Vascular Injury Causes Neointimal Formation in Angiotensin II Type 1a Receptor Knockout Mice

Koichiro Harada, Issei Komuro, Takeshi Sugaya, Kazuo Murakami, Yoshio Yazaki

Abstract—Many studies using small-animal models suggest that angiotensin II (Ang II) plays an important role in neointimal formation after vascular injury. In the present study, we examined whether Ang II type 1 receptor (AT₁)-mediated Ang II signaling is indispensable for the development of injury-induced neointimal formation using AT₁ knockout (KO) mice. Reverse transcriptase–polymerase chain reaction analysis revealed that AT₁ mRNA was not detectable in both uninjured and injured carotid arteries of KO mice, whereas the AT₁ gene was expressed in uninjured carotid arteries of wild-type (WT) mice. At 14 days after injury, AT₁ mRNA levels were increased by 1.5-fold in injured arteries of WT mice. Although AT₂ mRNA was not detectable in uninjured arteries, expression of AT₂ gene was induced in both animal groups at 2 weeks after injury. Vascular injury induced neointimal formation in KO mice as well as in WT mice. There were no significant differences between WT and KO mice in the extent of histological findings such as increased cross-sectional areas of the neointima and the media, the number of proliferating smooth muscle cells, and the amount of collagen and fibronectin. Treatment with subpressor doses of Ang II after injury enhanced the growth of neointima in WT mice but not in KO mice. Moreover, treatment with the selective AT₁ antagonist CV-11974 before injury significantly decreased the formation of neointima in only WT mice, whereas treatment with the selective AT₂ antagonist PD-123319 before injury had no effects in both animal groups. These results suggest that AT₁-mediated Ang II signaling is not essential for the development of neointimal formation, although it may modify it. (Circ Res. 1999;84:179-185.)

Key Words: angiotensin ■ angioplasty ■ stenosis ■ carotid artery

Vascular injury induces migration of smooth muscle cells (SMCs) from the media to the intima and subsequent proliferation of SMCs within the intima. These vascular responses are proposed to be critical processes of neointimal formation and induce narrowing of the vascular lumen, resulting in arteriosclerosis. Many studies have demonstrated that angiotensin-converting enzyme (ACE) inhibitors prevent neointimal formation in the injury model of small animals, whereas other agents that have blood-pressure–lowering effects fail to prevent it. Injured arteries, mRNA and/or protein levels of ACE, angiotensinogen, renin, and mRNA and/or binding activity of angiotensin II (Ang II) receptors have been reported to be increased. In addition, it has been reported that Ang II infusion into rat after arterial injury induces proliferative response in SMCs in the neointima, suggesting that the renin-angiotensin system (RAS) plays an important role in neointimal formation. However, ACE inhibitors did not sufficiently inhibit neointimal formation after angioplasty in human trials as well as in swine and nonhuman primates. These results suggest that the RAS is not important for the neointimal formation of some species or that the pathway of Ang II production is different among animal species.

The effects of Ang II are exerted through specific 7-transmembrane Ang II receptors. At present, Ang II receptors are divided into 2 subtypes, AT₁ and AT₂, and AT₁ is further subdivided into AT₁a and AT₁b. It is generally accepted that most of the well-known Ang II functions in the cardiovascular system are mediated through AT₁. It has also been reported that the predominant Ang II receptor in SMCs is the AT₁ subtype and that AT₁ stimulation plays a major role in the development of neointimal formation in injured arteries. In contrast, AT₂ expression is abundant during the fetal stage but is rapidly decreased soon after birth in most adult organs including the vessels. Interestingly, AT₂ is reexpressed in pathological situations such as neointimal formation induced by vascular injury. There are reports suggesting that AT₂ is also involved in injury-induced neointimal formation, because blockade of AT₂ with a specific antagonist was effective in preventing neointimal formation after injury. However, Nakajima et al have reported that AT₂ elicited growth inhibition of vascular SMCs in opposi-
tion to the effects of AT\(_1\). Thus, the relative importance of Ang II receptor subtypes in the neointimal formation is still controversial.

We and others have recently generated AT\(_{1a}\) knockout (KO) mice by gene targeting.\(^{23,24}\) In the present study, we report on an injury model of murine carotid artery by which we can obtain reproducible and circumferential intimal response and on the pathophysiological roles of the RAS in the injury-induced neointimal formation using AT\(_{1a}\) KO mice. AT\(_1\) mRNA levels were increased in only injured arteries of wild-type (WT) mice, whereas expression of AT\(_2\) mRNA was induced by injury in both kinds of mice. Vascular injury induced neointimal formation in KO mice as well as WT mice. Prolonged Ang II treatment after injury enhanced neointimal formation in only WT mice. In this model, a selective AT\(_1\) antagonist significantly inhibited neointimal formation in only WT mice. These results suggest that although Ang II could modify neointimal formation through AT\(_1\), vascular injury can induce neointimal formation without the AT\(_1\)-mediated Ang II signaling pathways.

## Materials and Methods

### Neointimal Formation of Murine Carotid Arteries

All protocols were approved by local institutional guidelines for animal care of Tokyo University. AT\(_{1a}\) KO mice (n=44) and WT mice (n=44) 18 to 22 weeks old from the same genetic background were used in the present study.\(^{25}\) Animals were housed under climate-controlled conditions with a 12-hour light/dark cycle and were provided standard food and water ad libitum. To reproducibly obtain neointimal formation, arterial injury was produced by modification of previously described methods under a dissecting microscope.\(^{25}\) In brief, mice were anesthetized with sodium pentobarbital (50 mg/kg IP). The curved flexible angioplasty wire (0.35-mm diameter) was introduced into the left common carotid artery via the external carotid artery and was passed along the vessel 3 times. Subsequently, a polyethylene tube (PE10) was immediately introduced into artery and passed along the vessel 3 times. The ratio of the polyethylene tube to artery was 1.2±0.09. The tube was then removed, and the external carotid artery was tied off proximal to the incision hole with the proximal ligature. All these procedures were performed within 10 minutes. There were no operative deaths in both kinds of animals. The identical injury procedure was performed for each animal to obtain reproducibly the same injury. All injured vessels showed a circumferential (360°) intimal response. The right carotid artery was sham-operated and used as a control. The segments of injured and uninjured carotid artery were excised at 7 (n=5) and 14 days (n=5) after vascular injury. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis was performed using mRNA prepared from 4 different pools of arterial segments for each group.

### RT-PCR Analysis

Total RNA was prepared from the murine carotid artery using RNA STAT-60 (TEL-TEST, Inc) followed by digestion with DNase. We first performed conventional RT-PCR analysis to detect whether Ang II receptor genes are expressed in arteries. The competitive RT-PCR analysis was also performed to quantify AT\(_1\) and AT\(_2\) mRNA using the deletion-mutated cRNA as described previously.\(^{26-28}\) The amplification efficiencies of target and competitor transcripts are equal under optimal concentrations of competitor transcripts. Because the primers used for the amplification of AT\(_1\) correspond to common sequences between AT\(_{1a}\) and AT\(_{1b}\), both AT\(_{1a}\) and AT\(_{1b}\) mRNA were amplified.\(^{27,28}\) To verify that equal amounts of RNA were subjected to RT-PCR, GAPDH mRNA was also amplified with the specific primers.\(^{27,28}\) Denaturing (94°C for 45 seconds), annealing (58°C for 1 minute), and extension (72°C for 1 minute) reactions were performed for 30 cycles. To examine the activation of the RAS in this injury model, we also quantified ACE mRNA levels using the same method as described previously.\(^{28}\) Expression levels of ACE mRNA were increased by 2.4-fold in this model of WT carotid arteries at 14 days after injury (data not shown). The range of concentrations of sample RNA and internal control–deleted cRNA, as well as the number of amplification cycles, was selected from within the exponential phase.

### Histological Studies

At 2 weeks after injury, carotid arteries were fixed by perfusion of 10% formalin under physiological pressure. Fixed tissues were embedded in paraffin, sectioned at 4 μm thickness, and stained with hematoxylin-eosin (H&E) for overall morphology or by the van Gieson method for collagen. Immunohistochemical stainings for α-smooth muscle actin, proliferating cell nuclear antigen (PCNA), and fibronectin were carried out on paraffin sections by using anti-human α-smooth muscle actin with EPOS system (Dako, Inc), anti-human PCNA with EPOS system (Dako), and anti-human fibronectin (Sigma Chemical Co) with LSAB kit (Dako), respectively. 3,3′-Diaminobenzidine tetrahydrochloride was used as chromogen, and the sections were counterstained with only hematoxylin. Areas of intima and media were selected (4 fields randomly), photographed, and determined with the image analysis software as described previously.\(^{27}\) For quantification of SMC proliferation after vascular injury, all PCNA-labeled cells were counted separately in the intima and media of 4 subsequent cross sections [The replication index: (PCNA-positive nuclei/total nuclei)]×100]. To determine the degree of extracellular matrix accumulation, we selected 5 fields randomly and calculated the ratio of von Gieson–stained or fibronectin immunoreactive area divided by total neointima area as described previously.\(^{27}\)

### Chronic Administration of Subpressor Dose of Ang II

An osmotic minipump (model 2002, Alza Corp) was implanted subcutaneously into mice after injury (n=3). Subpressor dose of Ang II (100 ng · kg\(^{-1}\) · min\(^{-1}\))\(^{27}\) or saline alone was administered for 2 weeks. To monitor the hemodynamic effects of Ang II treatment at 2 weeks after implantation, arterial blood pressure was directly measured in conscious mice under unrestrained conditions as described previously.\(^{27}\) After blood pressure was recorded, carotid arteries were excised and subjected to additional analysis.

### Statistical Analyses

All results are expressed as mean±SEM. Multiple comparisons between 3 or more groups were carried out by 2-way ANOVA and Fisher exact test for post hoc analyses. Statistical significance was accepted at a value of P<0.05.

## Results

### RT-PCR Analysis of AT\(_1\) and AT\(_2\) mRNA in Murine Carotid Arteries

To examine whether AT\(_1\) and AT\(_2\) mRNA are expressed in carotid arteries, we first performed RT-PCR analysis. RT-PCR analysis using mRNA of carotid arteries revealed that AT\(_1\) gene was expressed in WT mice, whereas AT\(_1\) mRNA...
was not detectable in arteries of KO mice (Table). AT2 mRNA was not detectable in uninjured arteries of both animal groups (Table). The findings in WT mice were in good agreement with the previous study of rat carotid arteries. Next, we quantified changes of the AT1 and AT2 mRNA levels after vascular injury by competitive RT-PCR analysis (Figure 1). In WT mice, AT1 mRNA levels were significantly higher in injured arteries than in uninjured arteries (1.5-fold; *P<0.05) (Figure 1A). AT1 expression was also not detected in injured arteries of KO mice. Expression levels of AT2 mRNA were increased in arteries of both mice by vascular injury, and there was no significant difference in the amount of AT2 mRNA levels between WT and KO arteries (Figure 1B).

### Effects of Arterial Injury on Neointimal Formation

At 2 weeks after operation, sham treatment did not induce intimal thickening in mice of either group. Unexpectedly, neointimal thickening was observed in arteries of both WT and KO mice (Figure 2). The extent of neointimal thickening was comparable between the two groups. The results suggest that AT1 and AT2 receptors may play a role in the formation of neointima after vascular injury.
vascular injury induced neointimal formation not only in WT mice but also in KO mice (Figure 2A), and there were no significant differences in cross-sectional areas of neointima or media between WT and KO mice (Figure 2B and 2C), indicating that vascular injury can induce neointimal formation without the AT1-mediated signaling. To determine the nature of the cells present in the vessel wall at 2 weeks after injury, we performed immunohistochemical analysis using an antibody against \(\alpha\)-smooth muscle actin. Almost all cells in the neointima showed immunoreactivity with the antibody against \(\alpha\)-smooth muscle actin (data not shown). To determine the SMC proliferation in injured arteries, immunohistochemical analysis was performed with an antibody against PCNA in serial sections. The percentage of replicating SMCs in the neointima as demonstrated by the immunoreactivity for PCNA was markedly increased at 7 days after injury (WT, 61.2%; KO, 63.7%) and decreased at 14 days after injury (WT, 25.7%; KO, 21.3%) (Figure 2D). There were no significant differences in these changes between WT and KO mice (Figure 2D).

### Histological Analysis

To further characterize the composition of the intimal lesion, we measured the amount of extracellular matrix (ECM) proteins such as collagen and fibronectin by the van Gieson staining and immunohistochemistry using specific antibody, respectively. Expression of fibronectin has been reported to be induced by Ang II. Expression of fibronectin has been reported to be induced by Ang II. The deposition of collagen and fibronectin was detected in the neointima after injury in KO as well as WT mice (Figure 3A). There were no statistically significant differences in the area of these ECM productions between KO and WT mice (Figure 3B).

### Effects of Ang II Treatment on Neointimal Formation

In the present study, the expression of the AT\(_2\) gene was induced by arterial injury in both kinds of mice (Table and Figure 1B). It has been reported that AT\(_2\) is involved in neointimal formation after vascular injury. To examine this possibility, we continuously administered subpressor doses of Ang II into mice for 2 weeks immediately after injury. Prolonged treatment with Ang II had no effects on blood pressure or heart rate (data not shown). The Ang II treatment enhanced the growth of neointima in only WT mice but not in KO mice (Figure 4). The results obtained in WT mice suggest that AT\(_1\) stimulation can enhance injury-induced neointimal formation, and KO mice results suggest that AT\(_2\) is not involved in injury-induced neointimal formation.
Effects of Treatment with AT₁ or AT₂ Antagonists on Neointimal Formation

Finally, we administered the AT₁ antagonist CV-11974 or the AT₂ antagonist PD-123319 into mice from 60 minutes before the injury through the 2 weeks that followed. With some minor variation, prolonged treatment with CV-11974 significantly inhibited (78 ± 6% inhibition; \( P < 0.01 \)) neointimal formation in only WT mice (Figures 5 and 6c). This result is consistent with the previous observation in the carotid injury model of rat.18 CV-11974 had no effects on neointima formation in KO mice (Figures 5 and 6e). Prolonged treatment with PD-123319 had no inhibitory effects on injury-induced neointimal formation in both kinds of mice (Figures 5, 6d, and 6f), suggesting that AT₂ is not involved in injury-induced neointimal formation.

Discussion

Genetically altered mice have considerable advantages to address the role of specific molecules. In the present study, we examined the specific role of Ang II in injury-induced neointimal formation using genetically AT₁a-deleted mice. Unexpectedly, vascular injury induced neointimal formation and the deposition of collagen and fibronectin in AT₁a KO mice as well as WT mice. AT₁ mRNA levels were increased by 1.5-fold in only WT mice but not in KO mice. The expression of the AT₂ gene in the artery was induced by vascular injury in both kinds of animals. Administration of Ang II immediately after injury enhanced neointimal formation in only WT mice. An AT₁ antagonist, not an AT₂ antagonist, inhibited neointimal formation in WT mice, whereas both antagonists had no inhibitory effects in KO mice. These results suggest that vascular injury can induce neointimal formation in the mice that genetically lack AT₁-mediated Ang II signaling pathways, although Ang II plays an important role in neointimal formation through AT₁ but not AT₂.

Many animal models of injury-induced neointimal formation have been reported. Although Lindner et al.25 developed the injury model of the murine carotid artery, which closely resembles that of the rat, this model induces only a negligible intimal response and has not been widely used. We established a new vascular injury model by which we can reproducibly induce circumferential neointimal lesions. We passed a polyethylene tube along the vessel, following their original procedures. In our model, formation of the neointima can be induced rapidly and measured reliably.
It has been reported that all components of the RAS were found in the vessel wall and the neointima and that although ACE inhibitors or AT1 antagonists effectively inhibited neointimal formation in rat models, ACE inhibitors were insufficient to reduce restenosis in human trials, as well as in porcine and nonhuman primate models. It has been reported that enzymes other than ACE can generate injury-induced neointimal formation, and that although AT1 antagonists were insufficient to reduce restenosis in human trials, suggesting that Ang II generation through ACE-independent pathways may participate in neointimal formation. However, more recent studies using a porcine coronary artery model of vascular restenosis have demonstrated that chronic blockade of Ang II at the receptor level was also insufficient to inhibit intimal hyperplasia after injury, suggesting that the importance of Ang II for neointimal formation is dependent on animal models and that Ang II is not a major mediator of intimal thickening, at least in larger animals including humans.

To determine whether Ang II is indispensable for injury-induced neointimal formation in rodents, we examined genetically AT1-deleted mice. At 14 days after injury, almost the same degree of the neointimal lesion was observed in both KO and WT mice. Neointimal formation in KO mice was associated with the accumulation of SMCs, collagen, and fibronectin in the neointima, and all these changes were indistinguishable between WT and KO mice. These results suggest at least 2 possibilities. One is that Ang II is not involved in injury-induced neointimal formation in mice. Another possibility is that there are at least 2 different pathways, AT1-dependent and AT2-independent pathways, in injury-induced neointimal formation, and that although AT1 plays a pivotal role in injury-induced neointimal formation, the AT2-independent pathway substitutes fully for the AT1-dependent pathway in KO mice. Pharmacological studies using Ang II and an AT1 antagonist suggest that the latter possibility may be correct. Because infusion of Ang II enhanced and treatment with an AT1 antagonist inhibited the neointimal formation after the arterial injury in WT mice, Ang II may play a critical role in injury-induced neointimal formation through AT1. It is unknown at present how injury induced neointimal formation in KO mice in which AT1-evoked signalings are completely deleted. It has been reported that besides Ang II, many growth factors and vasodilatory peptides such as platelet-derived growth factor, basic fibroblast growth factor, transforming growth factor-β1, endothelin-1, and catecholamines are induced by injury and are implicated in the process of neointimal formation. These factors may influence the migration and proliferation of SMCs and production of ECM through similar signaling mechanisms. It remains to be determined what factors are activated by vascular injury in AT1 KO mice.

The role of AT2 in the neointimal formation has not yet been established. In the present study, the neointimal formation after vascular injury in carotid arteries was accompanied by an increase in levels of AT2 mRNA in KO mice as well as in WT mice. However, infusion of Ang II into KO mice that have only AT2 and treatment with AT2 antagonist of both mice had no effects on the ratio of neointima to media. These results suggest that at least in this model, AT2 is not involved in neointimal formation after vascular injury.

In summary, we demonstrated using a AT1 KO mice model that AT1-mediated Ang II signaling is not indispensable for the injury-induced neointimal formation and that signaling pathways other than those provoked by Ang II can fully induce responses to injury in the absence of AT1. The identification of the AT2-independent signaling pathways will provide new insights into the development of novel therapeutic strategies for preventing neointimal formation.

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**References**


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