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### Article

The role of the renin-angiotensin system (RAS) in angiogenesis is little known. Here, we show that the angiotensin II (ATII) type 1 (AT1) receptor plays an important role in ischemia-induced angiogenesis. Well-developed collateral vessels and angiogenesis were observed in wild-type (WT) mice in response to hindlimb ischemia, whereas these responses were reduced in ATII type 1a receptor knockout ( $AT1a^{-/-}$ ) mice. Ischemia-induced angiogenesis was also impaired in WT mice treated with the AT1 receptor blocker TCV-116. These effects were not due to reduced systemic blood pressure (SBP), because hydralazine treatment preserved angiogenesis in WT mice although it reduced SBP to a level similar to that of  $AT1a^{-/-}$  mice. Infiltration of inflammatory mononuclear cells (MNCs), including macrophages and T lymphocytes, was suppressed in the ischemic tissues of  $AT1a^{-/-}$  mice compared with WT mice. Double immunofluorescence staining revealed that infiltrated macrophages and T lymphocytes expressed VEGF, and the expression of VEGF and monocyte chemoattractant protein-1 was also decreased in  $AT1a^{-/-}$ . Finally, the impaired angiogenesis in  $AT1a^{-/-}$  mice was rescued by intramuscular transplantation of MNCs obtained from WT mice, further indicating the importance of MNC infiltration in ischemia-induced angiogenesis. Thus, the ATII-AT1 receptor pathway promotes early angiogenesis by supporting inflammatory cell infiltration and angiogenic cytokine expression.

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# Evidence for the importance of angiotensin II type 1 receptor in ischemia-induced angiogenesis

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The role of the renin-angiotensin system (RAS) in angiogenesis is little known. Here, we show that the angiotensin II (ATII) type 1 (AT1) receptor plays an important role in ischemia-induced angiogenesis. Well-developed collateral vessels and angiogenesis were observed in wild-type (WT) mice in response to hindlimb ischemia, whereas these responses were reduced in ATII type 1a receptor knockout (*AT1a*<sup>-/-</sup>) mice. Ischemia-induced angiogenesis was also impaired in WT mice treated with the AT1 receptor blocker TCV-116. These effects were not due to reduced systemic blood pressure (SBP), because hydralazine treatment preserved angiogenesis in WT mice although it reduced SBP to a level similar to that of *AT1a*<sup>-/-</sup> mice. Infiltration of inflammatory mononuclear cells (MNCs), including macrophages and T lymphocytes, was suppressed in the ischemic tissues of *AT1a*<sup>-/-</sup> mice compared with WT mice. Double immunofluorescence staining revealed that infiltrated macrophages and T lymphocytes expressed VEGF, and the expression of VEGF and monocyte chemoattractant protein-1 was also decreased in *AT1a*<sup>-/-</sup>. Finally, the impaired angiogenesis in *AT1a*<sup>-/-</sup> mice was rescued by intramuscular transplantation of MNCs obtained from WT mice, further indicating the importance of MNC infiltration in ischemia-induced angiogenesis. Thus, the ATII-AT1 receptor pathway promotes early angiogenesis by supporting inflammatory cell infiltration and angiogenic cytokine expression.

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## Introduction

The renin-angiotensin system (RAS) plays essential roles in the maintenance of vascular homeostasis (1). Recent large-scale clinical trials demonstrated that angiotensin-converting enzyme (ACE) inhibitors reduced not only the mortality rate due to cardiovascular disease but also the rate of tumor-related death (2). Because tumor growth depends on angiogenesis (3, 4), ACE inhibitors might inhibit angiogenesis and thereby retard tumor growth. However, there are several controversial reports regarding the effects of ACE inhibitors on angiogenesis. For example, the ACE inhibitor lisinopril is reported to suppress diabetic retinopathy, which is characterized by pathological angiogenesis on the retina (5). Likewise, Volpert et al. (6) showed that captopril suppresses tumor angiogenesis and growth. In contrast, Fabre et al. (7) reported that quinapril augments angiogenesis in a rabbit model of hindlimb ischemia, leaving the precise role of the RAS in angiogenesis unclear. Furthermore, in these previous studies, ACE inhibitors were used to suppress the functions of the RAS. However, there are limitations regarding the use of ACE inhibitors to examine the role of the RAS in vivo. First, ACE inhibitors suppress not only angiotensin II (ATII) syn-

thesis but also the breakdown of bradykinin (8). This would stimulate endothelial nitric oxide release (8), which in turn might promote angiogenesis (7, 9, 10). Second, because ATII is produced not only by ACE, but also by other enzymes, such as chymase (11), it is not possible to evaluate the role of ATII in angiogenesis in vivo using ACE inhibitors alone.

There are two major subtypes of ATII receptors, AT1 and AT2. In mice, the AT1 receptor is further subdivided into AT1a and AT1b receptors (12). Most of the well-known ATII functions in the cardiovascular system are mediated through the AT1 receptor (13, 14), especially through the AT1a receptor in rodents (15). We and other investigators recently generated AT1a knockout (*AT1a*<sup>-/-</sup>) mice that showed reduced systemic blood pressure (SBP) (16, 17). Accordingly, we used these mice to examine the role of the AT1a receptor in angiogenesis, employing a well-established mouse model of ischemia-induced angiogenesis.

## Methods

**Animals.** To obtain AT1a-deficient heterozygous (*AT1a*<sup>+/-</sup>) mice that have C57BL/6 background, a germline chimera derived from TT2 embryonic stem cells with a targeted

mutation of the *AT1a* gene as described previously (16) was backcrossed for five generations with C57BL/6 mice (18). The resulting *AT1a*<sup>-/-</sup> F<sub>5</sub> mice were then intercrossed to generate the homozygous (*AT1a*<sup>-/-</sup>) mice (16, 18). The *AT1a*<sup>-/-</sup> mice were then inbred to obtain an appropriate number of animals for the present study. As wild-type (WT; *AT1a*<sup>+/+</sup>) mice, C57BL/6-strain mice were obtained from Clea Japan Inc. (Tokyo, Japan). Male mice at the age of 8–10 weeks were used.

**Mouse model of angiogenesis.** The study protocols were approved by the Institutional Animal Care and Use Committee of Kurume University School of Medicine. We used a mouse model of angiogenesis, in which the entire left femoral artery and vein were excised surgically (10, 19). When hindlimb ischemia was induced, new blood vessels grew into the ischemic limb (10, 19). We prepared this model in *AT1a*<sup>-/-</sup> mice and WT mice and employed various pharmacological agents to determine whether ischemia-induced angiogenesis was affected by the deficiency of the AT1a receptor. In brief, mice were subjected to unilateral hindlimb ischemia under anesthesia with sodium pentobarbital (50 mg/kg intraperitoneally) (10). Before surgery and on postoperative days 3, 7, 14, 21, 28, and 35, body weight and SBP were determined. SBP was determined using a tail-cuff pressure analysis system (TK370C; UNICOM, Tokyo, Japan) in the conscious state. Capillary angiogenesis, collateral vessel formation (arteriogenesis), and limb blood flow were examined by the following methods.

**Laser Doppler blood flow analysis.** We measured hindlimb blood flow using a laser Doppler blood flow (LDBF) analyzer (Moor LDI; Moor Instruments, Devon, United Kingdom) as described previously (10). Before and on postoperative days 3, 7, 14, 21, 28, and 35, we performed LDBF analysis over the legs and feet. Blood flow (i.e., blood cell movement) was displayed as changes in the laser frequency using different color pixels. After scanning, the stored images were analyzed to quantify blood flow, and mean LDBF values of the ischemic and nonischemic legs were calculated. To avoid data variations due to ambient light and temperature, hindlimb blood flow was expressed as the ratio of left (ischemic) to right (nonischemic) LDBF.

**Angiographic score.** On postoperative day 14, under pentobarbital anesthesia (50 mg/kg intraperitoneally), the peritoneal cavity was opened, and a 26-gauge soft-tip catheter was inserted through the abdominal aorta. The lower limbs were gently perfused with 0.5 ml of warm saline containing heparin (10 U/ml). Post-mortem angiography was then performed by injecting 0.3 ml of contrast media through the catheter at a perfusion pressure of 80–90 mmHg. X-ray angiograms were taken using a mammography system (Senographe 500T; GE Medical Systems, Paris, France), and the extent of collateral vessel formation was quantified by angiographic score as described previously (20).

**Capillary density.** Capillary density within the ischemic thigh adductor skeletal muscles was analyzed to obtain specific evidence of vascularity at the level of microcir-

ulation. Three pieces of ischemic muscles were harvested from each animal, sliced, and fixed in methanol. Tissues were embedded in paraffin, and multiple tissue slices, 5 μm in thickness, were prepared. Capillary endothelial cells (ECs) were identified by immunohistochemical staining with a rat anti-mouse CD31 mAb (PharMingen, San Diego, California, USA). Fifteen random microscopic fields from three different sections in each tissue block were examined for the presence of capillary ECs, and capillary density was expressed as the number of capillaries per high power field (×400).

**Effects of hydralazine on ischemia-induced angiogenesis in WT mice.** *AT1a*<sup>-/-</sup> mice had lower SBP than WT mice (16, 17). We thus examined whether the lower SBP itself influenced ischemia-induced angiogenesis. We examined the effects of the reduction of SBP by hydralazine (Novartis Pharmaceuticals Corp., Tokyo, Japan) on angiogenesis in nine WT mice. SBP was reduced by oral hydralazine (50 mg/kg/d) in WT mice; our preliminary experiments showed that the SBP in WT mice treated with this dose of hydralazine was similar to that of *AT1a*<sup>-/-</sup> mice. On day 10 of hydralazine treatment, hindlimb ischemia was induced, and the ischemic/normal LDBF ratio was examined up to 35 days after surgery.

**Effects of TCV-116 on ischemia-induced angiogenesis in WT mice.** We examined whether selective pharmacological blockade of the AT1 receptor by candesartan cilexetil (TCV-116; Takeda Chemical Industries, Osaka, Japan) (12, 13, 21) in WT mice mimicked the altered angiogenic response observed in *AT1a*<sup>-/-</sup> mice. TCV-116 is a specific AT1 receptor antagonist, and its AT1-binding affinity is 80 times greater than that of losartan and 10 times greater than that of EXP 3174, the active form of losartan (12, 13). TCV-116 has been used to block the major vascular effects of ATII in previous *in vivo* studies (21–23). In preliminary experiments, we examined the dose-response effects of oral TCV-116 on SBP in WT mice. We used 5 mg/kg/d of TCV-116, because this dose reduced SBP in WT mice to a level similar to that of *AT1a*<sup>-/-</sup> mice. TCV-116 was administered via drinking water to 15 WT mice. On day 10 of the administration, mice were subjected to hindlimb ischemia, and ischemic/normal LDBF ratios were examined up to 35 days after surgery.

**Effects of PD123319 on ischemia-induced angiogenesis in *AT1a*<sup>-/-</sup> mice.** To further test the biological role of ATII in the ischemia-induced angiogenesis, we examined the effects of PD123319, a selective AT2 receptor antagonist, on angiogenesis in *AT1a*<sup>-/-</sup> mice with hindlimb ischemia. PD123319 has been used to block the biological effects of the ATII–AT2 receptor pathway in various *in vivo* experiments. We chose the dose of PD123319 (30 mg/kg/d) that effectively suppressed AT2 receptor function in previous studies (24). PD123319 was administered using an osmotic pump (ALZA Corp., Palo Alto, California, USA) to five *AT1a*<sup>-/-</sup> mice, which were then subjected to surgical hindlimb ischemia. Ischemic/normal LDBF ratios were examined up to 35 days after surgery.

**Histological analysis of the inflammatory responses.** ATII has been shown to play a proinflammatory role (25–27). We thus examined whether the extent of inflammatory reactions in the ischemic tissues differed between WT and *AT1a*<sup>-/-</sup> mice. Five-micrometer-thick multiple sections prepared from paraffin-embedded tissues of the ischemic limbs were used for histological analysis. Leukocyte infiltration was examined by hematoxylin and eosin (H&E) staining as well as immunohistochemical staining using an anti-mouse CD45 mAb (PharMingen), a common leukocyte antigen.

**VEGF expression in inflammatory mononuclear cells.** Inflammatory mononuclear cells (MNCs), mainly macrophages and T lymphocytes, release various angiogenic cytokines including VEGF (19, 28). Therefore, we also examined VEGF expression in MNCs in ischemic tissues using a double immunofluorescence staining technique. Cryostat sections, 5  $\mu$ m in thickness, from ischemic tissues were mounted on silicone-coated slides. They were then incubated overnight at 4°C with an anti-mouse VEGF mAb (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), and with either rat anti-mouse F4/80 mAb or rat anti-mouse CD3 mAb (Serotec Inc., Raleigh, North Carolina, USA) in a moist chamber. The slides were then incubated for 30 minutes at 37°C with an FITC-conjugated anti-goat IgG secondary antibody (Zymed Laboratories Inc., South San Francisco, California, USA) to detect VEGF. Then they were further incubated for 30 minutes at 37°C with TRITC-conjugated anti-rat IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) to detect macrophages or T lymphocytes. The slides were examined and photographed using a laser confocal microscope (Noran Instruments Inc., Middleton, Wisconsin, USA). The infiltrated macrophages, T lymphocytes, and VEGF-positive leukocytes per high power field ( $\times 400$ ) were then counted.

**Western blot analysis.** Protein extracts were obtained from homogenized ischemic skeletal muscles. One hundred micrograms of protein per sample was separated on a 12.5% polyacrylamide gel and electroblotted onto PVDF membranes (Trans-blot; Bio-Rad Laboratories Inc., Hercules, California, USA). The membrane was blocked with 10% nonfat dry milk in PBS with 0.2% Tween (T-PBS) and then probed with 1:100 of a goat polyclonal anti-mouse VEGF antibody (clone P-20) or with 1:100 of a goat polyclonal anti-mouse monocyte chemoattractant protein-1 (MCP-1) (clone M-18; Santa Cruz Biotechnology Inc.) for 3 hours at room temperature. After incubation with the primary antibody, the membrane was washed three times in T-PBS and then incubated for 1 hour with 1:5000 of anti-goat IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc.). The membrane was then washed in T-PBS, and antigen-antibody complexes were visualized using an enhanced chemiluminescence kit (ECL; Amersham Biosciences, Buckinghamshire, United Kingdom) at room temperature, followed by exposure to x-ray films (Hyperfilm; Amersham Biosciences).

**Isolation of peripheral blood MNCs from WT or *AT1a*<sup>-/-</sup> mice and implantation into the ischemic hindlimb of *AT1a*<sup>-/-</sup> mice.** Peripheral blood was obtained from WT and *AT1a*<sup>-/-</sup> mice. MNCs were isolated by centrifugation through a Histopaque-1083 density gradient (Sigma Chemical Co., St. Louis, Missouri, USA). In 14 *AT1a*<sup>-/-</sup> mice, left hindlimb ischemia was induced, and WT mouse-derived MNCs ( $4 \times 10^5$  cells per animal,  $n = 8$ ) or *AT1a*<sup>-/-</sup> mouse-derived MNCs ( $4 \times 10^5$  cells per animal,  $n = 6$ ) were implanted into the thigh adductor muscles in the ischemic limb on postoperative day 3. In five additional mice, neutralizing anti-mouse VEGF mAb (R&D Systems Inc., Minneapolis, Minnesota, USA) was continuously administered by a subcutaneously implanted osmotic pump (ALZA Corp.), and ischemia-induced angiogenesis was examined in *AT1a*<sup>-/-</sup> mice that had been subjected to WT-derived MNC transplantation. The ischemic/normal LDBF ratio was examined up to 35 days after surgery.

**Peritonitis model for *in vivo* leukocyte transendothelial migration assay.** To examine the effects of the AT1a receptor deficiency on *in vivo* leukocyte transendothelial migration, we employed an additional inflammation model: a murine peritonitis model induced by injecting oyster glycogen (intraperitoneally). This is an established model used to examine the degree of leukocyte transendothelial migration in response to inflammation *in vivo* (29, 30). *AT1a*<sup>-/-</sup> ( $n = 10$ ) and WT mice ( $n = 10$ ) were lightly anesthetized with pentobarbital (30 mg/kg intramuscularly), and 2 ml of 0.1 vol% oyster glycogen type II (Sigma Chemical Co.) in sterile saline was injected into the peritoneal cavity. Four hours after the injection, the abdominal wall was cut and the peritoneal cavity was opened with special care to avoid blood contamination. The peritoneal cavity was washed with 2 ml of warm heparinized (10 U/ml) saline; then peritoneal fluid was collected and centrifuged at 400 g for 10 minutes. The pellets were resuspended in PBS (1 ml), and the total number of transmigrated leukocytes was calculated using a hemocytometer (K-800; Sysmex, Kobe, Japan).

**Statistics.** All values are presented as the mean  $\pm$  SE. All data were subjected to ANOVA followed by Fisher's analysis for comparison between any two means. Probabilities of less than 0.05 were considered to be statistically significant.

## Results

**Unilateral hindlimb ischemia.** All 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 1). Immediately after the left femoral artery and vein were resected, the ratio of ischemic (left) to normal (right) hindlimb blood flow decreased from 1.0 to  $0.12 \pm 0.01$  in WT and  $0.12 \pm 0.02$  in *AT1a*<sup>-/-</sup> mice ( $P =$  not significant [NS]). Thus, the severity of induced ischemia was comparable in the two groups.

**Table 1**

Blood pressure and body weight of mice in each experimental group

SBP (mmHg) (n)	Before	Day 3	Day 7	Day 14	Day 21	Day 28	Day 35
WT (10)	94 ± 2	95 ± 1	96 ± 2	98 ± 2	98 ± 2	98 ± 3	99 ± 2
<i>AT1a</i> <sup>-/-</sup> (9)	72 ± 4 <sup>A</sup>	71 ± 4 <sup>A</sup>	73 ± 4 <sup>A</sup>	73 ± 3 <sup>A</sup>	73 ± 4 <sup>A</sup>	74 ± 4 <sup>A</sup>	74 ± 4 <sup>A</sup>
WT + TCV-116 (8)	70 ± 2 <sup>A</sup>	68 ± 2 <sup>A</sup>	69 ± 2 <sup>A</sup>	72 ± 1 <sup>A</sup>	68 ± 1 <sup>A</sup>	69 ± 1 <sup>A</sup>	69 ± 1 <sup>A</sup>
WT + Hyd (9)	70 ± 2 <sup>A</sup>	71 ± 1 <sup>A</sup>	70 ± 2 <sup>A</sup>	70 ± 1 <sup>A</sup>	71 ± 1 <sup>A</sup>	72 ± 1 <sup>A</sup>	71 ± 1 <sup>A</sup>
<i>AT1a</i> <sup>-/-</sup> + PD123319 (5)	82 ± 2 <sup>A</sup>	NM	79 ± 3 <sup>A</sup>	79 ± 2 <sup>A</sup>	82 ± 3 <sup>A</sup>	83 ± 2 <sup>A</sup>	84 ± 2 <sup>A</sup>
<i>AT1a</i> <sup>-/-</sup> + WT-MNCs (8)	72 ± 1 <sup>A</sup>	NM	74 ± 2 <sup>A</sup>	75 ± 1 <sup>A</sup>	76 ± 0 <sup>A</sup>	74 ± 1 <sup>A</sup>	76 ± 2 <sup>A</sup>
<i>AT1a</i> <sup>-/-</sup> + <i>AT1a</i> <sup>-/-</sup> -MNCs (6)	73 ± 2 <sup>A</sup>	NM	75 ± 2 <sup>A</sup>	74 ± 2 <sup>A</sup>	78 ± 2 <sup>A</sup>	79 ± 3 <sup>A</sup>	77 ± 1 <sup>A</sup>
<i>AT1a</i> <sup>-/-</sup> + WT-MNCs + VEGF mAb (5)	74 ± 1 <sup>A</sup>	NM	78 ± 1 <sup>A</sup>	76 ± 1 <sup>A</sup>	76 ± 1 <sup>A</sup>	75 ± 1 <sup>A</sup>	76 ± 1 <sup>A</sup>
Body weight (g) (n)	Before	Day 3	Day 7	Day 14	Day 21	Day 28	Day 35
WT (10)	24.2 ± 0.5	24.3 ± 0.4	24.1 ± 0.3	24.7 ± 0.4	24.5 ± 0.4	24.9 ± 0.4	25.7 ± 0.4
<i>AT1a</i> <sup>-/-</sup> (9)	24.9 ± 0.5	25.2 ± 0.6	25.1 ± 0.5	25.6 ± 0.6	25.2 ± 0.5	25.4 ± 0.5	26.0 ± 0.4
WT + TCV-116 (8)	21.6 ± 0.4	22.8 ± 0.5	22.2 ± 0.4	24.0 ± 0.4	24.6 ± 0.5	25.5 ± 0.5	25.5 ± 0.5
WT + Hyd (9)	22.7 ± 0.3	23.1 ± 0.5	23.8 ± 0.4	24.7 ± 0.4	25.5 ± 0.5	25.2 ± 0.6	25.8 ± 0.5
<i>AT1a</i> <sup>-/-</sup> + PD123319 (5)	23.1 ± 0.4	NM	23.4 ± 0.4	24.5 ± 0.2	25.6 ± 0.2	25.9 ± 0.2	25.9 ± 0.2
<i>AT1a</i> <sup>-/-</sup> + WT-MNCs (8)	23.2 ± 0.6	NM	23.9 ± 0.8	26.0 ± 0.7	26.6 ± 0.6	26.8 ± 0.6	27.2 ± 0.6
<i>AT1a</i> <sup>-/-</sup> + <i>AT1a</i> <sup>-/-</sup> -MNCs (6)	21.9 ± 0.2	NM	23.1 ± 0.2	24.4 ± 0.3	25.6 ± 0.2	25.8 ± 0.2	26.3 ± 0.1
<i>AT1a</i> <sup>-/-</sup> + WT-MNCs + VEGF mAb (5)	22.3 ± 0.3	NM	24.6 ± 0.5	24.4 ± 0.4	25.2 ± 0.2	25.9 ± 0.3	26.3 ± 0.1

VEGF mAb, neutralizing anti-VEGF mAb. NM, not measured. <sup>A</sup>*P* < 0.01 vs. WT mice. SBP did not differ among the groups of *AT1a*<sup>-/-</sup>, WT + TCV-116, WT + Hyd, *AT1a*<sup>-/-</sup> + PD123319, *AT1a*<sup>-/-</sup> + WT-MNCs, *AT1a*<sup>-/-</sup> + *AT1a*<sup>-/-</sup>-MNCs, and *AT1a*<sup>-/-</sup> + WT-MNCs + VEGF mAb. Body weight did not differ among all the groups.

**Serial 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 induction of ischemia (Figure 1, a and b). In contrast, the LDBF ratio of *AT1a*<sup>-/-</sup> mice remained impaired during the follow-up period, and the ratio of ischemic/normal LDBF was persistently low compared with WT mice (*P* < 0.05) (Figure 1b).

**Angiographic score.** We next examined whether the reduced LDBF ratio was associated with the number of angiographically visible collateral vessels (arteriogenesis). On day 14, postmortem angiography revealed a lower number of collateral vessels in the thigh adductor muscle area of *AT1a*<sup>-/-</sup> mice compared with WT mice (Figure 1c). Quantitative analysis of collateral vessels revealed that the angiographic score was significantly lower in *AT1a*<sup>-/-</sup> mice than in WT mice (Figure 1d).

**Tissue capillary density.** To investigate the extent of angiogenesis at the microcirculation level, we measured capillary density in histologic sections harvested from the ischemic tissues. Figure 1e shows representative photomicrographs of tissue immunostained with CD31. Quantitative analysis revealed that on postoperative day 14 the capillary density was significantly reduced in *AT1a*<sup>-/-</sup> mice compared with WT mice (Figure 1f).

**Effects of hydralazine on angiogenesis in WT mice.** SBP was lower in *AT1a*<sup>-/-</sup> mice than in WT mice (16, 17). We therefore examined the effects of the lower SBP itself on ischemia-induced angiogenesis. We administered hydralazine to WT mice to lower their SBP to a level similar to that of *AT1a*<sup>-/-</sup> mice (Table 1). The recovery of the LDBF ratio in hydralazine-treated WT mice after ischemia was completely preserved compared with nontreated WT mice (Figure 2). Thus, the reduced SBP itself did not impair angiogenesis in this mouse model.

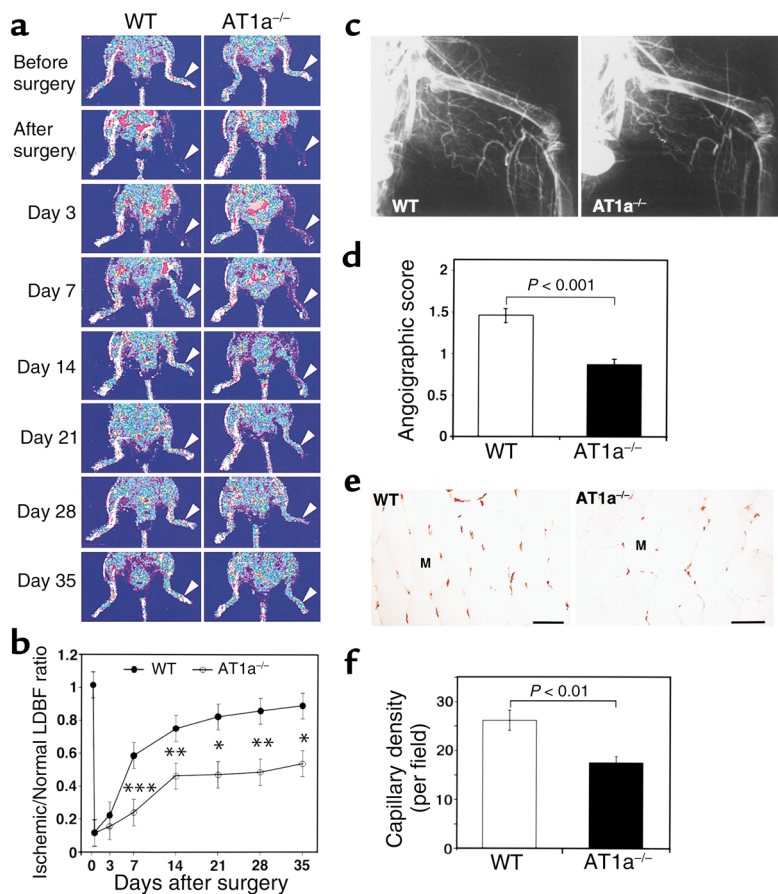
**Effects of TCV-116 on angiogenesis in WT mice.** We examined whether a pharmacological blockade of the AT1

receptor (by TCV-116) in WT mice would mimic the reduced angiogenic response observed in *AT1a*<sup>-/-</sup> mice. WT mice (*n* = 15) were treated with TCV-116, a selective AT1 receptor antagonist. TCV-116 reduced the SBP of WT mice to a level similar to that of *AT1a*<sup>-/-</sup> mice (Table 1). TCV-116 impaired ischemia-induced angiogenesis in WT mice as assessed by the LDBF ratio, which was similar to the LDBF values observed in *AT1a*<sup>-/-</sup> mice (Figure 2).

**Effects of PD123319 on angiogenesis in *AT1a*<sup>-/-</sup> mice.** In *AT1a*<sup>-/-</sup> mice, the ATII-AT2 receptor pathway is still functioning and may potentially affect angiogenesis. Accordingly, we tested the effects of the selective AT2 receptor antagonist PD123319 on ischemia-induced angiogenesis in *AT1a*<sup>-/-</sup> mice. PD123319 did not alter the SBP of *AT1a*<sup>-/-</sup> mice (*n* = 5) (Table 1). After induction of limb ischemia, PD123319 did not influence the extent of angiogenesis in *AT1a*<sup>-/-</sup> mice, as assessed by the ischemic/normal limb LDBF ratio (Figure 2).

**Tissue inflammatory responses.** The RAS plays proinflammatory roles (18, 25), and inflammation is an early trigger for ischemia-induced angiogenesis (31). We therefore examined histology of ischemic tissues on postoperative days 3 and 7. H&E staining and CD45 immunostaining revealed a marked infiltration of inflammatory leukocytes in WT mice. In contrast, the number of infiltrated leukocytes was lower in the ischemic tissues of *AT1a*<sup>-/-</sup> mice (Figure 3a). Moreover, the number of CD45-positive leukocytes was lower in *AT1a*<sup>-/-</sup> mice than in WT mice (Figure 3b).

To further determine the type of infiltrated leukocytes, we stained tissue sections with F4/80 mAb (for macrophages), anti-CD3 mAb (for T lymphocytes), and anti-CD45 mAb (for common leukocyte antigen). The quantitative analysis revealed that the number of infiltrated macrophages and T lymphocytes was again lower in *AT1a*<sup>-/-</sup> mice than in WT mice on days 3 and 7 (Figure 3d).



**Figure 1**

Angiogenesis was impaired in the ischemic hindlimb of *AT1a*<sup>-/-</sup> mice. (a) A low perfusion signal (dark blue) was observed in the ischemic hindlimb of an *AT1a*<sup>-/-</sup> mouse, whereas a high perfusion pattern (red to orange) was detected in a control WT mouse. (b) Computer-assisted quantitative analyses of hindlimb blood perfusion demonstrated a significant reduction in the ischemic/normal hindlimb blood flow ratio in *AT1a*<sup>-/-</sup> mice ( $n = 11$ ) compared with WT animals ( $n = 13$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , WT vs. *AT1a*<sup>-/-</sup>. (c) Postmortem angiograms showed markedly reduced collateral vessel formation in *AT1a*<sup>-/-</sup> mouse compared with WT mouse. (d) Quantitative analysis revealed a low angiographic score in *AT1a*<sup>-/-</sup> mice examined on postoperative day 14 ( $n = 5$  in each group). (e) Immunostaining of ischemic tissues with anti-CD31 mAb (brown) showed markedly reduced capillary density in an *AT1a*<sup>-/-</sup> mouse compared with a WT mouse. (f) Quantitative analysis revealed a reduced capillary density in *AT1a*<sup>-/-</sup> mice examined on postoperative day 14 ( $n = 5$  in each group).

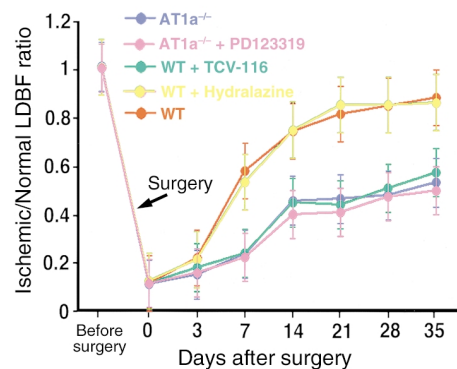
*Inflammatory leukocytes express angiogenic cytokine VEGF.* Inflammatory leukocytes release angiogenic cytokines including VEGF, which promote neovascularization (19, 28). To examine the relationship between leukocytes and VEGF expression, we performed double immunofluorescence staining for VEGF and leukocyte markers (CD3, F4/80, and CD45). There were VEGF<sup>-</sup> and F4/80<sup>-</sup> double positive macrophages as well as VEGF<sup>-</sup> and CD3<sup>-</sup> double positive T lymphocytes in ischemic tissues (Figure 3c). The number of VEGF-positive macrophages and T lymphocytes was lower in *AT1a*<sup>-/-</sup> mice than in WT mice on days 3 and 7 (Figure 3d). Double immunofluorescence staining showed that both macrophages and T lymphocytes were the major MNCs expressing VEGF. Skeletal myocytes in the ischemic tis-

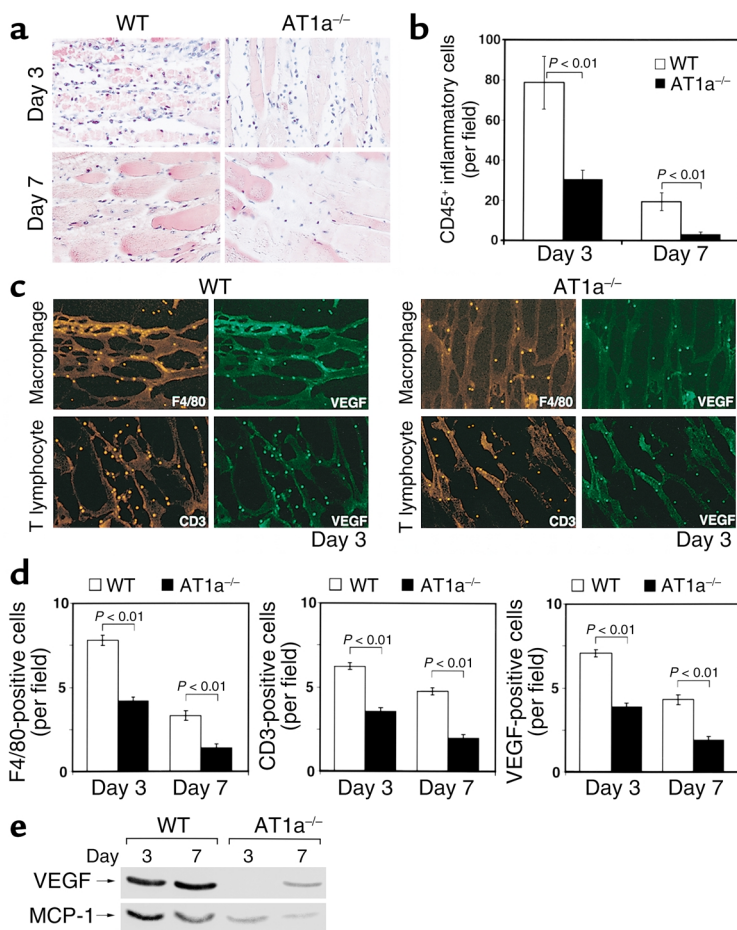
sues also expressed VEGF, but the extent of VEGF expression was comparable in WT mice and *AT1a*<sup>-/-</sup> mice. There were only a few VEGF<sup>-</sup> and CD45<sup>-</sup> double positive polymorphonuclear leukocytes (PMNs) (i.e., negative for both F4/80 and CD3) in ischemic tissues.

Because VEGF and MCP-1 are the major cytokines responsible for ischemia-induced angiogenesis as well as arteriogenesis (28–30, 32, 33), and because MCP-1 can recruit monocytes, we performed Western blot analysis to examine the endogenous expression of VEGF and MCP-1 in the ischemic tissues of WT mice and *AT1a*<sup>-/-</sup> mice. Western blot analysis revealed a marked reduction in the expression of both VEGF and MCP-1 proteins in *AT1a*<sup>-/-</sup> mice compared with WT mice on postoperative days 3 and 7 (Figure 3e).

**Figure 2**

Effects of hydralazine and ATII receptor blockers on ischemia-induced angiogenesis. No impairment in ischemic/normal hindlimb LDBF ratio was observed in WT mice treated with hydralazine (WT + Hydralazine) ( $P = NS$  vs. WT). Administration of the AT1 receptor blocker TCV-116 (5 mg/kg/d) impaired ischemia-induced angiogenesis in WT mice (WT + TCV-116) ( $P < 0.01$  vs. WT), so that the LDBF values were comparable to those observed in *AT1a*<sup>-/-</sup> mice. Administration of the AT2 receptor blocker PD123319 (30 mg/kg/d) did not alter ischemia-induced angiogenesis in *AT1a*<sup>-/-</sup> mice (*AT1a*<sup>-/-</sup> + PD123319) ( $P = NS$  vs. *AT1a*<sup>-/-</sup>).





**Figure 3**

Inflammatory responses and expression of angiogenic cytokines in ischemic tissues. (a) H&E staining revealed marked infiltration of inflammatory leukocytes in the ischemic tissues of WT mice but not in those of *AT1a*<sup>-/-</sup> mice. (b) Quantitative analysis revealed reduced inflammatory cell infiltration in *AT1a*<sup>-/-</sup> mice examined on days 3 and 7 after induction of limb ischemia (*n* = 4 in each group). (c) Double immunofluorescence staining of the ischemic tissues revealed that macrophages and T lymphocytes expressed VEGF protein. VEGF showed green fluorescence, whereas cell surface markers (F4/80 and CD3) were stained in red. Skeletal myocytes in the ischemic tissues also moderately expressed VEGF protein. (d) Quantitative analysis revealed that the number of infiltrating macrophages and T lymphocytes was significantly smaller in *AT1a*<sup>-/-</sup> mice than in WT mice. The number of VEGF-positive inflammatory cells was also significantly lower in *AT1a*<sup>-/-</sup> mice than in WT mice. (e) Western blot analysis revealed that the expression of VEGF and MCP-1 proteins in the ischemic tissues was weaker in *AT1a*<sup>-/-</sup> mice than in WT mice on days 3 and 7 after induction of limb ischemia.

*In vivo leukocyte migration assay using a peritonitis model.* We examined whether leukocyte infiltration was reduced in another model of inflammatory response in *AT1a*<sup>-/-</sup> mice. Intraperitoneal administration of oyster glycogen induces peritonitis, which is an established inflammation model in mice (29). In this model, peritonitis was associated with a marked accumulation of inflammatory leukocytes in the peritoneal cavity of WT mice ( $1.8 \times 10^6 \pm 0.2 \times 10^6$  cells, *n* = 10). However, in *AT1a*<sup>-/-</sup> mice (*n* = 10), there were only  $1.1 \times 10^6 \pm 0.1 \times 10^6$  cells (*P* < 0.01 vs. WT mice). Thus, the AT1a receptor deficiency was associated with reduced transendothelial migration of leukocytes in the peritonitis model.

*Effects of the implantation of WT mouse- or AT1a<sup>-/-</sup> mouse-derived peripheral blood MNCs on angiogenesis in AT1a<sup>-/-</sup> mice.* Because ischemic tissues of *AT1a*<sup>-/-</sup> mice contained fewer inflammatory leukocytes with VEGF expression compared with WT mice, we examined whether impaired angiogenesis in *AT1a*<sup>-/-</sup> mice could be rescued by implantation of WT mouse- or *AT1a*<sup>-/-</sup> mouse-derived peripheral blood MNCs. LDBF analysis showed that implantation of WT-derived MNCs into the ischemic hindlimb significantly restored the ischemic/normal LDBF ratio in *AT1a*<sup>-/-</sup> mice compared with nontransplanted *AT1a*<sup>-/-</sup> mice (Figure 4). In contrast, implantation of *AT1a*<sup>-/-</sup> mouse-derived MNCs

into the ischemic limb restored the ischemic/normal LDBF ratio slightly in *AT1a*<sup>-/-</sup> mice, but the values were not significantly different from those of *AT1a*<sup>-/-</sup> mice (Figure 4). Finally, under the treatment with a neutralizing anti-VEGF mAb, implantation of WT mouse-derived MNCs into the ischemic hindlimb no longer restored the angiogenesis in *AT1a*<sup>-/-</sup> mice, suggesting an important role of VEGF produced by MNCs in the rescue of ischemia-induced angiogenesis.

## Discussion

Angiogenesis, arteriogenesis, and blood flow recovery in response to hindlimb ischemia were significantly impaired in *AT1a*<sup>-/-</sup> compared with WT mice. Moreover, pharmacological blockade of the AT1 receptor by TCV-116 suppressed the ischemia-induced angiogenesis in WT mice. The impaired angiogenesis was not due to the reduced SBP, since hydralazine treatment did not influence angiogenesis. Therefore, the present study, for the first time to our knowledge, provides *in vivo* evidence that the ATII-AT1 receptor pathway is important for ischemia-induced angiogenesis. Our findings further extend previous reports indicating that ATII functions as a proangiogenic molecule (32–34). For instance, ATII induces VEGF expression in vascular smooth muscle cells, which stimulates EC proliferation, migration, and angiogenesis (32, 33). ATII also enhances VEGF-mediated angiogenesis (35).

ATII functions through *AT1a* and *AT2* receptors in mice, and the RAS is activated in *AT1a*<sup>-/-</sup> mice (16, 17). Therefore, in *AT1a*<sup>-/-</sup> mice, the ATII-AT2 receptor pathway is still functioning and could affect angiogenesis. Therefore, we tested the effects of the selective AT2

receptor antagonist PD123319 on ischemia-induced angiogenesis in *AT1a*<sup>-/-</sup> mice. PD123319 did not influence the extent of angiogenesis in *AT1a*<sup>-/-</sup> mice as assessed by the LDBF ratio, indicating that the ATII-AT2 receptor pathway may play a minimum role in modulating ischemia-induced angiogenesis in *AT1a*<sup>-/-</sup> mice. Our results are consistent with a previous report showing that the expression of AT2 receptor was low in vascular tissues and thus the ATII functioned through AT2 receptor only weakly within skeletal muscles (36).

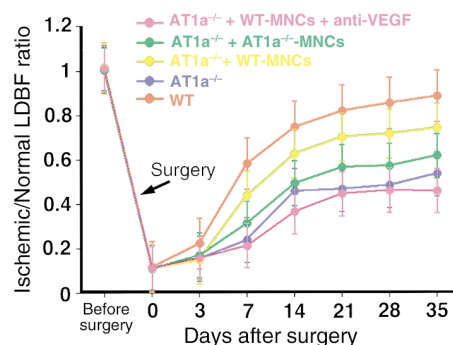
We next sought to explore the potential mechanism(s) for the impaired angiogenesis in *AT1a*<sup>-/-</sup> mice. During the examination of tissue histology of the ischemic skeletal muscles, we found a marked infiltration of inflammatory leukocytes in WT mice, whereas the number of leukocytes was lower in *AT1a*<sup>-/-</sup> mice. Moreover, the number of infiltrated macrophages and T lymphocytes was significantly lower in *AT1a*<sup>-/-</sup> mice than in WT mice on days 3 and 7. In the present study, transendothelial migration of leukocytes was also suppressed in *AT1a*<sup>-/-</sup> mice in the glycogen-induced peritonitis model. Our findings are consistent with previous studies indicating that ATII supported leukocyte transendothelial migration via an AT1 receptor-dependent, but blood pressure-independent, mechanism (37–39). Because inflammation is an early key process for ischemia-induced angiogenesis and arteriogenesis (31), it is conceivable that the deficiency of the *AT1a* receptor may have resulted in reduced inflammatory reactions, and that, consequently, angiogenesis was impaired. In this regard, recent studies showed that the RAS exists not only in the vascular wall but also in immune-privileged tissues and cells (26, 27), which play important proinflammatory roles (18, 25).

Infiltrated inflammatory leukocytes release angiogenic cytokines including VEGF, which promote neovascularization (19, 28). In fact, previous studies showed that the infiltration of VEGF-positive T lymphocytes and macrophages plays an important role in ischemia-induced and tumor-related angiogenesis (19, 28, 40, 41). In the present study, infiltrated macrophages and T lymphocytes expressed VEGF intensively, and the number of VEGF-positive macrophages and T lymphocytes was lower in *AT1a*<sup>-/-</sup> mice than in WT mice. There were only a few VEGF- and CD45-double positive PMNs lacking F4/80 and CD3 in ischemic tissues. The latter result was consistent with a report by Koehne et al. (42), who showed a lack of hypoxic stimulation of VEGF expression in PMNs. Skeletal myocytes in the ischemic tissues occasionally expressed moderate amounts of VEGF, but the extent of VEGF expression was almost comparable between WT mice and *AT1a*<sup>-/-</sup> mice. Taken together, infiltrated MNCs (i.e., macrophages and T lymphocytes) released VEGF protein, which might have facilitated ischemia-induced angiogenesis.

In the present study, arteriogenesis (i.e., angiographically visible collateral vessel formation) was also impaired in *AT1a*<sup>-/-</sup> mice compared with WT mice. Arte-

riogenesis is mainly mediated by the chemokine MCP-1 (43, 44), which is a key molecule for the recruitment of monocytes and macrophages. Recent studies documented that not only angiogenesis but also arteriogenesis in response to tissue ischemia depended on macrophage infiltration (31, 40, 41, 43). We therefore examined the tissue contents of both VEGF and MCP-1 in the ischemic hindlimb tissues of WT mice and *AT1a*<sup>-/-</sup> mice. Western blot analysis revealed a marked reduction in the expression of both VEGF and MCP-1 proteins in *AT1a*<sup>-/-</sup> mice compared with WT mice on postoperative days 3 and 7. This indicates that reduced macrophage infiltration and repression of angiogenic cytokines, such as VEGF and MCP-1, may account at least in part for the reduced macrophage infiltration as well as suppressed arteriogenesis in *AT1a*<sup>-/-</sup> mice.

To further determine the functional role of the infiltration of MNCs in angiogenesis, we tested whether implantation of WT mouse-derived MNCs or *AT1a*<sup>-/-</sup> mouse-derived MNCs into the ischemic tissues could rescue the impaired angiogenesis in *AT1a*<sup>-/-</sup> mice. We previously showed that direct tissue implantation of bone marrow MNCs or human cord blood-derived endothelial precursor cells augmented neovascularization in vivo (45, 46). LDBF analysis showed that implantation of WT mouse-derived MNCs into the ischemic limb significantly restored the ischemic/normal LDBF ratio in *AT1a*<sup>-/-</sup> mice. In contrast, implantation of *AT1a*<sup>-/-</sup> mouse-derived MNCs into the ischemic limb slightly restored the LDBF ratio in *AT1a*<sup>-/-</sup> mice, but the values were not significantly different from those of *AT1a*<sup>-/-</sup> mice. These findings suggest that inflammatory MNC infiltration is indeed an important event for the ischemia-induced angiogenesis. Moreover, the results suggest that the expression of *AT1a* receptor on MNCs is also important for inflammatory cell-mediated angiogenesis.



**Figure 4** Impacts of the implantation of WT mouse- or *AT1a*<sup>-/-</sup> mouse-derived MNCs into the ischemic hindlimbs of *AT1a*<sup>-/-</sup> mice on angiogenesis. The impaired LDBF ratio in *AT1a*<sup>-/-</sup> mice was partially but significantly restored after implantation of WT mouse-derived MNCs into the ischemic hindlimb of *AT1a*<sup>-/-</sup> mice ( $P < 0.01$  vs. *AT1a*<sup>-/-</sup>). In contrast, implantation of *AT1a*<sup>-/-</sup> mouse-derived MNCs slightly improved the LDBF ratio in *AT1a*<sup>-/-</sup> mice, but the difference was not significant (vs. *AT1a*<sup>-/-</sup>). Neutralizing anti-VEGF mAb treatment abolished the WT-MNC-mediated rescue of impaired angiogenesis in *AT1a*<sup>-/-</sup> mice ( $P = NS$  vs. *AT1a*<sup>-/-</sup>).



Finally, we examined whether VEGF released from transplanted WT-derived MNCs played a role in the rescue of angiogenesis in *AT1a*<sup>-/-</sup> mice. We tested the effects of a neutralizing anti-VEGF mAb on angiogenesis in *AT1a*<sup>-/-</sup> mice subjected to WT-derived MNC implantation. The anti-VEGF mAb treatment attenuated the WT-MNC-mediated rescue of the impaired angiogenesis in *AT1a*<sup>-/-</sup> mice. This result further indicates that the implantation of WT-derived MNCs rescued the angiogenesis in *AT1a*<sup>-/-</sup> mice mainly via a VEGF-dependent pathway.

There are several limitations to our study. First, the dose of TCV-116 was higher than the clinical dose (47). However, this dose was necessary to reduce the SBP of WT mice to a level similar to that of *AT1a*<sup>-/-</sup> mice, and this dose has been commonly used in animal models to block the AT1 receptor in vivo (21–23). Second, in several experimental groups, we measured only LDBF values to assess ischemia-induced angiogenesis. However, we and others previously demonstrated that tissue capillary density was well correlated with the ischemic/normal LDBF ratio in this mouse model of angiogenesis (10, 19). Thus, we believe that the LDBF ratio represents the extent of angiogenesis in the ischemic hindlimb. Third, Western blot analysis demonstrated lower expression of VEGF protein in homogenized tissue extracts in *AT1a*<sup>-/-</sup> mice than in WT mice. This method does not address the possibility that the expression of VEGF in skeletal muscles may differ between WT and *AT1a*<sup>-/-</sup> mice. However, this possibility is less likely, since our immunohistochemical staining revealed a comparable expression of VEGF in skeletal myocytes. Moreover, skeletal myocytes express only low amounts of AT1 and AT2 receptors (36, 48), and thus skeletal muscle VEGF expression may be little influenced by ATII.

In summary, our findings suggest that the ATII-AT1 receptor pathway plays an important role in ischemia-induced angiogenesis in vivo. The mechanism is likely related to the ATII-mediated inflammatory MNC infiltration. These are newly discovered functions of the ATII-AT1 receptor pathway in vivo. Moreover, our results have several clinical implications. First, there may be a therapeutic window for AT1 receptor antagonists in patients with acute ischemic diseases. Because angiogenesis is an important survival mechanism for tissues exposed to ischemia (49), the use of an AT1 receptor blocker early after ischemia may suppress subsequent angiogenesis, resulting in exacerbation of ischemia. Second, the suppression of inflammatory cell infiltration by the AT1 receptor blockage would provide a unique strategy against angiogenic disorders, including malignant tumors. In fact, recent studies suggest that infiltration of macrophages and T lymphocytes promote tumor-related angiogenesis (28, 50).

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