Attenuation of Cuff-Induced Neointimal Formation by Overexpression of Angiotensin II Type 2 Receptor-Interacting Protein 1

Teppei Fujita, Masaki Mogi, Li-Juan Min, Jun Iwanami, Kana Tsukuda, Akiko Sakata, Hideki Okayama, Masaru Iwai, Clara Nahmias, Jitsuo Higaki, Masatsugu Horiuchi

Abstract—Recently, we have cloned angiotensin II type 2 receptor–interacting protein 1 (ATIP1) as a novel protein that interacts specifically with the C-terminal tail of the angiotensin II type 2 receptor; however, the pathophysiological roles of ATIP1 in vascular remodeling are still unknown. Here, we generated ATIP1-transgenic (ATIP1-Tg) mice expressing mouse ATIP1 and investigated the role of ATIP1 in vascular remodeling using these transgenic mice. ATIP1-Tg mice exhibited no significant difference in blood pressure compared with wild-type (WT) mice. Angiotensin II type 2 receptor mRNA expression in the femoral artery was increased in injured femoral arteries, reaching a peak at 7 days after operation in WT mice, and a similar result of angiotensin II type 2 receptor expression was observed in ATIP1-Tg mice. In ATIP1-Tg mice, neointimal formation of the femoral artery was increased in injured femoral arteries, reaching a peak at 7 days after operation in WT mice, and a similar result of angiotensin II type 2 receptor expression was observed in ATIP1-Tg mice.

Key Words: ATIP1  vascular remodeling  cell proliferation  oxidative stress  ERK

Angiotensin (Ang) II exerts its important physiological functions through 2 distinct receptor subtypes, type 1 (AT1) and type 2 (AT2) receptors. Recently, new emerging concepts of regulation of these receptors have been highlighted.1 Several novel receptor-interacting proteins that regulate the functions of AT1 and AT2 receptors have been cloned. These associated proteins could contribute not only to Ang II receptor functions but also to influencing the pathogenesis of cardiovascular diseases.

We have recently cloned AT2 receptor-interacting protein 1 (ATIP1) as a protein that interacts specifically with the C-terminal tail of the AT2 receptor, using a yeast 2-hybrid system,2 and it has been shown recently to cooperate with the AT2 receptor to transinactivate receptor tyrosine kinases independent of G proteins. In Chinese hamster ovary cells expressing the human AT2 receptor, ATIP1 inhibits growth factor–induced extracellular signal–regulated kinase (ERK) 2 activation and DNA synthesis and attenuates insulin receptor autophosphorylation.3 In contrast, AT2 receptor-binding protein of 50 kDa, which is identical to ATIP1, is reported by Wruck et al4 to potentially act as a membrane-associated Golgi protein that dictates delivery of the AT2 receptor to the cell surface. Knocking down of AT2 receptor-binding protein of 50 kDa using small-interference RNA reduced the cell surface expression of the AT2 receptor by translocation of this receptor from the Golgi apparatus and attenuated its antiproliferative effects. ATIP1 was found to be identical to a ubiquitously expressed tumor suppressor protein localized in mitochondria.4 Therefore, ATIP1 seems to act as a novel early component of the growth-inhibitory signaling cascade of the AT2 receptor.

We reported that neointimal formation and the proliferation of vascular smooth muscle cells (VSMCs) induced by polyethylene cuff placement on the femoral artery were greater in AT2 receptor–null mice than in wild-type (WT) mice,5 indicating that AT2 receptor stimulation attenuates vascular remodeling. Furthermore, AT2 receptor deletion from apolipoprotein E–deficient mice enhances atherosclerosis mainly through the inhibition of oxidative stress,6 indicating that AT2 receptor stimulation also attenuates atherosclerosis. Recently, we have reported that, in rodent neurons, AT2...
receptor stimulation enhanced neural differentiation and inhibited neural damage via translocation of ATIP1 into the nucleus, indicating that ATIP1 may play an important role in tissue repair, such as improvement of vascular remodeling. However, the roles of ATIP1 in vascular remodeling in vivo have never been investigated. These results led us to generate ATIP1 gene-transgenic mice and to explore the functions of ATIP1 in vascular remodeling induced by cuff placement on the femoral artery.

Materials and Methods
This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All of the animal studies were reviewed and approved by the Ehime University Animal Studies Committee.

Generation of ATIP1-Transgenic Mice
Adult male ATIP1-transgenic (ATIP1-Tg) mice were generated similarly, as described previously. Transgenic mice were identified by PCR using 5'-AAA CTG ACC AAC GGA GAC CT-3' as the forward primer and 5'-TTC CCA TGA GAG GGT CAG TC-3' as the reverse primer.

Animals and Treatment
The animals were housed in a room where lighting was controlled (12 hours on and 12 hours off) and the temperature was kept at 25°C. They were given a standard diet (MF, Oriental Yeast) and water ad libitum. Systolic blood pressure was measured in conscious mice by a noninvasive tail-cuff system (MK-1030, Muromachi Co Ltd) with or without Ang II infusion via an intraperitoneally implanted osmotic minipump (model 1002, Alza) at a dose of 1.44 mg/kg per day.

Cuff-Induced Vascular Remodeling Model
ATIP1-Tg mice (from 8 to 10 weeks old) and WT littermate mice were used in the following experiments. Some ATIP1-Tg mice were treated with an AT2 receptor antagonist, PD123319, via an intraperitoneally implanted osmotic minipump around the femoral artery under anesthesia with IP injection of 60 mg/kg of pentobarbital sodium in saline, and IP injection (30 mg/kg) 18 hours before sampling, followed by IP treatment again (30 mg/kg) 12 hours before sampling, as described previously.5,11,12 The femoral arteries were obtained 1 week after cuff placement. BrdUrd incorporation in paraffin-embedded sections was monitored using computer imaging software (Densitograph, ATTO Corp).

Preparation of VSMCs
VSMCs were isolated from the thoracic aorta of adult male WT and ATIP1-Tg mice by the explant method.9 Cells were cultured with DMEM (Life Technologies, Inc), containing 10% FBS. Cells at passage 4 to 8 were used for the experiment.

Dihydroethidium Staining
Superoxide generation in cryostat frozen sections and cultured VSMCs was analyzed using fluorogenic dihydroethidium (5 μmol/L), as described previously.10 Intensity of fluorescence was analyzed and quantified using computer imaging software (Densitograph, ATTO Corp).

5-Bromodeoxyuridine Incorporation Assay
5-Bromodeoxyuridine (BrdUrd) was administered by SC (100 mg/kg) and IP injection (30 mg/kg) 18 hours before sampling, followed by IP injection again (30 mg/kg) 12 hours before sampling, as described previously.5,11,12 The femoral arteries were obtained 1 week after cuff placement. BrdUrd incorporation in paraffin-embedded sections was evaluated using a BrdUrd staining kit (Zymed-Laboratory).

Detection of Cell Proliferation In Vitro
Cell proliferation was evaluated by Cell Counting Kit-8 (Dojindo Laboratories), which is based on using tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (monosodium salt), according to the manufacturer’s protocol.

Real-Time RT-PCR
Total RNA was extracted from the pool of 4 different femoral arteries. In this experiment, both femoral arteries were sampled in a mouse. Real-time quantitative RT-PCR was performed with Premix Ex Taq (Takara Bio Inc). PCR primers were as follows: 5'-CGA GTG ACG CGT CGT TAG CC-3' (forward) and 5'-GTT CCA TGA GAG GGT CAG TC-3' (reverse) for TNF-α, 5'-AAA CTG ACC AAC GGA GAC CT-3' (forward) and 5'-TTC CCA TGA GAG GGT CAG TC-3' (reverse) for ATIP, and 5'-ATG TCG GCT CAC AAC GGA GAC CT-3' (forward) and 5'-TTC GACTTC ACA CAC ATT GC-3' (reverse) for GAPDH. mRNA expression of TNF-α was shown by a ratio of TNF-α/GAPDH.

Immunoblot Analysis
Total proteins were prepared from femoral arteries under different experimental conditions. The proteins were subjected to SDS-PAGE and immunoblotted with an antibody against ERK1/2 (Cell Signaling Technology, Inc) or β-tubulin (Sigma-Aldrich, Inc). The bands of proteins were visualized with an enhanced chemiluminescence system (GE Healthcare). Densitometric analysis was performed using National Institutes of Health image software.15

Statistical Analysis
All of the values are expressed as means±SEMs in the text and figures. The data were evaluated by ANOVA, followed by posthoc analysis for multiple comparisons. Differences with P<0.05 were considered to be significant.

Results
ATIP1-Tg Mice Showed Attenuated Neointimal Formation Without Change of Blood Pressure
ATIP1 was generally overexpressed in tissues, especially in the cardiovascular tissues such as the heart, aorta, lung, kidney, and brain, in ATIP1-Tg mice (Figure S1; please see the online data supplement at http://hyper.ahajournals.org). Overexpression of ATIP1 protein was confirmed by Western blot in the thoracic aorta of ATIP1-Tg mice (Figure S2). Next, we compared blood pressure and heart/body weight ratio between ATIP1-Tg and WT mice. Systolic blood pressure at the baseline did not differ in ATIP1-Tg mice compared with WT mice. Moreover, administration of Ang II increased blood pressure and heart/body weight ratio in both WT and ATIP1-Tg mice; however, these increases were not significantly different between them (Table). mRNA expressions of AT1 and AT2 receptors at the baseline were not remarkably different between them. Inflammatory vascular injury was induced by polyethylene cuff placement around the femoral artery under anesthesia with IP injection of 60 mg/kg of pentobarbital sodium in saline, and IP injection (30 mg/kg) 18 hours before sampling, followed by IP treatment again (30 mg/kg) 12 hours before sampling, as described previously.5,11,12 The femoral arteries were obtained 1 week after cuff placement. BrdUrd incorporation in paraffin-embedded sections was monitored using computer imaging software (Densitograph, ATTO Corp).

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</table>

Ang II was administered via an intraperitoneally implanted osmotic minipump at a dose of 1.44 mg/kg per day. HW indicates heart weight; BW, body weight; NS, not significant.

2H-tetrazolium (monosodium salt), according to the manufacturer’s protocol.
changed in the thoracic aorta of ATIP1-Tg mice (Figure 1). These expressions were increased 7 days and 14 days after cuff placement in WT mice. In ATIP1-Tg mice, the level and change of expression of both AT$_1$ and AT$_2$ receptors after cuff placement were similar to those in WT mice (Figure 1). However, neointimal formation at 14 days after cuff placement was significantly attenuated in ATIP1-Tg mice (Figure 2A). Interestingly, pretreatment with PD123319, an AT$_2$ receptor antagonist, could not cancel the effect of a reduction of neointimal formation (Figure 2A). Cuff placement induces proliferation of VSMCs and neointimal formation in the femoral artery. Therefore, we next measured cell proliferation using BrdUrd incorporation. BrdUrd-positive cell number was significantly reduced in ATIP1-Tg mice compared with that in WT mice (Figure 2B). In contrast, the number of apoptotic cells in the arteries detected by TUNEL staining did not show a difference between the groups (data not shown). Next, we assessed cell proliferation using VSMCs prepared from ATIP1-Tg mice. In normal culture condition with 10% FBS, VSMCs prepared from ATIP1-Tg mice exhibited marked attenuation of cell proliferation compared with VSMCs from WT mice (Figure 2C).

**ATIP1-Tg Mice Showed Reduced Oxidative Stress and Inflammation in Injured Arteries**

To assess the involvement of oxidative stress in the attenuation of neointimal formation in ATIP1-transgenic mice, the production of superoxide anion in the intima and media of injured arteries was evaluated by dihydroethidium staining. Superoxide anion production was not changed at the basal level and increased in the WT medium after cuff placement, whereas the small increase in superoxide anion production was significantly less in the medium of injured arteries in ATIP1-Tg mice (Figure 3A and 3B). In VSMCs, although Ang II and thrombin enhanced superoxide anion production in WT mice, overexpression of ATIP1 attenuated the superoxide anion production induced by both Ang II and thrombin (Figure 3C and 3D). Proinflammatory cytokines such as TNF-$\alpha$ are related to inflammation-induced vascular injury. Therefore, we assessed the expression of TNF-$\alpha$ in the injured arteries. TNF-$\alpha$ mRNA expression was increased in WT mice; however, this increase was approximately half in ATIP1-Tg mice, as shown in Figure 4.

**Failure of Response to the Proliferative Signal in ATIP1-Tg Mice**

Finally, we investigated the proliferative response, such as ERK phosphorylation, in the injured femoral artery 7 days after cuff placement in ATIP1-Tg mice. As shown in Figure 5, ERK was phosphorylated after cuff placement in WT mice; however, such ERK phosphorylation was significantly attenuated in ATIP1-Tg mice.

**Discussion**

The present study demonstrated that ATIP1 plays an important role in cuff-induced vascular remodeling through attenuation of cell proliferative response, oxidative stress, and

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**Figure 1.** Comparison of mRNA expression of AT$_1$ and AT$_2$ receptors in the femoral artery before and 7 or 14 days after cuff placement.

**Figure 2.** A, Neointimal formation in injured femoral artery after cuff placement in WT and ATIP1-Tg mice. Some ATIP1-Tg mice were treated with PD123319 at a dose of 30 mg/kg per day. Top, Representative photos of areas of media and neointima in cross-sections of the femoral artery measured at 14 days after cuff placement with elastica van Gieson staining. Bottom, Histogram analysis of the neointimal area in injured femoral artery. Values are means±SEM (n=8 for each group). B, Cell proliferation in injured femoral artery after cuff placement in WT and ATIP1-Tg mice. Cell proliferation was measured as the ratio of BrdUrd-positive nuclei/total nuclei in the femoral artery at 7 days after cuff placement. Values are means±SEM (n=8 for each group). C, Cell proliferation in VSMCs prepared from WT and ATIP1-Tg mice. Cell proliferation was measured by cell counting kit described in the Materials and Methods section. Values are means±SEM (n=8 for each group).
expression of inflammatory cytokines using ATIP1-Tg mice. Previously, we and other groups demonstrated that AT\(_2\) receptor stimulation attenuates vascular remodeling and atherosclerosis through the inhibition of AT\(_1\) receptor signaling involving oxidative stress and inflammatory response\(^5\)\(^,\)\(^6\)\(^,\)\(^16\) and via activation of vasodilation signaling, such as bradykinin and endothelial NO synthase.\(^17\)\(^,\)\(^18\) Based on our results in this and previous articles,\(^5\)\(^,\)\(^11\) we could expect that these preventive effects of AT\(_2\) receptor stimulation could be enhanced, at least in part, by ATIP1 binding to the AT\(_2\) receptor, resulting in attenuation of neointimal formation. However, we cannot conclude that this beneficial effect of ATIP1 overexpression on vascular remodeling is completely dependent on AT\(_2\) receptor signaling, because it is possible that ATIP1 could directly attenuate growth-promoting signaling.

AT\(_1\) and AT\(_2\) receptor levels did not show significant changes in WT and ATIP1-Tg mice before or after vascular injury. Treatment with the AT\(_2\) receptor blocker PD123319 did not change neointimal formation in ATIP1-Tg mice. These results apparently suggest that ATIP1 overexpression decreased neointimal formation, independent of AT\(_2\) receptor activation. However, we cannot exclude the involvement of AT\(_2\) receptor activation in the decrease of neointimal formation in ATIP1-Tg mice. Neointimal formation and superoxide anion production were less in ATIP1-Tg mice; therefore, we were afraid that the effects of PD123319 were not obvious. In other words, in examination of the effects of PD123319, it is difficult to obtain clear results in ATIP-Tg mice. We have reported previously that AT\(_2\) receptor activation increased binding of Src homology 2 domain-containing protein-tyrosine phosphatase 1 (SHP-1) and ATIP and triggers own-stream signaling.\(^7\) Therefore, the binding of SHP-1 and ATIP1 could be markedly attenuated by PD123319, and it could be possible that we could not observe additional effects of AT\(_2\) receptor–mediated inhibitory effects on neointimal formation. Further investigation is necessary to evaluate the association of ATIP1 with the AT\(_2\) receptor in vivo and in

**Figure 3.** Production of superoxide anion in injured artery after cuff placement and VSMCs in WT and ATIP1-Tg mice. A, Representative photos of dihydroethidium staining of femoral arteries 7 days after cuff placement. B, Comparisons of fluorescence intensity in media and intima. Values are means±SEMs (n=8 for each group). C, Representative photos of dihydroethidium staining of vascular smooth muscle cells with or without incubation of Ang II (10\(^{-7}\) M) or thrombin (2 U/mL). D, Comparisons of fluorescence intensity in cells. Values are means±SEMs (n=5 for each group).

**Figure 4.** Expression of inflammatory cytokine, TNF-\(\alpha\), in injured femoral artery after cuff placement in ATIP1-Tg mice. Tissue samples were prepared from arteries 7 days after cuff placement. Values are means±SEMs (n=8 for each group).

**Figure 5.** Phosphorylation of ERK in injured femoral artery in WT and ATIP1-Tg mice with or without cuff placement. Tissue samples were prepared from arteries 7 days after cuff placement. A, Representative immunoblots of phosphorylated and total ERK in 3 different pooled samples are shown. B, Histogram analysis of the ERK activation in injured femoral artery. Values are means±SEMs (n=3 for each group).
vitro and to examine whether these preventive effects of ATIP1 on vascular remodeling are because of enhanced AT₂ receptor signaling by binding with ATIP1 or because of ATIP1 itself.

In the intima and media of ATIP1-Tg mice after cuff injury, oxidative stress was markedly attenuated compared with that in WT mice. ATIP1 is identical to mitochondrial tumor suppressor gene 1, for which mutation or copy number variants are found in human malignant tumors.⁴,¹⁹,²⁰ Therefore, attenuation of oxidative stress may be related to mitochondrial function in mice with overexpression of ATIP1, at least in part independent of the AT₂ receptor–mediated reduction of proliferative response. However, the functional effects of ATIP1 in the mitochondria are totally unknown. Furthermore, oxidative stress influences the mitogen-activated protein kinase signaling pathways, such as the ERK, c-Jun N-terminal kinase, p38, and big mitogen-activated protein kinase 1 pathways.²¹ Therefore, we have speculated that oxidative stress is at least partly involved in ERK activation. Further investigation in detail should be necessary to assess the relation between ERK and oxidative stress, including mitochondrial function in ATIP-Tg mice, in the future.

SHP-1 is also reported to have unique roles in AT₂ receptor–induced signaling.²,³,¹²,²² Recently, we reported a new signaling mechanism in AT₂ receptor–induced neural differentiation and protection via formation of a complex of ATIP1 and SHP-1.⁷ SHP-1 tyrosine phosphatase is also an early transducer of the AT₂ receptor signaling pathway.²³ We reported that AT₂ receptor stimulation–mediated SHP-1 activation inhibits nuclear factor-κB activation⁶ and signal transducers and activators of transcription (STATs),²⁴ in addition to inactivation of ERK. Although the role of SHP-1 and the effect of the interaction between ATIP1 and SHP-1 via AT₂ receptor stimulation were not investigated in the present study, other AT₂ receptor–induced signaling may be involved in the preventive effect of vascular remodeling observed in ATIP1-Tg mice. The AT₂ receptor is known to activate the bradykinin/NO system in the vasculature.¹⁸ Moreover, we reported recently that the AT₂ receptor transactivates methyl methanesulfonate sensitive 2,¹³ resulting in improvement of VSMC DNA damage.²⁵ It is necessary to elucidate the detailed mechanism of the involvement of ATIP1 in AT₂ receptor–mediated improvement of vascular remodeling.

**Perspectives**

Taking these results together, ATIP1 could play a role in the attenuation of vascular remodeling. We expect that ATIP1 could be a novel therapeutic target in the future to prevent vascular damage by inhibiting oxidative stress and inflammatory response. More detailed analysis of the functions of ATIP1 in the vasculature, transcriptional regulation of ATIP1, and functional regulation of ATIP1, such as by phosphorylation and dephosphorylation, would help to explore the more sophisticated regulation of the renin-Ang system.

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**Disclosures**

None.

**References**


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**Online Supplement Figures**

**Figure S1**
Expression of ATIP1 mRNA in ATIP1-Tg and wild-type mice. Tissues were excised from mice at 12 weeks of age, homogenized, and mRNA level was measured by real-time quantitative RT-PCR. mRNA expression of ATIP1 was shown by a ratio of ATIP1/GAPDH.

**Figure S2**
Expression of ATIP1 protein in ATIP1-Tg mice. Tissues were excised from mice at 12 weeks of age, and protein level was measured by western blot with an antibody against the internal domain (ID) of ATIP, which is a rabbit polyclonal antibody as previously described.¹