Possible Role of Angiotensin-Converting Enzyme 2 and Activation of Angiotensin II Type 2 Receptor by Angiotensin-(1–7) in Improvement of Vascular Remodeling by Angiotensin II Type 1 Receptor Blockade

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Abstract—Cross talk between the angiotensin-converting enzyme (ACE)/angiotensin II (Ang II)/Ang II type 1 (AT1) receptor axis and the ACE2/Ang-(1–7)/Mas axis plays a role in the pathogenesis of cardiovascular remodeling. Furthermore, possible stimulation of the Ang II type 2 (AT2) receptor by Ang-(1–7) has been highlighted as a new pathway. Therefore, we examined the possibility of whether the ACE2/Ang-(1–7)/Mas axis and Ang-(1–7)/AT2 receptor axis are involved in the inhibitory effects of AT2 receptor blockers on vascular remodeling. Wild-type, Mas-knockout, and AT2 receptor knockout mice were used in this study. Vascular injury was induced by polyethylene-cuff placement around the mouse femoral artery. Some mice were treated with azilsartan, an AT1 receptor blocker, or Ang-(1–7). Neointimal formation 2 weeks after cuff placement was more marked in Mas-knockout mice compared with wild-type mice. Treatment with azilsartan or Ang-(1–7) attenuated neointimal area, vascular smooth muscle cell proliferation, increases in the mRNA levels of monocyte chemoattractant protein-1, tumor necrosis factor-α, and interleukin-1β, and superoxide anion production in the injured artery; however, these inhibitory effects of azilsartan and Ang-(1–7) were less marked in Mas-knockout mice. Administration of azilsartan or Ang-(1–7) attenuated the decrease in ACE2 mRNA and increased AT2 receptor mRNA but did not affect AT1 receptor mRNA or the decrease in Mas mRNA. The inhibitory effect of Ang-(1–7) on neointimal formation was less marked in AT2 receptor knockout mice compared with wild-type mice. These results suggest that blockade of the AT1 receptor by azilsartan could enhance the activities of the ACE2/Ang-(1–7)/Mas axis and ACE2/Ang-(1–7)/AT2 receptor axis, thereby inhibiting neointimal formation. (Hypertension. 2014;63:e53-e59.) ● Online Data Supplement

Key Words: angiotensin ◼ angiotensin-converting enzyme 2 ◼ inflammation ◼ oxidative stress ◼ receptors ◼ remodeling

It has been suggested that the angiotensin-converting enzyme (ACE) 2/angiotensin-(1–7) (Ang-(1–7))/Mas pathway exerts an antagonistic action in many physiological and pathophysiological processes in several systems and organs, including cardiovascular remodeling, via opposing the classical ACE/Ang II/Ang II type 1 (AT1) receptor axis–mediated action. Recent studies have suggested that Ang-(1–7), generated by ACE2, has vasoprotective and atheroprotective effects in several animal models. Ang-(1–7) is synthesized from Ang I and Ang II mainly via ACE2 activity. The effects of Ang-(1–7) could appear as the balance between the ACE2/Ang-(1–7)/Mas axis and the ACE/Ang II/AT1 receptor axis, which seems to be switched on by ACE2. Accordingly, it is possible that the increase in Ang-(1–7) level during ACE inhibition and AT1 receptor blockade could result in Mas receptor activation and the induction of cardioprotective and renoprotective effects. In addition, it has been suggested that AT1 receptor blockade directly activates the ACE2/Ang-(1–7)/Mas pathway. A previous report suggested that AT1 receptor stimulation regulated ACE2 and Ang-(1–7) expression in the aorta of spontaneously hypertensive rats. AT1 receptor stimulation downregulated ACE2 via the extracellular signal-regulated kinase/p38 mitogen-activated protein kinase pathway mediated by AT1 receptor stimulation. These results suggest that AT1 receptor stimulation inhibited ACE2 expression, whereas AT1 receptor blockade increased it. Consistent with these observations, we demonstrated that in AT1 receptor knockout mice, mRNA expression and immunostaining of ACE2 and

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Mas in the injured artery were greater, with less neointimal formation than in wild-type (WT) mice, and that increases in ACE2 expression and neointimal formation in the injured artery were also observed with treatment with an AT₁ receptor blocker (ARB), olmesartan, suggesting that AT₁ receptor blockade by ARBs seemed to alter the balance between the ACE2/Ang-(1–7)/Mas axis and the ACE/Ang II/AT₁ receptor axis to improve vascular remodeling.⁹

Ang II type 2 (AT₂) receptor stimulation is known to be involved in the beneficial effects of ARBs on cardiovascular remodeling. In addition to cross talk between the AT₁ receptor and Mas, the role of the AT₂ receptor in terms of the effects mediated by the ACE2/Ang-(1–7)/Mas pathway, distinguishing the Mas and AT₂ receptor signaling pathways, has been highlighted.¹⁰ Previously, it was reported that Ang-(1–7) exerts a role as a vasodepressor via AT₁ receptor activation in the presence of partial AT₁ receptor blockade¹¹ and that the vasoprotective and atheroprotective effects of Ang-(1–7) are mediated by restoration of nitric oxide bioavailability via both the Mas and AT₂ receptors.⁴ It is reported that AT₂ receptor expression in the femoral artery was increased after cuff placement.¹² These results led us to examine the possibility that the Ang-(1–7)/AT₁ receptor pathway is involved in the inhibitory effects of ARB on vascular remodeling using Mas-knockout (MasKO) mice and AT₂ receptor knockout (AT₂KO) mice.

Methods
This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. The experimental protocol was approved by the Animal Studies Committee of Ehime University.

Animals and Treatment
AT₂KO (C57BL/6J background)¹³ mice and MasKO mice (C57BL/6J background) aged 10 to 11 weeks, weighing 25 to 30 g, were used. WT (C57BL/6J) mice were used as control. Animal treatment and targeted disruption of the Mas gene are presented in Methods in the online-only Data Supplement.

Morphometric Analysis and Immunohistochemical Staining
The femoral arteries were taken 14 days after cuff placement and fixed with 10% neutral-buffered formalin. Paraffin-embedded cross-sections were prepared as described previously.¹² Samples were examined with a Zeiss Axioskop 2 microscope (Carl Zeiss, Oberkochen, Germany) equipped with a computer-based imaging system.¹⁰

Dihydroethidium Staining
Superoxide generation in cryostat frozen section was evaluated using fluorogenic dihydroethidium (5 μmol/L), as described previously.¹⁵ The intensity of fluorescence was analyzed and quantified using computer imaging software (Densitograph, ATTO Corp).

Quantitative Real-Time Polymerase Chain Reaction
Please see the online-only Data Supplement.

Statistical Analysis
All values are expressed as mean±SD in the text and figures. Data were evaluated by ANOVA. If a statistically significant effect was found, post hoc analysis was performed to detect the difference between the groups. Values of P<0.05 were considered to be statistically significant.

Results
Effect of Administration of Azilsartan or Ang-(1–7) on Neointimal Formation, Cell Proliferation, Inflammatory Cytokines, and oxidative Stress in Injured Artery Induced by Cuff Placement in WT and Mas-Deficient Mice
To explore the involvement of the ACE2/Ang-(1–7)/Mas axis in vascular remodeling, we used a vascular injury model induced by polyethylene-cuff placement around the femoral artery in WT and MasKO mice. As shown in Figure 1A and Figure S1 in the online-only Data Supplement, the neointimal area 14 days after cuff placement was significantly larger in MasKO mice compared with WT mice. Proliferating cell nuclear antigen labeling index was also higher in both the intima and media in MasKO mice (Figure 1B; Figure S2). We examined the possibility that the inhibitory effect of ARB on vascular injury would involve activation of the ACE2/Ang-(1–7)/Mas axis. Figure S3 shows the dose-dependent effect of azilsartan on neointimal formation. We used a nonhypotensive dose of azilsartan (0.3 mg/kg per day) in the following experiments. We compared the effect of azilsartan on neointimal formation between WT and MasKO mice and observed that treatment with azilsartan decreased neointimal area in the injured artery more markedly in control WT mice than in MasKO mice (76.1% reduction in WT mice, 64.3% reduction in MasKO mice; Figure 1A; Figure S1). Basal levels of proliferating cell nuclear antigen labeling index, mRNA levels of monocyte chemoattractant protein-1, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), and superoxide anion production before cuff placement did not differ between WT and MasKO mice (data not shown). The inhibitory effect of azilsartan on proliferating cell nuclear antigen labeling index was also more marked in WT mice than in MasKO mice (Figure 1B; Figure S2). The mRNA levels of monocyte chemoattractant protein-1, TNF-α, and IL-1β in the injured artery determined 7 days after cuff placement were more markedly increased in MasKO mice. Treatment with azilsartan attenuated the increases in mRNA levels of these cytokines; however, these inhibitory effects of azilsartan were less marked in MasKO mice (Figure 2). To assess the involvement of oxidative stress in the attenuation of neointimal formation in azilsartan-treated WT and MasKO mice, the production of superoxide anion in the intima and media of injured arteries was evaluated by dihydroethidium staining (Figure 3; Figure S4). Superoxide anion production in the intima and media 7 days after cuff placement was significantly greater in MasKO mice than in WT mice. Treatment with azilsartan and Ang-(1–7) decreased superoxide anion production in the injured artery in WT mice. These inhibitory effects of azilsartan and Ang-(1–7) were less marked in MasKO mice. Next, we assessed the expressions of mRNA of oxidative stress markers including NAD(P)H (nicotinamide adenine dinucleotide phosphate) oxidase subunits, p22phox, p40phox, p47phox, p67phox, gp91phox, Nox1, Nox4, and Rac1 (Figure S5). We observed that mRNA expressions of p22phox and gp91phox were higher in MasKO mice and that treatment with azilsartan or Ang-(1–7) significantly decreased mRNA expressions of p22phox, p40phox, p47phox, p67phox, gp91phox, Nox1, and Rac1, whereas these effects of azilsartan or Ang-(1–7) were...
Administration of Ang-(1–7) at a nonhypotensive dose decreased neointimal formation, proliferating cell nuclear antigen (PCNA) labeling index, expression of mRNA of monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), and superoxide anion production in the injured artery, and these effects of Ang-(1–7) were more marked in MasKO mice than in control WT mice (Figures 1A, 1B, 2, and 3).

Effect of Administration of Azilsartan or Ang-(1–7) on mRNA Levels of ACE, ACE2, Mas, AT1 Receptor, and AT2 Receptor in Injured Artery Induced by Cuff Placement in WT and Mas-Deficient Mice

We examined the changes in mRNA levels of ACE, ACE2, Mas, AT1 receptor, and AT2 receptor in injured artery induced by cuff placement in WT and Mas-knockout (MasKO) mice. Artery samples were taken 7 days after operation for measurement of the expression of MCP-1 (A), TNF-α (B), and IL-1β (C). Values are mean±SEM of 5 samples in each group. *P<0.05 vs WT control mice. †P<0.05 vs MasKO control mice.
levels were markedly decreased in the injured artery in WT mice, whereas ACE mRNA was not significantly changed, as previously reported (data not shown).9 The changes in mRNA levels of ACE and ACE2 in the femoral artery of MasKO mice were not significantly different from those in WT mice. Expression of Mas mRNA was undetectable in the femoral artery of MasKO mice. We next examined the effects of administration of azilsartan or Ang-(1–7) on these mRNA levels. Expression of ACE mRNA in WT and MasKO mice did not change with these treatments. However, ACE2 mRNA level in the injured artery was higher in azilsartan- and Ang-(1–7)-treated WT mice compared with untreated WT mice, whereas Mas mRNA level was not significantly changed by treatment with azilsartan or Ang-(1–7). In MasKO mice, ACE2 mRNA level in the injured artery was also higher in the azilsartan- and Ang-(1–7)-treated groups. AT1 receptor mRNA level was not different among all groups. Expression of AT2 receptor mRNA increased in the injured artery compared with the noninjured artery in both WT and MasKO mice. In addition, we observed that treatment with azilsartan or Ang-(1–7) increased AT2 receptor mRNA level in the injured artery in both WT and MasKO mice compared with noninjured mice.

Possible Role of Ang-(1–7) in Vascular Remodeling via Angiotensin II Type 2 Receptor Stimulation

As shown in Figures 1 to 3, administration of Ang-(1–7) did not effectively blunt neointimal formation, inflammation, or oxidative stress in MasKO mice compared with the effects in WT mice. These results support the possibility that Ang-(1–7) could activate the AT2 receptor in this mouse model of vascular remodeling, as previously reported,12 and that AT2 receptor stimulation plays a critical role in the improvement of vascular injury. To further explore the involvement of the Ang-(1–7)/AT2 receptor pathway in vascular remodeling after cuff placement, we also used AT2KO mice and examined the effect of Ang-(1–7) on neointimal formation (Figure 5; Figure S6). The neointimal area after cuff placement was larger in AT2KO mice than in WT mice, as we previously reported.16 Interestingly, we observed that the inhibitory effect of Ang-(1–7) on neointimal formation was less marked in AT2KO mice compared with WT mice (64.8% reduction in WT mice, 51.0% reduction in AT2KO mice). Mas mRNA level was not significantly changed in AT2KO mice compared with WT mice (data not shown).

Figure 4. Effect of administration of azilsartan (Azil) or angiotensin-(1–7) (Ang-(1–7)) on the expression of Ang-converting enzyme (ACE) (A), ACE2 (B), Mas (C), Ang II type 1 (AT1) receptor (D), and Ang II type 2 (AT2) (E) receptor in injured artery induced by cuff placement in wild-type (WT) and Mas-deficient (MasKO) mice. Cuff placement was performed using WT and MasKO mice, and artery samples were taken at 7 days after cuff placement for measurement of superoxide production. In situ production of superoxide was detected with dihydroethidium as fluorescence intensity. Values are mean±SEM of 5 samples in each group. *P<0.05 vs WT control mice. †P<0.05 vs MasKO control mice.

Figure 3. Effect of administration of azilsartan (Azil) or angiotensin-(1–7) (Ang-(1–7)) on superoxide anion production in injured artery induced by cuff placement in wild-type (WT) and Mas-knockout (MasKO) mice. Azil and Ang-(1–7) were administered as described in the Methods section. Artery samples were taken 7 days after cuff placement for measurement of superoxide production. Values are mean±SEM of 5 samples in each group. *P<0.05 vs WT control mice. †P<0.05 vs MasKO control mice.
Figure 5. Effect of administration of angiotensin-(1–7) (Ang-(1–7)) on neointimal formation in injured artery induced by cuff placement in Ang II type 2 receptor knockout (AT2KO) mice. Ang-(1–7) was administered intraperitoneally at a dose of 0.5 mg/kg per day using an osmotic minipump. Artery samples were taken 14 days after cuff placement for measurement of neointimal area. Values are mean±SEM of 10 samples in each group. *P<0.05 vs wild-type (WT) control mice. †P<0.05 vs AT2 KO control mice.

Discussion

We demonstrated that neointimal formation, cell proliferation, inflammation, and oxidative stress in MasKO mice after cuff placement were enhanced compared with those in WT mice, that the effect of AT1 receptor blockade by azilsartan was attenuated in MasKO mice, and that Ang-(1–7) treatment decreased neointimal formation after cuff placement even in MasKO mice; however, its effect on neointimal formation in MasKO mice was decreased compared with that in WT mice. We also observed that the effect of Ang-(1–7) on neointimal formation in AT2KO mice was less marked than that in WT mice. This experimental model actually reproduced the phenotype of small quantitative differences versus WT mice, with the neointimal area behaving in otherwise the same way to Ang-(1–7) in both WT and AT2KO mice. We speculate that the effects of Ang-(1–7) could be exerted simultaneously in concert with both Mas and AT2 receptor stimulation. Accordingly, we proposed that blockade of the AT1 receptor by azilsartan could enhance the activities of the ACE2/Ang-(1–7)/Mas axis and ACE2/Ang-(1–7)/AT2 receptor axis, thereby inhibiting neointimal formation.

ACE2 and its product Ang-(1–7) have been reported to exert antiatherosclerotic properties, including decrease in inflammation and cell proliferation. For instance, chronic Ang-(1–7) infusion induced improvement of endothelial cell function and inhibited atherosclerotic lesion formation in apolipoprotein E-deficient mice. Furthermore, a recent study demonstrated that long-term Ang-(1–7) treatment dose dependently inhibited early atherosclerotic lesion formation through vascular smooth muscle cell proliferation and migration in apolipoprotein E-deficient mice. One important mechanism of vascular remodeling is via oxidative stress induced by the ACE2/Ang II/AT1 receptor axis, and the ACE2/Ang-(1–7)/Mas axis has also been reported to be involved in the regulation of oxidative stress. For instance, it has been reported that MasKO mice from 2 different genetic backgrounds showed a reduction in superoxide dismutase and catalase activity, suggesting impaired antioxidant properties in these animals. In addition, it is reported that the vasoprotective and atheroprotective effects of Ang-(1–7) are mediated by the restoration of nitric oxide bioavailability in apolipoprotein E-deficient mice. In the present study, we demonstrated that neointimal formation after cuff placement was significantly greater in MasKO mice than in WT mice, with an increase in inflammation, oxidative stress, and cell proliferation. However, Ang-(1–7) treatment decreased neointimal formation, with attenuated inflammation, oxidative stress, and cell proliferation. These results suggest an important role of the ACE2/Ang-(1–7)/Mas axis in vascular remodeling, including counteraction of inflammation and oxidative stress. Recently, Rompe et al demonstrated that a direct AT1 receptor stimulation reduces TNF-α–induced IL-6 expression by inhibition of nuclear factor-κB activity. Therefore, it is important to assess the nuclear factor-κB activity to understand in more detail the upstream of monocyte chemoattractant protein-1, TNF-α, and IL-1β in our experimental condition. Additional detailed investigation is necessary to investigate the role of ACE2/Ang-(1–7)/Mas and possible Ang-(1–7)/AT2 receptor axis in terms of preventing inflammation.

In our previous study using ACE2KO mice, it was shown that activation of the ACE2/Ang-(1–7)/Mas axis is at least partly involved in the beneficial effects of an ARB, olmesartan, on vascular remodeling. It has also been suggested that AT1 receptor stimulation regulated ACE2 and Ang-(1–7) expression in the aorta of spontaneously hypertensive rats. Furthermore, it has been reported that an ARB, olmesartan, decreased neointimal formation and increased ACE2 immunostaining in the aorta after balloon injury in spontaneously hypertensive rats. Koka et al reported that Ang II downregulated ACE2 via the extracellular signal-regulated kinase/p38 mitogen-activated protein kinase pathway mediated by AT1 receptor stimulation. In the present study, we also observed that the mRNA of ACE2 and Mas in the injured artery was markedly decreased in WT mice, and administration of azilsartan attenuated the decrease in ACE2 mRNA. In contrast, the inhibitory effects of azilsartan on vascular remodeling were attenuated in MasKO mice, despite restoration of the decreased ACE2 mRNA. Taking these findings together, AT1 receptor blockade by ARBs seems to alter the balance between the ACE2/Ang-(1–7)/Mas axis and the ACE2/Ang II/AT1 receptor axis, with improvement in vascular remodeling. Consistently, an ARB, olmesartan, has been reported to increase ACE2 expression in injured aorta and femoral artery. Furthermore, we reported that AT1 receptor null mice showed an increase in ACE2 expression in the injured femoral artery after cuff placement. In the present study, azilsartan also increased ACE2 expression in the injured femoral artery, in addition to an increase in AT1 receptor expression. These results suggest that activation of the ACE2/Ang-(1–7)/Mas axis and Ang-(1–7)/AT1 receptor axis is at least partly involved in the beneficial effects of ARBs.

Furthermore, we observed that the inhibitory effect of Ang-(1–7) on neointimal formation was less marked in AT2KO mice compared with WT mice. Recently, the Ang-(1–7)/AT2 receptor axis has been highlighted as a...
new pathway.10 A previous report has demonstrated that Ang-(1–7) evoked a vasodepressor effect in conscious Wistar-Kyoto rats and spontaneously hypertensive rat in the presence of partial AT1 receptor blockade, suggesting possible stimulation of the AT1 receptor by Ang-(1–7).11 Another report has also demonstrated that chronic Ang-(1–7) treatment produced vasoprotective and atheroprotective effects in the apolipoprotein E–deficient mouse model of atherosclerosis as a result of increased nitric oxide bioavailability via both Mas and AT1 receptors.4 Our previous study showed that expression of the AT1 receptor is upregulated in the injured femoral artery after cuff placement.12 In addition, AT2 receptor stimulation is known to be involved in the effects of ARBs on vascular remodeling. These results, including our previous results, suggest that the Ang-(1–7)/AT2 receptor activity, and this mechanism may be involved in the beneficial effects of ARBs.

In summary, our results indicate possible involvement of both the ACE2/Ang-(1–7)/Mas axis and the Ang-(1–7)/AT2 receptor pathways in the actions mediated by azilsartan in the context of vascular remodeling. Ang-(1–7) seemed to show counterregulation against vascular remodeling via the AT2 receptor and Mas. In addition, blockade of the AT2 receptor may enhance the activity of the Ang-(1–7)/AT2 receptor axis, as well as the ACE2/Ang-(1–7)/Mas axis activity, and this mechanism may be involved in the beneficial effects of ARBs.

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Disclosures
None.

References


### Novelty and Significance

#### What Is New?
- Neointimal formation in Mas-knockout mice after cuff placement was greater than that in wild-type mice, and the effect of angiotensin II (Ang II) type 1 receptor blockade by azilsartan was attenuated in Mas-knockout mice compared with that in wild-type mice. Ang-(1–7) treatment decreased neointimal formation after cuff placement even in Mas-knockout mice; however, its inhibitory effect on neointimal formation in Mas-knockout mice was less marked than that in wild-type mice. The inhibitory effect of Ang-(1–7) on neointimal formation in Ang II type 2 receptor knockout mice was also less marked than that in wild-type mice.

#### What Is Relevant?
- We showed possible involvement of the Ang-(1–7)/Ang II type 2 receptor pathway in the inhibitory action of azilsartan on vascular remodeling. This observation suggests a new mechanism of action of Ang II type 1 receptor blockers.

#### Summary
- Our results suggest a possible role of the Ang-(1–7)/Ang II type 2 receptor axis in the regulation of vascular remodeling.
Possible Role of Angiotensin-Converting Enzyme 2 and Activation of Angiotensin II Type 2 Receptor by Angiotensin-(1–7) in Improvement of Vascular Remodeling by Angiotensin II Type 1 Receptor Blockade
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Possible Role of ACE2 and Activation of AT2 Receptor by Angiotensin-(1-7) in Improvement of Vascular Remodeling by AT1 Receptor Blockade

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Short title: ACE2/Ang-(1-7)/AT2 axis in vascular remodeling

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Expanded Methods

Animals and treatment
AT2KO (C57BL/6J background)\(^1\) mice and MasKO mice (C57BL/6J background) at 10-11 weeks of age, weighing 25 to 30 g, were used. WT (C57BL/6J) mice were used for control. The animals were housed in a room where lighting (12 h on, 12 h off) and room temperature (25 °C) were controlled. They were given free access to standard laboratory chow (CE2 rodent diet, CLEA Japan, Inc., Tokyo, Japan) and water. To induce vascular injury, a polyethylene tube (2-mm-long: PE 90, Becton Dickinson, Franklin Lakes, NJ) was placed loosely around the left femoral artery, as described previously.\(^2\) An ARB, azilsartan (provided by Takeda Pharmaceutical Company, Osaka, Japan), was administered at a dose of 0.3 mg/kg per day in chow. In some experiments, Ang-(1-7) was administered intraperitoneally at a dose of 0.5 mg/kg/day using an osmotic minipump (Alzet model 1002, Durect Corp., Cupertino, California, USA) at the same time as cuff placement. In the control group, saline was administered with an osmotic minipump. Blood pressure was measured by the indirect tail-cuff method (BP-98A, Softron Co., Ltd., Tokyo, Japan).

Generation of Mas1 knockout mice
MasKO mice (C57BL/6J background) at 10-11 weeks of age were used in the present study. Targeted disruption of the Mas gene was as follows: Genomic clones containing fragments of the MAS1 gene were isolated from a C57BL/6 (RP23: 309H20) BAC clone. A 13.37Kb region used to construct the targeting vector was first subcloned and confirmed by sequencing. The Neo cassette replaced 4.83Kb of the gene including exon 9 and ~3Kb upstream of exon 9 including a repetitive sequence between 5.86Kb, the long homology arm located 5’ to exon 9, and 2.69Kb, the short homology arm located 3’ to exon 9. The targeting vector was confirmed by restriction analysis and by sequencing. A pGK-gb2 loxP/FRT Neo cassette was inserted into the gene as described above. The targeting construct was introduced into embryonic stem (ES) cells. These clones were identified as homologous recombinants by PCR and Southern blotting. Male chimeric offspring were bred with C57BL6 female mice, and germ line transmission of the mutant Mas1 allele was identified by PCR of genomic DNA.

Quantitative RT-PCR
Pooled samples of 7-10 arteries for the group without cuff placement, and 4-5 arteries for the group with cuff placement were used. Total RNA was extracted from the femoral arteries 7 days after cuff placement. Real-time quantitative reverse-transcription polymerase chain reaction (PCR) was performed with a Premix Ex Taq (Takara Bio Inc., Shiga, Japan). The PCR primers were as follows: angiotensin converting enzyme (ACE), 5'-AGAGTACAACCAGATCCTGCTAGAC-3' (forward) and 5'-TCCAGCTTTCCATGCCCATAAG-3' (reverse); ACE2, 5'-GCACTCTCAGCAGACAAGAACAA-3' (forward) and 5'-ATTTCATCCAATCCCTGGCTCAAGT-3' (reverse); Mas, 5'-GACTAACGATGCCCACCGATGC-3' (forward) and 5'-GTTCTTGCCCTGGGACTTCCTC-3' (reverse); monocyte chemoattractant protein-1 (MCP-1), 5'-TTAACGCCCACTCACCTTGCTG-3' (forward) and 5'-GCTTTGGGATCCACCTGCTGC-3' (reverse); tumor necrosis factor-α (TNF-α), 5'-CGAGTGACAAGCTGTAGGC-3' (forward) and 5'-GGTGAGGAGCACGTGC-3' (reverse); interleukin-1β (IL-1β), 5'-CAACGACAAAATACCTGTGGCCT-3' (forward) and 5'-GCTTGGGATCCACCTGCTGC-3' (reverse); angiotensin II (Ang II) type 1 (AT₁) receptor, 5'-GTTCTGCTACACGTGCAGTGC-3' (forward) and 5'-CATCAGCCAGATGATGATGC-3' (reverse); Ang II type 2 (AT₂) receptor, 5'-CCAGACAGCCTGTGAGATG-3' (forward) and 5'-CTGCCATGAGTGTGCAGAG-3' (reverse); p22phox, 5'-TGCTCAGCTGCTGCTGATAGATG-3' (forward) and 5'-CTCCAGGAGACAGATGACTGAC-3' (reverse); p40phox, 5'-TTTGAGAGGCAGACTCAGCCA-3' (forward) and 5'-CTCCAGGAGACAGATGACTGAC-3' (reverse); p47phox, 5'-TGAGCAGCTCCAGACAGA-3' (forward) and 5'-GGTGAAAGGGCTGCTTCTTG-3' (reverse); p67phox, 5'-CAGACAAAAACCCAGAAA-3' (forward) and 5'-AGGGTGAATCCGAAGCTCAA-3' (reverse); gp91phox, 5'-TGGGATCACAGGAATTGTCA-3' (forward) and 5'-CTTCCACATCTTCGAGTC-3' (reverse); NOX1, 5'-TGGCTAAATCCCATCCAGTC-3' (forward) and 5'-CCAAAGCTCCTCTGTCTTG-3' (reverse); NOX4,
5'-GAGTCACTCCATTGTCATCG-3' (forward) and 
5'-TCCCCATCTGTTTGAAGTGGG-3' (reverse); Rac1,
5'-CCAGTGAATCTGGGCCTATG-3' (forward) and 
5'-ACAGTGGTGTCCACTTGAGC-3' (reverse); glyceraldehyde-3-phosphate 
dehydrogenase (GAPDH), 5'-ATGTAGGCCATGAGGTCCAC-3' (forward) and 
5'-TGCGACTTTCAACAGCACTC-3' (reverse).
References
Figure S1. Effect of administration of azilsartan (Azil) or angiotensin (Ang)-(1-7) on neointimal formation in injured artery induced by cuff placement in wild-type (WT) and Mas-deficient (MasKO) mice. Azil and Ang-(1-7) were administered as described in “Methods”. Artery samples were taken 14 days after cuff placement for measurement of neointimal area. Representative photos are shown.
Figure S2. Effect of administration of azilsartan (Azil) or angiotensin (Ang)-(1-7) on cell proliferation in injured artery induced by cuff placement in wild-type (WT) and Mas-deficient (MasKO) mice. Azil and Ang-(1-7) were administered as described in “Methods”. Artery samples were taken 7 days after cuff placement for immunostaining of proliferating cell nuclear antigen (PCNA) as described in “Methods”. Representative photos of injured femoral artery in cross-sections after PCNA staining are shown.
Figure S3
Figure S3. Dose-dependent effect of azilsartan (Azil) on neointimal formation in injured artery induced by cuff placement in wild-type (WT) mice. Azil was administered as described in “Methods”. (A) Representative photos of neointimal formation in cross-sections of femoral artery. (B) Histogram analysis of dose-dependent effect of azilsartan on neointimal formation. Values are mean ± SEM of 10 samples. *p<0.05 vs. WT control mice.
Figure S4. Effect of administration of azilsartan (Azil) or angiotensin (Ang)-(1-7) on superoxide anion production in injured artery induced by cuff placement in wild-type (WT) and Mas-deficient (MasKO) mice. Azil and Ang-(1-7) were administered as described in “Methods”. Artery samples were taken 7 days after cuff placement for measurement of superoxide production. In situ production of superoxide was detected with dihydroethidium, as described in “Methods”. Representative images for detection of superoxide production in femoral artery with or without cuff placement are shown.
Figure S5
Figure S5. Effect of administration of azilsartan (Azil) or angiotensin (Ang)-(1-7) on the mRNA expression of oxidative stress makers in injured artery induced by cuff placement in wild-type (WT) and Mas-deficient (MasKO) mice. Azil and Ang-(1-7) were administered as described in “Methods”. Artery samples were taken 7 days after cuff placement for measurement of mRNA expression of oxidative stress makers. (A) p22phox, (B) p40phox, (C) p47phox, (D) p67phox, (E) gp91phox, (F) NOX1, (G) NOX4 and (H) Rac1. Values are mean ± SEM of 4 samples in each group. *p<0.05 vs. WT control mice. †p<0.05 vs. MasKO control mice.
Figure S6. Effect of administration of angiotensin (Ang)-(1-7) on neointimal formation in injured artery induced by cuff placement in Ang II type 2 receptor-deficient (AT2KO) mice. Ang-(1-7) was administered intraperitoneally at a dose of 0.5 mg/kg/day using an osmotic minipump at the same time as cuff placement. In the control group, saline was administered with an osmotic minipump. Artery samples were taken 14 days after cuff placement for measurement of neointimal area. Representative photos of neointimal formation in cross-sections of femoral artery are shown.