

# Involvement of the Renin–Angiotensin System in the Development of Vascular Damage in a Rat Model of Arthritis

## Effect of Angiotensin Receptor Blockers

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**Objective.** To explore the involvement of the renin–angiotensin system (RAS) in the development of vascular damage in adjuvant-induced arthritis (AIA) in rats.

**Methods.** Angiotensin II (Ang II; 0.25 or 1.0 mg/kg/day) was infused in control rats and rats with AIA for 21 days, and the impact of systemic inflammation on Ang II–induced hypertension, endothelial dysfunction, and vascular hypertrophy was evaluated. Expression of angiotensin II type 1 receptor (AT<sub>1</sub>R) and angiotensin-converting enzyme (ACE) in the aortas of rats with AIA were examined by real-time polymerase chain reaction (PCR) and Western blot analyses. Losartan (3 mg/kg/day) or irbesartan (5 mg/kg/day), both of which are AT<sub>1</sub>R blockers, was administered orally to rats with AIA for 21 days. In situ superoxide production in aortas was assessed according to the fluorogenic oxidation of dihydroethidium to ethidium. The expression and activity of NAD(P)H oxidases in aortas were examined by real-time PCR analysis and lucigenin chemiluminescence assay. Endothelial function in rats with AIA treated in vivo or ex vivo with AT<sub>1</sub>R blockers was also determined.

**Results.** The Ang II–induced hypertensive response, endothelial dysfunction, and vascular hypertro-

phy were exacerbated in rats with AIA. Expression of AT<sub>1</sub>R and ACE was increased in the aortas of rats with AIA. Both losartan and irbesartan decreased the levels of superoxide and the expression and activity NAD(P)H oxidases in the aortas of rats with AIA. The endothelial dysfunction in AIA was improved by the in vivo or ex vivo treatment with AT<sub>1</sub>R blockers.

**Conclusion.** The locally activated RAS is involved in the increased vascular oxidative stress and endothelial dysfunction in AIA. Our findings have important implications for clinical approaches to the reduction of cardiovascular risk in patients with rheumatoid arthritis.

The risks of cardiovascular disease and death are higher among patients with rheumatoid arthritis (RA) than among the general population (1–5). RA patients have a marked increase in carotid atherosclerosis that is independent of the classic risk factors (6). Recent studies have shown that the prevalence of cardiovascular disease and preclinical atherosclerosis in RA is increased to an extent that is at least comparable to that of diabetes mellitus (7,8). It has been postulated that systemic inflammation plays an important role in the pathogenesis of cardiovascular disease in RA (9–11).

Endothelial cell injury is an early step in the process of atherosclerosis. Previous studies identified the presence of endothelial dysfunction in RA patients with high levels of inflammatory activity (12–15). Consistent with the findings in human studies, we have shown impaired endothelial function in rats with adjuvant-induced arthritis (AIA), a well-known animal model of RA (16,17). Reactive oxygen species, which likely contribute to the pathophysiology of endothelial dysfunction, were shown to be overproduced in the aorta of rats with AIA (16). NAD(P)H oxidases are major

Supported in part by grants-in-aid from the Ministry of Education, Science, Culture, and Technology of Japan (19591191 and 21591274 to Dr. Morita) and by Research Project grants from Kawasaki Medical School (19-302T, 20-309T, and 21-308 to Dr. Morita).

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Submitted for publication August 21, 2009; accepted in revised form January 28, 2010.

sources of reactive oxygen species in the vasculature (18), and these enzymes were found to be responsible for the vascular oxidative stress in AIA (16,17). We postulated that further studies using the same model would provide additional understanding of the pathophysiology of vasculopathies in systemic inflammatory diseases.

It has been well established that activation of the renin-angiotensin system (RAS) plays an important role in the physiology and pathophysiology of the cardiovascular system. Angiotensin II (Ang II) regulates blood pressure and electrolyte homeostasis and contributes to the inflammatory response in the vascular wall (19). Ang II enhances the formation of cardiac and vascular reactive oxygen species through stimulation of NAD(P)H oxidase (20–22), causing endothelial dysfunction. Indeed, it has been demonstrated that RAS blockers, such as angiotensin-converting enzyme (ACE) inhibitors and angiotensin II type 1 receptor (AT<sub>1</sub>R) blockers, improve endothelial dysfunction in animal models and in patients with various vascular disease states, such as hypercholesterolemia and diabetes mellitus (23–25). Such agents, moreover, reduce both cardiovascular morbidity and mortality in these patients (26,27). These findings indicate the critical involvement of the RAS in the development of vascular damage caused by classic risk factors.

A role of the RAS in the pathophysiology of vasculopathies in RA or systemic inflammatory diseases has yet to be clearly established. Eight weeks of treatment with quinapril, an ACE inhibitor, in patients with RA was shown to have no significant effects on the endothelial vascular responses (28). However, treatment with ramipril, another ACE inhibitor, was found to improve the endothelial dysfunction in RA patients without affecting systemic inflammation (29). No data have previously been reported that explore the role of the RAS in the development of vascular damage in animal models of systemic inflammation, and data on the effects of AT<sub>1</sub>R blockers are also needed. Thus, we set out to determine whether RAS activation is involved in early vascular damage in the rat AIA animal model of RA.

## MATERIALS AND METHODS

**Induction and treatment of AIA in rats.** Six-week-old male Lewis rats were obtained from Japan SLC. Complete adjuvant was prepared by suspending heat-killed *Mycobacterium butyricum* (Difco) in mineral oil at a concentration of 10 mg/ml. Rats were injected intradermally with 100  $\mu$ l of the adjuvant at the base of the tail. The animals developed arthritis 10 days later. The normal nonarthritic and the arthritic rats

were divided into 3 treatment groups (5–6 rats per group): the control group received saline infusion, the Ang II low group received 0.25 mg/kg/day of Ang II, and Ang II high group received 1.0 mg/kg/day of Ang II. Briefly, the rats were anesthetized, and an osmotic pump containing 200  $\mu$ l of either saline or Ang II (Sigma-Aldrich) was implanted subcutaneously. Ang II was administered at a dosage of 0.25 mg/kg/day or 1.0 mg/kg/day for 21 days after the onset of arthritis.

Systolic arterial blood pressure was measured with a tail-cuff system using a pulse transducer (BP98-A; Softron). Rats were monitored for signs of arthritis, and the posterior limbs were individually scored (0–3 scale). Scores were assigned based on the extent of erythema or swelling or the severity of joint rigidity present in each posterior limb (maximum score of 6 per rat). Paw volume was measured with a water-displacement plethysmometer (MK-101CMP; Muromachi). Twenty-one days after the onset of arthritis, the rats were killed, and the thoracic aortas were isolated for further experiments.

In a separate experiment, 6-week-old male Lewis rats ( $n = 24$ ) were divided into 4 groups (6 rats per group): a normal control group, an AIA group, an AIA group treated with losartan, and an AIA group treated with irbesartan. Rats with AIA were administered 3 mg/kg of losartan (Sigma-Aldrich) or 5 mg/kg of irbesartan (Dainippon Sumitomo Pharma) by gavage once daily for 21 days after the onset of arthritis.

All procedures were performed in accordance with our institutional guidelines for animal research and were approved by the Animal Care and Use Committee of Kawasaki Medical School (no. 07-011).

**Endothelium-dependent vascular responses in rats with AIA.** The aorta was dissected out and cut into 3.0-mm rings, which were then placed in an organ bath. Endothelial vascular function was evaluated as described previously (16). Briefly, the aortic rings were suspended under 1g of tension and were precontracted by adding  $3 \times 10^{-7}M$  norepinephrine. After the contraction force had reached a plateau, acetylcholine ( $10^{-9}$ – $10^{-5}M$ ; an endothelium-dependent vasodilator) or sodium nitroprusside ( $10^{-9}$ – $10^{-5}M$ ; an endothelium-independent vasodilator) was added incrementally to the bath. In some experiments, the rings were exposed to *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) ( $10^{-4}M$ ; a nitric oxide synthase inhibitor) for 30 minutes before incubation with acetylcholine. The force of isometric contraction was measured using a force-displacement transducer (model MTOB-1Z; Labo Support). Responses to acetylcholine were expressed as a percentage of the precontracted tension induced by norepinephrine.

**Assessment of vascular wall hypertrophy.** The thoracic aorta was embedded in paraffin, and 4- $\mu$ m cross-sections were cut starting 5 mm from the aortic arch. Sections were stained with hematoxylin and eosin. To quantify wall thickness, the distance from the internal elastic lamina to the external lamina and the lumen diameter were measured at a minimum of 4 locations in the aortic section, and the ratio of the average medial thickness to the lumen diameter was calculated. We also evaluated the cross-sectional wall area. The perimeters of the internal and external laminae were traced, and the area inside each respective perimeter was determined. The lumen area represented the area enclosed by the internal elastic

lamina. The media area was obtained by subtracting the lumen area from the area encompassed by the external elastic lamina, and the ratio of media area to the lumen area was calculated. All measurements were performed using National Institutes of Health Image software (online at: <http://rsb.info.nih.gov/nih-image/>).

**Real-time quantitative polymerase chain reaction (PCR) analysis.** Total RNA was extracted with TRIzol reagent using the single-step method. Reverse transcriptase reactions were performed using a Ready-to-Go T-Primed First-Strand kit (Amersham Biosciences) for synthesis of the first-strand complementary DNA (cDNA). Real-time quantitative PCR was performed using an ABI Prism 7700 sequence detection system (Applied Biosystems). The sequences of primers and probes used for NAD(P)H oxidase components (p22<sup>phox</sup> and gp91<sup>phox</sup>), AT<sub>1</sub>R, and ACE have been described previously (30–32). The cDNA from serially diluted samples was amplified using Premix Ex Taq (Takara Bio). The threshold cycle is the PCR cycle at which an increase in the fluorescent emission above the baseline signal is first detected. Since the value of the threshold cycle decreases proportionally with increased target quantity, it was used to determine the relative amount of cDNA in each sample and to evaluate the levels of p22<sup>phox</sup>, gp91<sup>phox</sup>, AT<sub>1</sub>R, and ACE messenger RNA (mRNA), which were normalized to the quantity of GAPDH mRNA, as described previously (17,33).

**Western immunoblotting.** The expression of AT<sub>1</sub>R in aortas isolated from rats was assessed by Western blotting in the same manner as described previously (16,17). Anti-AT<sub>1</sub>R antibody (Abcam) and anti-GAPDH antibody (Santa Cruz Biotechnology) were used as the primary antibodies.

**Determination of ACE activity.** Aortic ACE activity was measured in a fluorescence assay using a commercial ACE activity assay kit (Life Laboratory). Briefly, samples of aorta were homogenized in an assay buffer and then clarified by centrifugation at 8,000g for 10 minutes at 4°C. ACE activity against a synthetic substrate (benzyloxycarbonyl-phenyl-alanyl-leucine) was determined using a colorimetric method. The product was measured fluorometrically at 320 nm of excitation and 405 nm of emission with a fluorocolorimeter, and the results were expressed relative to the control values in normal rats. All measurements were performed in duplicate.

**In situ measurement of superoxide (O<sub>2</sub><sup>-</sup>) by confocal fluorescence microscopy.** The oxidative fluorescent indicator dihydroethidium was used to evaluate in situ O<sub>2</sub><sup>-</sup> generation, as described previously (17). Briefly, fresh aortic rings embedded in OCT compound were cut into 20- $\mu$ m-thick sections, submerged in 2  $\mu$ M dihydroethidium (Sigma-Aldrich) in phosphate buffered saline (PBS), and incubated at 37°C for 30 minutes. At the end of this incubation period, the slides were washed with PBS and maintained at 4°C. Fluorescence was detected with a 585-nm long-pass filter by using a laser scanning confocal microscope (Leica Microsystems). The mean fluorescence intensity of aortic tissues (total of 24 locations in the aortic section from 6 rats per group) was analyzed with Leica TCS-NT system software (Leica Microsystems), and the results were expressed relative to the control values in normal rats.

**Measurement of NAD(P)H oxidase activity.** NAD(P)H oxidase activity in isolated aortas was assessed by lucigenin chemiluminescence after addition of NADPH in the same

manner as described previously (22). Briefly, 100  $\mu$ g of protein from aortic samples was diluted in modified HEPES buffer (140 mM NaCl, 5 mM KCl, 0.8 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM HEPES, and 1% glucose, pH 7.2). Dark-adapted lucigenin (5  $\mu$ M), with or without NADPH (100  $\mu$ M), was added just before reading. Lucigenin chemiluminescence was recorded for 3 minutes and was expressed as units per minute per milligram of weight (unit/minute/mg).

**Measurement of serum levels of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ).** Serum samples were collected 21 days after arthritis onset from rats with AIA treated or not treated with Ang II as well as from control rats. Serum concentrations of rat TNF $\alpha$  were determined by enzyme-linked immunosorbent assay (ELISA) using paired antibodies (R&D Systems) according to the manufacturer's instructions. The lower limit of detection for this cytokine was 62.5 pg/ml.

**Ex vivo treatment of aortas from rats with AIA with AT<sub>1</sub>R blockers.** The aortic rings isolated from rats with AIA were resuspended in RPMI 1640 (Life Technologies) supplemented with 1% heat-inactivated fetal calf serum. After incubating the aortas for 18 hours with or without 10  $\mu$ M losartan or irbesartan in 96-well flat-bottomed plates, the endothelium-dependent vascular responses were determined as described above.

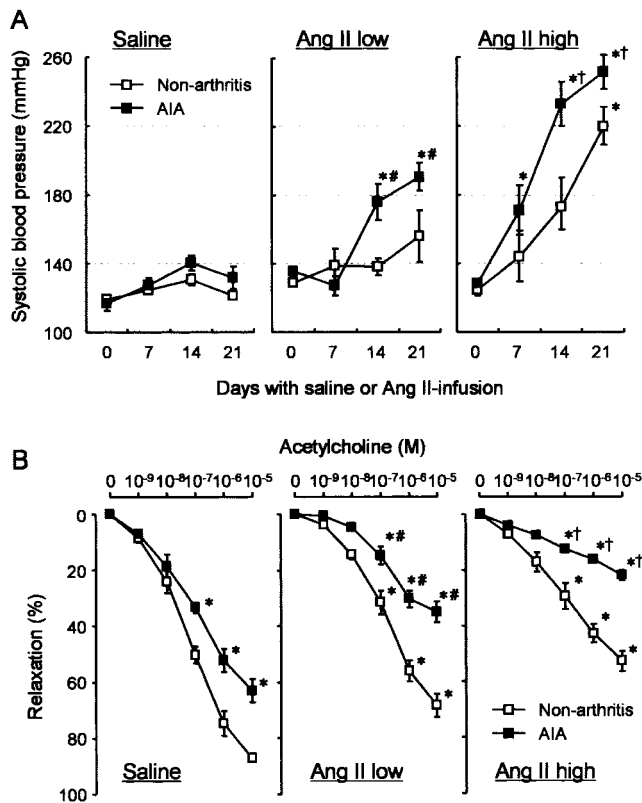
**Statistical analysis.** Data are expressed as the mean  $\pm$  SEM of the indicated number of samples studied. The Mann-Whitney U test was used to compare group data. *P* values less than 0.05 were considered statistically significant.

## RESULTS

**Exacerbation of Ang II-induced vascular responses in rats with AIA.** Treatment with Ang II induces hypertension, endothelial dysfunction, and vascular hypertrophy in animals (21,34). We first examined whether systemic inflammation in AIA affects Ang II-induced vascular responses in rats. Normal control rats and rats with AIA were treated with saline or exogenous Ang II at a dosage of 0.25 or 1.0 mg/kg/day for 21 days. The severity of clinical arthritis was not affected by Ang II infusion during the course of disease (results not shown). Of note, the hypertensive response to Ang II was more profound (*P* < 0.05) in rats with AIA than in nonarthritic rats (Figure 1A).

We next evaluated endothelium-dependent vasodilatory responses in rats with AIA treated with saline or Ang II (Figure 1B). Ang II resulted in a dose-dependent and significant depression of endothelium-dependent vasodilation. We found that the Ang II-induced endothelial dysfunction was significantly exacerbated (*P* < 0.05) in rats with AIA as compared with that in nonarthritic rats.

To evaluate vascular hypertrophy, we also examined the medial thickness of aortas from rats with AIA



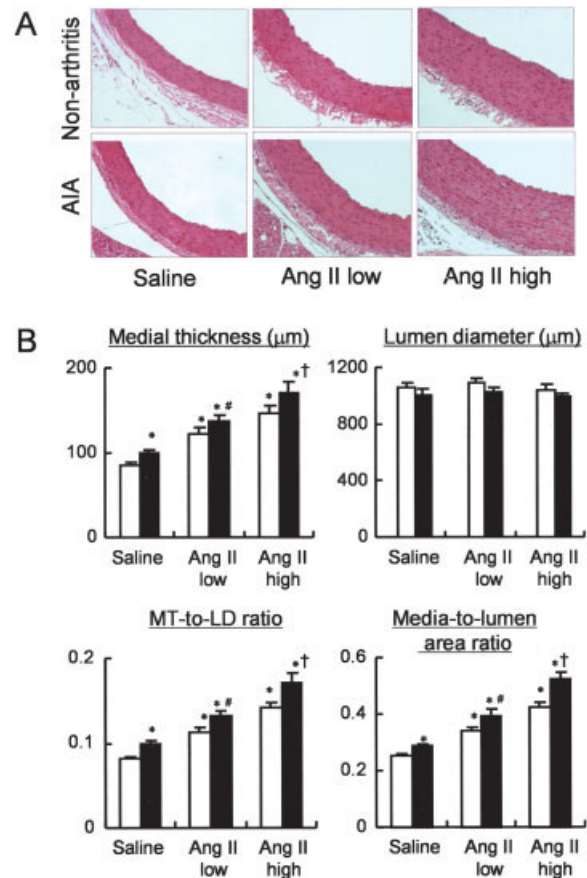
**Figure 1.** Exacerbation of angiotensin II (Ang II)-induced hypertension and endothelial dysfunction in rats with adjuvant-induced arthritis (AIA). Saline or Ang II (low-dose 0.25 mg/kg/day or high-dose 1.0 mg/kg/day) was administered to control rats and rats with AIA for 21 days after the onset of arthritis. **A**, Systolic blood pressure values obtained on the indicated days. **B**, Endothelium-dependent vasodilation induced by acetylcholine. Values are the mean  $\pm$  SEM of 5–6 rats per group. \* =  $P < 0.05$  versus saline-infused normal control rats; # =  $P < 0.05$  versus low-dose Ang II-infused rats without arthritis; † =  $P < 0.05$  versus high-dose Ang II-infused rats without arthritis.

treated with saline or Ang II (Figure 2). The medial thickness was slightly, but significantly ( $P < 0.05$ ), greater in saline-infused rats with AIA than in normal control rats. Treatment with Ang II resulted in dose-dependent and significant increases in medial thickness ( $P < 0.05$ ) but not lumen diameter. These effects were more profound ( $P < 0.05$ ) in rats with AIA than in nonarthritic control rats. Similar results were obtained when we evaluated the ratio of the medial thickness to the lumen diameter and when we evaluated the cross-sectional wall area. These results indicate that the Ang II-induced vascular hypertrophic responses in rats were significantly exacerbated in the presence of systemic inflammation.

In the aortas of rats with AIA, oxidative stress is

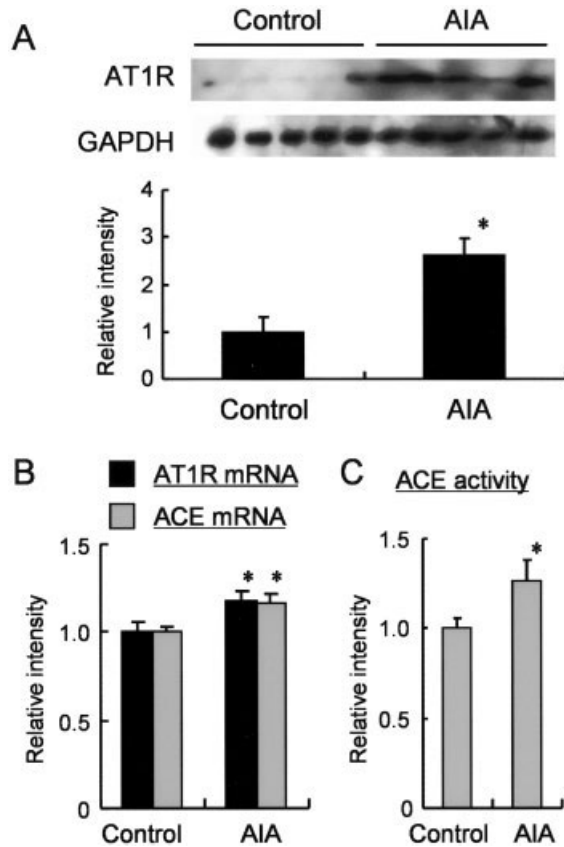
increased, and NAD(P)H oxidases are responsible for the vascular overproduction of  $O_2^-$  (16,17). In the present study, we found that mRNA expression and activity of NAD(P)H oxidases in rat aortas were increased by the induction of AIA as well as by the infusion of Ang II infusion, and the increase was further enhanced by the combination of both stimuli (results not shown).

Serum concentrations of lipids were measured 21 days after the onset of arthritis. There was no difference

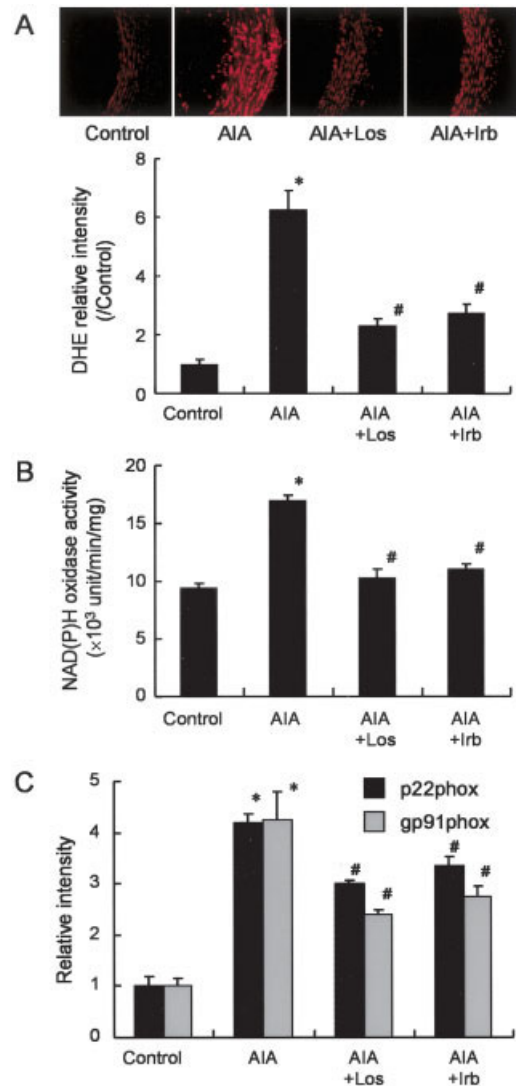


**Figure 2.** Aortic hypertrophy in rats with adjuvant-induced arthritis (AIA) infused with angiotensin II (Ang II; low-dose 0.25 mg/kg/day or high-dose 1.0 mg/kg/day). **A**, Representative images of hematoxylin and eosin-stained aortic cross-sections (original magnification  $\times 200$ ). **B**, Aortic hypertrophy in nonarthritic rats (open bars) and rats with AIA (solid bars), as indicated by the aortic medial thickness, the lumen diameter, the ratio of the medial thickness (MT) to the lumen diameter (LD), and the ratio of the media area to the lumen area. See Materials and Methods for details. Values are the mean and SEM of 5–6 rats per group. \* =  $P < 0.05$  versus aortas from saline-infused normal control rats; # =  $P < 0.05$  versus aortas from rats without arthritis infused with low-dose Ang II; † =  $P < 0.05$  versus aortas from rats without arthritis infused with high-dose Ang II.

between normal rats, untreated rats with AIA, and Ang II-treated rats with AIA in terms of serum levels of total cholesterol (mean  $\pm$  SEM  $83.5 \pm 1.9$  pg/ml in normal rats,  $68.7 \pm 0.5$  in untreated rats with AIA,  $83.0 \pm 2.6$  in rats treated with low-dose Ang II,  $69.5 \pm 0.7$  in rats with AIA that received low-dose Ang II,  $90.7 \pm 3.3$  in rats treated with high-dose Ang II, and  $99.8 \pm 6.0$  in rats with



**Figure 3.** Expression and activity of components of the renin-angiotensin system in aortas isolated from rats with or without adjuvant-induced arthritis (AIA). **A**, Western blot analysis of angiotensin II type 1 receptor (AT<sub>1</sub>R) in isolated rat aortas (top) and densitometric quantification of the corresponding bands (bottom). Densitometric quantification was performed using image analysis software from the National Institutes of Health. Values are the mean and SEM of 5 rats per group. GAPDH was included as a loading control for protein in the Western blots. **B**, AT<sub>1</sub>R and angiotensin-converting enzyme (ACE) mRNA expression in isolated rat aortas. Real-time polymerase chain reaction analysis was performed as described in Materials and Methods. Levels of AT<sub>1</sub>R and ACE mRNA were normalized to the level of GAPDH mRNA. **C**, ACE activity in isolated aortas ( $n = 6$  rats per group). Aortic ACE activity was measured in a fluorescence assay as described in Materials and Methods. Values are the mean and SEM of 6 rats per group and are expressed relative to the control values. \* =  $P < 0.05$  versus normal control rats.



**Figure 4.** Reduction of oxidative stress in the aortas of rats with adjuvant-induced arthritis (AIA) treated with angiotensin II type 1 receptor (AT<sub>1</sub>R) blockers. **A**, Superoxide ( $O_2^-$ ) production in aortic tissues of normal control rats and rats with AIA that were left untreated or were treated with an AT<sub>1</sub>R blocker: losartan (Los; 3 mg/kg/day) or irbesartan (Irb; 5 mg/kg/day), administered orally for 21 days after the onset of arthritis. In situ  $O_2^-$  generation was evaluated by fluorogenic oxidation of dihydroethidium (DHE) to ethidium, with imaging by confocal laser scanning microscopy (magnification  $\times 200$ ). Representative images are shown (top). The mean fluorescence intensity of the aortic tissues (bottom) was quantified as described in Materials and Methods. **B** and **C**, Activity and expression of NAD(P)H oxidase components in the aortas of normal control rats and rats with AIA that were left untreated or were treated with an AT<sub>1</sub>R blocker: losartan or irbesartan. Homogenates of the isolated aortas were incubated with NADPH, and  $O_2^-$  production was measured according to the lucigenin chemiluminescence (**B**). Real-time polymerase chain reaction analysis was performed to evaluate the mRNA levels of p22<sup>phox</sup> and gp91<sup>phox</sup> (**C**). Values are the mean and SEM of 6 rats per group. \* =  $P < 0.05$  versus control rats; # =  $P < 0.05$  versus untreated rats with AIA.

**Table 1.** Physical features of the rats\*

	Weight, gm	Systolic BP, mm Hg	Arthritis score	Paw volume, ml
Normal rats	307 ± 11	119 ± 2	0	1.70 ± 0.02
Rats with AIA				
Untreated	245 ± 11†	122 ± 2	5.8 ± 0.2	2.03 ± 0.09†
Losartan-treated	224 ± 8†	125 ± 4	5.7 ± 0.3	2.23 ± 0.04†
Irbesartan-treated	212 ± 4†	114 ± 3	5.6 ± 0.2	2.38 ± 0.11†

\* Rats with adjuvant-induced arthritis (AIA) were left untreated or were treated orally with losartan at a dosage of 3 mg/kg/day or with irbesartan at a dosage of 5 mg/kg/day for 21 days after the onset of arthritis. Body weight, systolic blood pressure (BP), arthritis score, and paw volume were measured 21 days after the onset of arthritis. The arthritis score and paw volume were assessed as described in Materials and Methods. Values are mean ± SEM of 6 rats per group.

†  $P < 0.05$  versus normal rats.

AIA that received high-dose Ang II). Serum triglyceride levels were significantly lower ( $P < 0.05$ ) in untreated rats with AIA than in normal rats, as we have previously reported (17), and the levels were decreased ( $P < 0.05$ ) by Ang II infusion (mean ± SEM 169.3 ± 11.3 pg/ml in normal rats, 55.8 ± 6.7 in untreated rats with AIA, 123.8 ± 10.7 in rats treated with low-dose Ang II, 38.2 ± 4.1 in rats with AIA that received low-dose Ang II, 109.3 ± 7.7 in rats treated with high-dose Ang II, and 35.2 ± 8.4 in rats with AIA that received high-dose Ang II).

We also attempted to measure serum levels of TNF $\alpha$ , which potentially affects endothelial dysfunction and vascular hypertrophy. However, serum concentrations of this cytokine in untreated rats with AIA or in Ang II-treated rats with AIA were below the sensitivity of ELISA used in our study.

**Increased expression of AT<sub>1</sub>R in the aortas of rats with AIA.** To examine the mechanism underlying the exacerbation of Ang II-induced vascular responses in rats with AIA, we assessed the expression of AT<sub>1</sub>R in their aortas. We found that both protein and mRNA levels of AT<sub>1</sub>R in the aortas of rats with AIA were significantly higher ( $P < 0.05$ ) than those in the aortas of control rats (Figures 3A and B). We further found that mRNA levels and enzymatic activity of ACE in the aortas were significantly higher ( $P < 0.05$ ) in rats with AIA than in normal rats (Figures 3B and C).

We also found in the present study that infusion of Ang II increased the mRNA expression levels of AT<sub>1</sub>R and ACE. Furthermore, Ang II-induced expression levels of both AT<sub>1</sub>R and ACE were significantly enhanced in rats with AIA as compared with control rats (results not shown).

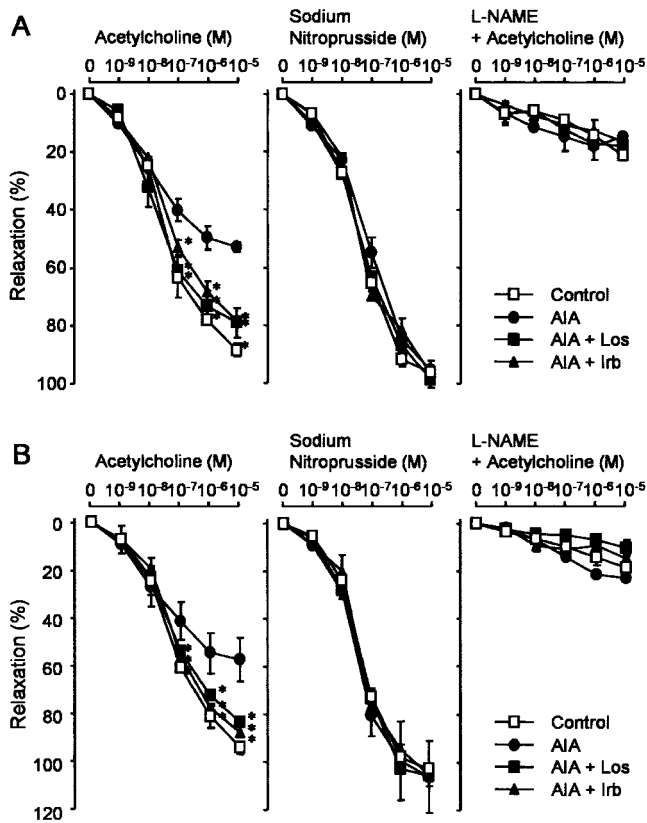
**Effect of AT<sub>1</sub>R blockers on enhanced oxidative stress in the aortas of rats with AIA.** To investigate whether RAS activation is involved with the enhanced

vascular oxidative stress, rats with AIA were treated for 21 days with 1 of 2 different compounds of AT<sub>1</sub>R blocker: losartan or irbesartan. Vascular O<sub>2</sub><sup>-</sup> production was evaluated by dihydroethidium conversion to ethidium and was imaged by confocal laser scanning microscopy (Figure 4A). Both losartan and irbesartan were found to decrease the levels of O<sub>2</sub><sup>-</sup> that were expressed in the tissue.

The physical features of the rats are summarized in Table 1. Despite the apparent antioxidative effect on the vasculature, neither losartan nor irbesartan affected the clinical severity of arthritis during the course of disease. Administration of losartan to rats with AIA did not alter blood pressure. Blood pressure in rats with AIA tended to be decreased following 21 days of treatment with irbesartan, but the reduction was not statistically significant ( $P = 0.065$ ). The mean body weight of rats with AIA was 80% of the weight of normal control rats and was not significantly affected by treatment with the AT<sub>1</sub>R blockers.

We next investigated the activity and expression of NAD(P)H oxidases in rat aortas. Consistent with our previous findings (16,17), NAD(P)H oxidase-derived O<sub>2</sub><sup>-</sup> production and expression of mRNA for NAD(P)H oxidase components p22<sup>phox</sup> and gp91<sup>phox</sup> were significantly higher in the aortas of rats with AIA than in the aortas of normal rats (Figures 4B and C). In the present study, we found that the activity and expression of NAD(P)H oxidases in the aorta of rats with AIA were decreased by treatment with the AT<sub>1</sub>R blockers (Figures 4B and C).

**AT<sub>1</sub>R blocker-induced improvement in endothelial dysfunction in rats with AIA.** We evaluated endothelium-dependent and endothelium-independent vasodilatory responses in rats with AIA treated with each of the AT<sub>1</sub>R blockers. Consistent with the results of our previous studies (16,17), the endothelium-



**Figure 5.** Effect of angiotensin II type 1 receptor ( $AT_1R$ ) blockers on endothelial dysfunction measured ex vivo in rats with adjuvant-induced arthritis (AIA). **A**, Analysis of thoracic aortic rings prepared from the aortas of normal control rats or rats with AIA that were left untreated or were treated with an  $AT_1R$  blocker: losartan (Los; 3 mg/kg/day) or irbesartan (Irb; 5 mg/kg/day), administered orally for 21 days after the onset of arthritis. Levels of endothelium-dependent vasodilation induced by acetylcholine and endothelium-independent vasodilation induced by sodium nitroprusside were determined. The effect of coadministration of  $N^G$ -nitro-L-arginine methyl ester (L-NAME; a nitric oxide synthase inhibitor [ $10^{-4}M$ ]) on acetylcholine-induced vasodilation is also shown. **B**, Analysis of thoracic aortic rings isolated from rats with AIA that were incubated for 18 hours in the presence or absence of  $10 \mu M$  losartan or irbesartan. Levels of endothelium-dependent and endothelium-independent vascular responses were determined as above. Values are the mean  $\pm$  SEM of 6 rats per group. \* =  $P < 0.05$  versus untreated rats with AIA.

dependent relaxation of the aortic ring was significantly depressed in rats with AIA as compared with normal control rats. We found that in vivo treatment with losartan or irbesartan mitigated the endothelial dysfunction in AIA ( $P < 0.05$ ) (Figure 5A). There was no difference in the endothelium-independent vascular responses among control rats, rats with AIA, and rats with AIA treated with  $AT_1R$  blockers (Figure 5A). We confirmed that coadministration of L-NAME consider-

ably inhibited the acetylcholine-induced relaxation, suggesting that it is indeed nitric oxide-dependent (Figure 5A).

We further explored whether ex vivo incubation of aortic rings with  $AT_1R$  blockers reduces endothelial dysfunction. Aortic rings isolated from rats with AIA were incubated for 18 hours in the presence or absence of losartan or irbesartan, and the endothelium-dependent and endothelium-independent vascular responses were determined. We found that ex vivo treatment with each of the  $AT_1R$  blockers significantly improved the endothelial dysfunction in AIA aortas ( $P < 0.05$ ) (Figure 5B).

## DISCUSSION

To our knowledge, this is the first study to evaluate the role of the RAS in vascular damage in an animal model of autoimmune/inflammatory disease. We hypothesized that RAS activation is involved in the development of vascular damage in AIA, and we obtained several pieces of evidence to support this hypothesis. First, Ang II-induced vascular responses were exacerbated in rats with AIA. Second, expression of  $AT_1R$  was significantly increased in the aortas of these rats. Third, vascular expression and activity of ACE, a key enzyme in the formation of Ang II, were enhanced in AIA. Fourth,  $AT_1R$  blockers improved endothelial dysfunction and reduced vascular oxidative stress in AIA. Based on these results, we conclude that local activation of the RAS in the vasculature plays a critical role in the increased vascular oxidative stress and endothelial dysfunction in the AIA model.

Morbidity and mortality from cardiovascular diseases are increased in patients with RA (1–5). However, evidence-based interventional strategies that will prevent the development of cardiovascular events have not yet been established. In the current study, we demonstrated that both losartan and irbesartan have potent vascular protective effects in the AIA animal model of RA. Our findings provide a scientific rationale for a trial of RAS inhibitors to reduce cardiovascular mortality in patients with RA.

A limited number of previous studies have demonstrated that  $AT_1R$  blockade exerts antiarthritic effects. In murine collagen-induced arthritis, olmesartan administered orally at a dosage of 10 mg/kg/day was shown to suppress arthritis (35). In a rat model of acute monoarthritis, losartan administered subcutaneously on alternate days at a dose of 15 mg/kg was shown to inhibit arthritis (36). However, in the present study, neither

AT<sub>1</sub>R blocker—losartan (3 mg/kg/day) or irbesartan (5 mg/kg/day)—influenced the clinical severity of arthritis or body weight in rats with AIA. These discrepancies could be explained by the differences in models, drugs, and drug doses. In human RA, no controlled study has demonstrated that AT<sub>1</sub>R blockers have significant antiarthritic effects.

Blood pressure values in rats with AIA were not significantly reduced by treatment with 3 mg/kg of losartan or 5 mg/kg of irbesartan, although these doses are almost equivalent to those currently used therapeutically in humans. This finding is actually consistent with those of previous studies (37–39) and could be explained by differences in species. It was shown in hypertensive rats that losartan administered orally at a dose of 5 or 10 mg/kg did not affect blood pressure, whereas a higher dose (30 mg/kg) of this drug did reduce blood pressure (37,38). Irbesartan administered orally to hypertensive rats at a dose of 3, 10, 30, or 100 mg/kg was shown to reduce blood pressure in a dose-dependent manner, although the hypotensive effects induced by lower doses (3 or 10 mg/kg) of this drug were weak and did not last for 24 or 48 hours (39). Our results suggest that the vascular protective effect of AT<sub>1</sub>R blockers in this model of arthritis can be independent of antihypertensive effects and probably occur with the use of lower doses.

The effects of AT<sub>1</sub>R blockers on improvement in endothelium-dependent vasodilation may be produced not only by their AT<sub>1</sub>R blockade effect, but also by their inverse agonistic effect against AT<sub>1</sub>R and their capacity for scavenging reactive oxygen species. Indeed, we found in the present study that *ex vivo* incubation of aortas from rats with AIA with the AT<sub>1</sub>R blockers improved endothelial dysfunction (Figure 5B). Renin and angiotensinogen are not present *in vitro*; thus, Ang II is not formed in this system. But, AT<sub>1</sub>R exists abundantly in the aorta, and its expression level was increased in the group with AIA. AT<sub>1</sub>R is a G protein-coupled receptor, and it can be activated through an Ang II-independent mechanism. Recent studies have shown that AT<sub>1</sub>R blockers have an inverse agonist effect (40). The binding of AT<sub>1</sub>R to the AT<sub>1</sub>R blocker not only blocks the potential entry of Ang II into its receptor pocket, but it also induces an inactive conformation of the receptor. Losartan is reported to have inverse agonism (41). In addition, irbesartan has also been shown to have this inverse agonistic effect (Dr. Shin-ichiro Miura, Department of Cardiology, Fukuoka University School of Medicine, Fukuoka, Japan: personal communication). These inverse agonistic effects may explain the results observed in our *ex vivo* experiments. Moreover, it has been

indicated that AT<sub>1</sub>R blockers possess unique *in vitro* properties as reactive oxygen species scavengers (e.g., hydroxyl radical scavenging and inhibition of the Fenton reaction) (42). This mechanism may also account for some of the effects of AT<sub>1</sub>R blockers in our model.

Ang II-induced hypertensive and vascular hypertrophic responses are potentiated in AIA. Ang II-induced endothelial dysfunction is exacerbated in the presence of inflammatory arthritis. The increased expression of AT<sub>1</sub>R in the aortas of rats with AIA may contribute in part to the exacerbation of Ang II-induced vascular responses in this model. In rabbits with hypercholesterolemia, enhanced arterial AT<sub>1</sub>R expression has been shown to lead to an elevated functional response in the vascular wall on Ang II stimulation (43). It is likely that patients with RA have a cluster of traditional and disease-specific risk factors that may rank them as individuals at high risk for the development of cardiovascular disease. The significant enhancement of Ang II-induced vascular responses in the AIA arthritis model reinforces the hypothesis that the coexistence of uncontrolled systemic inflammation and RAS activation carries a considerable risk of vasculopathy in patients with RA.

It has been shown that AT<sub>1</sub>R expression is enhanced by many agonists, such as insulin, low-density lipoprotein cholesterol, and progesterone (44–46). It remains to be determined which mediators are involved in the increased expression of AT<sub>1</sub>R in the aortas of the AIA model. Circulating proinflammatory cytokines possibly influence the vascular expression of AT<sub>1</sub>R. IL-6 is of particular interest, since this cytokine has been demonstrated to induce an up-regulation of AT<sub>1</sub>R gene and protein expression in rat cultured vascular smooth muscle cells and in mouse vascular tissue (47). Elucidation of this mechanism could be critically important to the establishment of strategies that involve neutralization of proinflammatory cytokines for the reduction of cardiovascular risk in RA patients.

In conclusion, our results indicate that the locally activated RAS is strongly involved in the increased vascular oxidative stress and endothelial dysfunction in autoimmune and inflammatory disease models. AT<sub>1</sub>R blockers have potent vascular protective effects in the AIA animal model of RA. We previously found the beneficial effects of statins on vascular function in the arthritis model (17). Based on these findings from animal models, it may be reasonable to suggest that both RAS blockers and statins are potential treatment options for the reduction of the risks of cardiovascular



damage in patients with RA, although further clinical studies to support this idea are needed.

#### AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Morita had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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