect the enhanced blood flow recovery observed in AT2-deleted mice (Figure 3).

Molecular Mechanisms Associated With Antiangiogenic Effect of AT2 Receptor Subtype

**Regulation of VEGF Protein Level**

In the nonischemic leg, the VEGF protein level was unaffected in either group. In the ischemic hindlimbs, no differences were observed between control and AT2-deficient mice. In contrast, Ang II enhanced VEGF protein content by 56% in the ischemic leg of Ang II–treated animals in reference to untreated wild-type animals (\( P < 0.01 \)). Ang II also raised VEGF level by 41% in Ang II–treated AT2-deleted mice when compared with untreated AT2-deficient mice (\( P < 0.05 \)) (Figure 4). In an effort to localize VEGF, we performed VEGF immunostaining and demonstrated a marked VEGF staining mainly in myocyte of ischemic legs in both wild-type and AT2-deficient mice (Figure 7).

**Regulation of eNOS Protein Level**

In the nonischemic leg, eNOS protein level was unaffected in either group. In the ischemic hindlimb, eNOS content was raised by 47% in AT2-deleted mice when compared with wild-type control (\( P < 0.05 \)). In addition, Ang II enhanced eNOS protein content by 60% in the ischemic leg of Ang II–treated animals in reference to untreated wild-type animals (\( P < 0.05 \)). Such an Ang II effect was abrogated in Ang II–treated AT2-deleted mice compared with untreated AT2-deleted mice (Figure 5). By using immunohistochemistry, we detected a positive eNOS staining mainly in capillaries of both control and AT2-deficient mice (Figure 7).

**Regulation of Angiopoietin-2 Protein Level**

In the nonischemic leg, no significant changes in angiopoietin-2 content were observed in either group. In control animals, angiopoietin-2 protein content in the ischemic leg was similar to that of nonischemic one (111±15% versus 100±17%, respectively; \( P > 0.05 \)). In addition, in the ischemic leg, angiopoietin-2 content was unchanged in AT2-deleted mice and control animals (117±31% versus 111±15%; \( P > 0.05 \)). Angiopoietin-2 protein level was also unaffected by Ang II treatment in both AT2-deleted mice and control animals (118±24% and 112±29%, respectively; \( P > 0.05 \) versus control animals).

**Relation Between AT2, Bcl-2, and TUNEL Labeling**

**Regulation of Bax Protein Level**

In the nonischemic legs, Bax level was unaffected in either group. In the control group, Bax was reduced by 50% in the ischemic hindlimb in color-coded images, normal perfusion is depicted in red, a marked reduction in blood flow of ischemic hindlimb is in blue. B, Quantitative evaluation of blood flow expressed as a ratio of blood flow in the ischemic foot to that in the normal foot. Values are mean±SEM, \( n = 5 \) per group. *\( P < 0.05 \) vs control mice. Experimental conditions are identical as given in legend to Figure 2.

**Figure 3. A,** Foot blood flow monitored in vivo by laser Doppler perfusion imaging in control and Agtr2+/− mice. In color-coded images, normal perfusion is depicted in red, a marked reduction in blood flow of ischemic hindlimb is in blue. B, Quantitative evaluation of blood flow expressed as a ratio of blood flow in the ischemic foot to that in the normal foot. Values are mean±SEM, \( n = 5 \) per group. *\( P < 0.05 \) vs control mice. Experimental conditions are identical as given in legend to Figure 2.

**Figure 4. A,** Left, Representative Western blot of VEGF and actin protein content in the ischemic legs of control and AT2-deficient mice with or without Ang II treatment. Right, Membrane with transferred total protein bands stained with Ponceau Red. B, Quantitative evaluation of VEGF protein levels expressed as a percentage of nonischemic control. Values are mean±SEM, \( n = 5 \) per group. *\( P < 0.05 \), **\( P < 0.01 \) vs nonischemic control mice; †\( P < 0.05 \), ††\( P < 0.01 \) vs ischemic control mice; and ‡\( P < 0.05 \) vs untreated Agtr2+/− mice. Experimental conditions are identical as given in legend to Figure 2.
ischemic hindlimb compared with the nonischemic one (data not shown). In the ischemic hindlimb, no significant differences were observed between control and AT2-deficient mice. However, Ang II treatment raised Bax protein content by 64% and 39% in the ischemic leg of Ang II-treated wild-type and AT2-deficient mice, respectively, when compared with that of untreated wild-type control (P<0.05) (Figure 6).

**Regulation of Bcl-2 Protein Level**

In the nonischemic leg, Bcl-2 levels was unaffected in either group. In the control group, Bcl-2 was reduced by 50% in the ischemic hindlimb compared with the nonischemic one (data not shown). In the ischemic leg, Bcl-2 protein content was increased by 211% of basal in AT2-deficient mice as compared with wild-type control (P<0.01). In addition, Ang II treatment induced a 50% increase in Bcl-2 level in Ang II-treated control compared with untreated wild-type mice (P<0.05). Such an Ang II effect was not observed in Ang II-treated AT2-deficient mice compared with untreated AT2-deleted mice (Figure 6). We also determined Bcl-2 expression by immunohistochemistry and observed a positive staining mainly in capillaries of both control and AT2-deficient mice (Figure 7).

**Ratio Bcl-2/Bax in Ischemic Leg**

The ratio of Bcl-2/Bax protein content, reflecting the antiapoptotic process was similar in wild-type animals treated or not with Ang II (1.01±0.3 and 1.08±0.02, respectively). Such a ratio was markedly increased in AT2-deficient mice treated or not with Ang II (2.7- and 3.5-fold, respectively; P<0.001 versus wild-type control) (Figure 6).

**TUNEL Labeling**

As previously reported, cell death was detected in hindlimbs by using TUNEL labeling. There was no staining in the nonischemic tissue (Figure 8). The number of cell death was 1.9-fold lower in ischemic leg of AT2-deficient mice in reference to wild-type control. Ang II treatment did not affect the cell death number in either group (Figure 8).

**Discussion**

The main results of this study are that the AT2 receptor subtype negatively modulates ischemia-induced angiogenesis and that this effect is associated with an activation of the apoptotic process. Although accumulating lines of evidence indicate the growth inhibitory or stimulating function of AT2 in cardiovascular tissues, little is known about its role in the angiogenic process. In the present study, we detected a marked increase in the levels of the AT2 receptor subtype in the ischemic tissue of control mice. Hypoxia and inflammation are likely candidates to account for this finding. Indeed,
cardiac tissues under ischemic and inflammatory conditions show elevated $\text{AT}_2$ expression. $^{22,23}$ $\text{AT}_2$ transcription is also markedly stimulated by cytokines in cultured cells. $^{17,24,25}$ Taken together, these studies highlight the putative involvement of $\text{AT}_2$ in ischemia-induced angiogenesis. The main mechanism of hypoxia-induced angiogenesis involves the rise in hypoxia-inducible factor-1α protein, resulting in increased expression of VEGF and subsequently in that of eNOS. $^{1,2}$ In control mice, 28 days after ligation, the levels of VEGF and eNOS were similar in ischemic and nonischemic legs, probably due to the end of a brief angiogenic process, whereas treatment with Ang II was associated with a marked increase in both VEGF and eNOS protein content at day 28. Likewise, the renin angiotensin system has been shown to modulate the angiogenic response to electrical stimulation in rat skeletal muscle through activation of the VEGF-dependent pathway. $^{26}$ Renin gene transfer in Dahl-Sensitive rats with low plasma renin activity also restored angiogenesis and VEGF expression on electrical stimulation. $^{27}$ More recently, Ang II has been found to upregulate VEGF expression and subsequently activates vessel growth via $\text{AT}_1$ in an angiogenesis assay in the rabbit cornea. $^{28}$ Similarly, the VEGF and eNOS protein levels were increased after Ang II treatment in $\text{AT}_2$-deficient mice, suggesting that Ang II regulated VEGF and eNOS protein content through an $\text{AT}_1$ receptor subtype–related pathway. We observed that skeletal muscle previously shown to upregulate VEGF expression in response to exercise and electrical stimulation constitutes the principal source of VEGF in this model of hindlimb ischemia, as previously described. $^{29}$ We also observed a marked $\text{AT}_1$-positive staining in skeletal muscle, supporting a role for $\text{AT}_1$ in VEGF regulation. In addition, eNOS staining was mainly observed into capillaries of ischemic legs, as previously described. $^{30}$ Because endothelial cells express high level of VEGF receptors, this suggests that activation of VEGF production from skeletal muscle may increase eNOS signaling in capillary via a paracrine pathway. Interestingly, the present study also uncovered that in $\text{AT}_2$-deleted mice, the vessel growth was specifically increased in the ischemic hindlimb with no effect on the nonischemic contralateral hindlimb. $\text{AT}_2$ receptor blockade enhanced the Ang II angiogenic effect in the rat subcutaneous sponge granuloma. $^{10}$ Consistent with these in vivo observations, cultured vascular smooth muscle cells transfected with an $\text{AT}_2$-expression vector also exhibit decreased rates of DNA synthesis. $^{31}$ Another study also reports an antiproliferative influence of the $\text{AT}_2$ receptor on cultured cells. $^{7}$ Taken together, these results suggest that the $\text{AT}_2$ receptor may inhibit neovessel growth in vivo. The increase in revascularization observed in the ischemic hindlimb of $\text{AT}_2$-deleted mice was associated with a marked increase in the cell survivor factor Bcl-2. $\text{Bcl-2}$ can prevent and delay apoptosis induced by a wide variety of stimuli, suggesting that $\text{Bcl-2}$ controls a distal step in the final common pathway for cell death. $^{32}$ Our present findings that the number of apoptotic cells was decreased in the ischemic hindlimbs of $\text{AT}_2$-deficient mice is supported by this view. The $\text{AT}_2$ receptor has been shown to induce apoptosis in PC12W cells and confluent R3T3 cells. $^{12}$ Activation of the $\text{AT}_2$ receptor also inhibits mitogen-activated protein kinase,
resulting in the inactivation of Bcl-2 and the induction of apoptosis. Hence, we can speculate that the AT<sub>2</sub> receptor may control vessel growth associated with tissue ischemia through the activation of the apoptotic reaction. AT<sub>1</sub> receptor may also directly or indirectly modulate several cellular pathways that play an important role in the regulation of angiogenesis. Endothelial NOS protein level was markedly elevated in the ischemic hindlimb of AT<sub>2</sub>-deficient mice. NO is a key regulatory factor for ischemia-induced angiogenesis. Indeed, angiogenesis, in the ischemic hindlimb, was impaired in eNOS-deficient mice and capillary growth was stimulated in rabbit receiving NO donor. These findings suggest that capillaries include the requisite elements for an autocrine AT<sub>2</sub> receptor–related pathway. It is also likely that part of the AT<sub>2</sub> deletion-induced increase in eNOS protein content could also reflect the increased number of capillary within the ischemic leg of AT<sub>2</sub>-deficient mice.

The vascular response to Ang II is exaggerated in AT<sub>1</sub> knockout mice through, in part, an increased vascular AT<sub>1</sub> receptor expression. Accordingly, we evidenced a slight increase in AT<sub>1</sub> mRNA and protein levels in the ischemic leg of AT<sub>2</sub>-deficient mice. Hence, we can also propose that the increase in the angiogenic process observed in AT<sub>2</sub>-deficient mice might result, in part, from an activation of AT<sub>1</sub> receptor–related pathway and a subsequent upregulation of eNOS. However, Ang II treatment did not affect the enhanced ischemia-induced angiogenesis observed in AT<sub>2</sub>-deficient mice, suggesting that AT<sub>1</sub> activation was not involved in such an effect. Nevertheless, it is noteworthy that vessel density in the ischemic leg of AT<sub>2</sub>-deficient mice was similar from that observed in the nonischemic one, suggesting that the genetic deletion of AT<sub>2</sub> normalized the ischemic leg vascularization. It is likely that other stimuli may negatively regulate the angiogenic reaction and maintain vessel growth within the ischemic leg to a physiological level (similar to that observed in the nonischemic one) despite the angiogenic stimuli of Ang II. In conclusion, the present study demonstrates for the first time to our knowledge an antiangiogenic effect of AT<sub>2</sub> receptor subtype in mice with surgically induced hindlimb ischemia. This antiangiogenic effect might be associated with an activation of the apoptotic process. Further studies are necessary to determine the exact mechanism of the antiangiogenic effect of AT<sub>2</sub>.

Acknowledgments

This study was supported by grants from INSERM and Université Paris 7. R. Tamarat was a recipient of a fellowship from Société Française de Pharmacologie. T. Inagami was supported by grants HL8205 from NIH.

References

hamster hearts and inhibit cell growth and fibrillar collagen metabolism. 


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Circ Res. 2002;90:1072-1079; originally published online April 25, 2002;
doi: 10.1161/01.RES.000019892.41157.24
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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