Possible Involvement of Angiotensin-Converting Enzyme 2 and Mas Activation in Inhibitory Effects of Angiotensin II Type 1 Receptor Blockade on Vascular Remodeling

Masaru Iwai, Hirotomo Nakaoka, Izumi Senba, Harumi Kanno, Tomozo Moritani, Masatsugu Horiuchi

Abstract—We explored the roles of angiotensin-converting enzyme 2 (ACE2), angiotensin-(1-7), and Mas activation in angiotensin II type 1 receptor blockade-mediated attenuation of vascular remodeling. Vascular injury was induced by polyethylene-cuff placement around the mouse femoral artery. After cuff placement, the mRNA level of both ACE2 and Mas was markedly decreased in wild-type mice, whereas ACE mRNA was not changed. Immunostaining of ACE2 and Mas was observed mainly in the media and was reduced in the injured artery. Administration of angiotensin-(1-7) decreased neointimal formation after cuff placement, whereas administration of [D-Ala(7)] angiotensin-(1-7), a Mas antagonist, increased it. Consistent with these results, we also demonstrated that neointimal formation induced by cuff placement was further increased in ACE2 knockout mice. In angiotensin II type 1a receptor knockout mice, mRNA expression and immunostaining of ACE2 and Mas in the injured artery were greater, with less neointimal formation than in wild-type mice. Increased ACE2 expression in the injured artery was also observed by treatment of wild-type mice with an angiotensin II type 1 receptor blocker, olmesartan. These results suggested that activation of the ACE2-angiotensin-(1-7)-Mas axis is at least partly involved in the beneficial effects of angiotensin II type 1 receptor blockade on vascular remodeling. (Hypertension. 2012;60:137-144.) • Online Data Supplement

Key Words: angiotensin-(1-7) ACE2 ■ Mas ■ receptor ■ vascular remodeling

Roles of angiotensin (Ang)-(1-7) in the cardiovascular system have been highlighted. Ang-(1-7) is synthesized from Ang I and Ang II mainly by Ang-converting enzyme (ACE) 2 and acts on the receptor protein, Mas. These factors construct a newly proposed bioactive pathway in the renin-Ang system called the ACE2-Ang-(1-7)-Mas axis. This axis exerts an antagonistic action against the classical renin-Ang system pathway with ACE-Ang II-Ang II type 1 (AT1) receptors.

We have reported previously that blockade of the AT1 receptor attenuated vascular remodeling caused by inflammatory vascular injury after polyethylene cuff placement, indicating involvement of Ang II in vascular remodeling. It has also been indicated that AT1 receptor blockade showed beneficial effects on cardiovascular remodeling in various experimental models. A previous study also detected ACE2 expression in the cardiovascular system. Therefore, it may be possible that Ang-(1-7) is produced locally and acts as a regulator in vascular injury, similar to Ang II, in addition to circulating Ang-(1-7). Because Ang-(1-7) can be synthesized from Ang II, it may be possible that Ang-(1-7) is involved in the inhibitory action of AT1 receptor blockade on vascular remodeling. Therefore, it may be possible that Ang-(1-7) is produced locally and acts as a regulator in vascular injury, similar to Ang II. We speculate that activation of the ACE2-Ang-(1-7)-Mas axis would contribute to the inhibitory effects of AT1 receptor blockade on vascular remodeling, dependent on crosstalk with the ACE-Ang II-AT1 receptor axis.

In the present study, we examined the possible involvement of ACE2 and Mas in vascular remodeling induced by inflammation with AT1 receptor blockade, using AT1 receptor knockout (AT1KO) mice and an AT1 receptor blocker (ARB).

Methods

Animals and Treatment

AT1 receptor–deficient (AT1KO; C57BL/6J background) mice and ACE2-deficient (ACE2KO; C57BL/6J background, provided by Otsuka Pharmaceutical Co, Ltd, Tokyo, Japan) mice at 10 to 11 weeks of age were used. Wild-type (WT; C57BL/6J) mice were used for control. Mice were housed in a room where lighting was controlled (12 hours on and 12 hours off) and room temperature was kept at 24°C. 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. The experimental protocol was approved by the Animal Studies Committee of Ehime University. An ARB, olmesartan (provided by Daiichi Sankyo Co, Ltd, Tokyo, Japan), was administered continuously at a dose of 1.0 mg/kg per day using an osmotic minipump (ALZET model 1002, Durect Corp, Cupertino, CA) implanted IP at the same time as cuff placement. In some experiments, Ang-(1-7) and a Mas antagonist, [D-Ala(7)]Ang-(1-7), were administered IP at a dose of 0.5 mg/kg per day using an osmotic minipump. Blood pressure was measured by the indirect tail-cuff method (BP-98A, Softron Co, Ltd, Tokyo, Japan).

Morphometric Analysis and Immunohistochemical Staining

The femoral arteries were taken at 14 days after cuff placement and fixed by perfusion with 10% neutral-buffered formalin. Paraffin-embedded cross-sections were prepared as described previously. Intimal area was measured using computer-imaging software (Densitograph, ATTO Corp, Tokyo, Japan) after elastica van Gieson staining. Immunostaining of ACE2 and Mas was performed with specific antibodies (anti-ACE2 and anti-Mas; LifeSpan Biosciences, Inc, Seattle, WA) using paraffin-embedded sections, followed by coloring with peroxidase and diaminobenzidine. To quantify the expression of ACE2 and Mas, an immunopositive area in femoral artery after immunostaining was measured using color extraction of computer-imaging software (Densitograph, ATTO Corp) and expressed as a ratio to total vascular area. Heart and aorta samples were taken from WT mice at 7 days. Paraffin-embedded cross sections were prepared, and immunostaining was performed similarly to those performed for femoral artery.

Quantitative RT-PCR

Please see the online-only Data Supplement.

Figure 1. Change in expression of angiotensin-converting enzyme (ACE), ACE2, and Mas in injured femoral artery after cuff placement in wild-type (WT) mice. Cuff placement was performed and artery samples were taken at 7 days. Total RNA was purified and mRNA level was assayed with quantitative real-time RT-PCR as described in the Methods section. Values are mean±SEM of pooled arteries (n=5 for cuff [−] and 4 for cuff [+]). *P<0.05 vs cuff (−).

Figure 2. Change in expression of angiotensin-converting enzyme (ACE), ACE2, and Mas in injured femoral artery after cuff placement in wild-type (WT) mice. Cuff placement was performed and artery samples were taken at 7 days. Total RNA was purified and mRNA level was assayed with quantitative real-time RT-PCR as described in the Methods section. Values are mean±SEM of pooled arteries (n=5 for cuff [−] and 4 for cuff [+]). *P<0.05 vs cuff (−).

Expression of ACE2 and Mas in Injured Artery Induced by Cuff Placement in WT (C57BL/6J) Mice

Figure 1 shows the mRNA levels in cardiovascular tissues of WT (C57BL/6J) mice. Although ACE2 mRNA was similar among these tissues, Mas mRNA was relatively higher in the femoral artery than in the heart and aorta (Figure 1). In immunostaining, ACE2 was stained positively in these tissues (Figure S1, available in the online-only Data Supplement). However, Mas was not clearly stained in heart and aorta (Figure S1). To examine the roles of the ACE2-Ang-(1-7)-Mas axis in vascular remodeling, we used a vascular injury model induced by polyethylene cuff placement on the femoral artery. Figure 2 shows the changes in mRNA levels for ACE, ACE2, and Mas in the femoral artery 14 days after cuff placement. Both ACE2 and Mas mRNA was markedly reduced in the injured artery after cuff placement, whereas ACE mRNA was not significantly changed. Such a change in ACE2 and Mas expression was also examined by immunostaining (Figure 3). Immunoreactive ACE2 was mainly expressed in the media (vascular smooth muscle) in the noninjured femoral artery.

Statistical Analysis

Values are expressed as mean±SEM in the text, and Figure data were analyzed by 1-way ANOVA. If a statistically significant effect was found, the results were further compared with Bonferroni multiple range tests. A value of P<0.05 was considered to indicate statistical significance.

Results

Figure 1. Expression of angiotensin-converting enzyme (ACE) 2 and Mas in femoral artery, aorta, and heart of wild-type (WT; C57BL/6J) mice. Total RNA was prepared from each tissue, and tissue content of mRNA was assayed by quantitative real-time RT-PCR as described in the Methods section. Femoral a indicates femoral artery. Values are mean±SEM in each group (n=3 for heart and aorta and 4 for femoral artery).
This ACE2 staining was markedly weaker in the injured artery. Mas was also expressed in the media and showed a similar change to ACE2 (Figure 3B). Quantification of immunopositive area showed similar changes observed in mRNA content after cuff placement (Figure 3C).

**Effects of Ang-(1-7) and Mas Antagonist on Neointimal Formation Induced by Cuff Placement**

To explore the involvement of Ang-(1-7) in neointimal formation, we examined the effects of Ang-(1-7) and [D-Ala(7)]-Ang-(1-7) (a Mas antagonist) on neointimal formation. As shown in Figure 4, the neointimal area 14 days after cuff placement was decreased by Ang-(1-7) treatment. In contrast, treatment with [D-Ala(7)]-Ang-(1-7) exaggerated neointimal formation. Mas antagonist also reversed the inhibition caused by Ang-(1-7); Figure 4). Both treatments did not affect the medial area (data not shown). Neither Ang-(1-7) nor [D-Ala(7)]-Ang-(1-7) affected blood pressure at this dose (control: 109.3±1.7 mmHg, Ang-(1-7): 108.6±2.4 mmHg; [D-Ala(7)]-Ang-(1-7): 109.1±3.2 mmHg, respectively). Treatment with Ang-(1-7) or the Mas antagonist did not significantly affect the mRNA content for ACE, ACE2, and Mas in injured artery (Figure S2).

**Neointimal Formation and Inflammatory Factors in Injured Artery Induced by Cuff Placement in ACE2-Deficient Mice**

To further examine the role of ACE2 in vascular remodeling after cuff placement, we also used ACE2-deficient (ACE2KO) mice (Figure 5). The neointimal area after cuff placement was larger in ACE2KO mice than in WT mice (Figure 5A). The change in mRNA level for ACE and Mas in the femoral artery of ACE2KO mice was not significantly different from that in WT mice (Figure 5A). ACE2 mRNA was at an undetectable level in the femoral artery of ACE2KO mice.

**Expression of ACE2 and Mas in Injured Artery Induced by Cuff Placement in AT1a Receptor-Deficient Mice**

Next, the possible relation between the ACE2-Ang-(1-7)-Mas axis and AT1 receptor stimulation was examined. In

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**Figure 3.** Immunostaining of angiotensin-converting enzyme (ACE) 2 (A) and Mas (B) in injured femoral artery of wild-type (WT) and angiotensin II type 1a (AT1a) receptor knockout (AT1aKO) mice after cuff placement. Cuff placement was performed and artery samples were taken at 14 days after operation. Formalin-fixed, paraffin-embedded sections were prepared and immunohistochemical staining was performed using anti-ACE2 and anti-Mas antibodies as described in the Methods section. A, Immunostaining of ACE2. B, Immunostaining of Mas. Representative staining are shown. C, Quantification of immunopositive area stained by anti-ACE2 and anti-Mas antibodies. Measurement of stained area was performed using computer-imaging software after color-extraction mode. The value was expressed as a ratio of immunopositive areatotal vascular area. Values are mean±SEM of 5 arteries in each group. *P<0.05 vs cuff (−).
ACE mRNA level was not different between AT1aKO and WT mice (Figure S3). Neointimal area was smaller between WT and AT1aKO mice (Figure 3A and Figure 3B). Immunostaining showed that immunoreactive ACE2 and Mas in the injured artery were also higher in AT1aKO mice than in WT mice. Expression of mRNA for ACE, ACE2, and Mas in injured artery were also higher in AT1aKO mice than in WT mice (Figure 6A). In contrast, ACE2-deficient (ACE2KO) mice. Cuff placement was performed and artery samples were taken at 7 days for mRNA measurement, at 14 days for measurement of neointimal area. Paraffin-embedded sections were prepared, and neointimal area was measured using computer-imaging software after elastica van Gieson staining. Values are mean±SEM of 6 samples in each group. *P<0.05 vs control. †P<0.05 vs Ang-(1-7) alone.

Neointimal area after cuff placement. Values are mean±SEM of 4 to 5 samples (n=4) for wild-type (WT) and 5 for ACE2KO. B, Expression of ACE and Mas mRNA in injured artery after cuff placement. Values are mean±SEM of pooled arteries in each group, n=4 for cuff (−) and n=3 for cuff (+). *P<0.05 vs WT mice. †P<0.05 vs Ang-(1-7) alone.

Figure 4. Effect of angiotensin (Ang)-(1-7) and [D-Ala(7)]-Ang-(1-7) on neointimal formation in femoral artery of wild-type (WT) mice after cuff placement. Cuff placement was performed and paraffin-embedded sections were prepared from injured artery at 14 days after cuff placement. Ang-(1-7) and a Mas antagonist were administered IP using an osmotic minipump at a dose of 0.5 mg/kg per day. Neointimal area was measured using computer-imaging software after elastica van Gieson staining. Values are mean±SEM of 6 samples in each group. *P<0.05 vs control. †P<0.05 vs Ang-(1-7) alone.

The injured artery after cuff placement, the mRNA level for ACE2 and Mas was higher in AT1a receptor-deficient (AT1aKO) mice than in WT mice (Figure 6A). In contrast, ACE mRNA level was not different between AT1aKO and WT mice. Expression of mRNA for ACE, ACE2, and Mas in the femoral artery without cuff placement was not different between WT and AT1aKO mice (Figure 6B). Immunostaining showed that immunoreactive ACE2 and Mas in the injured artery were also higher in AT1aKO mice than in WT mice (Figure 3A and Figure 3B). Neointimal area was smaller in AT1aKO mice than that in WT mice (Figure S3).

Effect of Olmesartan on Expression of ACE2 and Mas in Injured Artery Induced by Cuff Placement in WT Mice
We reported previously that administration of olmesartan decreased neointimal formation.9 Figure 7A shows the mRNA levels for ACE2 and Mas in the injured artery of WT mice treated with olmesartan. ACE mRNA level tended to be lowered by olmesartan, but this was not significant. However, ACE2 mRNA level in the injured artery was higher in the olmesartan group, whereas Mas mRNA level was not significantly changed by olmesartan. Level of mRNA for ACE, ACE2, and Mas in control artery without cuff placement was not changed by olmesartan (Figure 7B). Immunostaining of the injured artery reflected the change in ACE2 after cuff placement (Figure 7C). Treatment with olmesartan at the dose in this study did not significantly change blood pressure (without olmesartan, 96.4±1.7 mmHg; with olmesartan, 96.9±2.8 mmHg, respectively). This nonhypotensive dose of olmesartan inhibited neointimal formation (Figure 7D). Treatment of WT mice with [D-Ala(7)]-Ang-(1-7) in addition to olmesartan partially reversed the inhibitory effect of olmesartan on neointimal formation (Figure 7D). Treatment with olmesartan did not significantly affect the expression of Mas, as well as that of ACE2, in AT1aKO mice (Figure S4).

Discussion
Previous studies indicate that Ang-(1-7) is synthesized by ACE2 and acts as an important regulator in the cardiovascular system. Long-term treatment with Ang-(1-7) induced improvement of endothelial cell function and inhibited atherosclerotic lesion formation in apolipoprotein E–deficient mice.14 Moreover, increased circulating Ang-(1-7) improved lipid and glucose metabolism.15 On the other hand, it has been reported that ACE2 suppressed cardiac dysfunction.9 Inhibition of ACE2 or ACE2 deficiency showed impairment of cardiovascular function16,17 and an increase in atherosclerotic changes.18

In addition, Mas expression is observed in the heart, kidney, lung, liver, spleen, tongue, and skeletal muscle.15,19 Recent studies suggest that some kinases, including Akt, seem to be involved in Mas-mediated signaling.19–21 Mas-deficient mice showed impairment of cardiovascular function.22,23 These results suggest that activation of the ACE2-Ang-(1-7)-Mas axis has protective actions on the cardiovascular system. However, the involvement of the ACE2-Ang-(1-7)-Mas axis and its relationship to AT1 receptor-mediated actions in inflammatory vascular remodeling are not yet clear. In the present study, the organ distribution of Mas mRNA suggests that the femoral
artery might be one of the targets of Ang-(1-7; Figure 1). It is reported that Mas acts as a receptor for Ang-(1-7).3 Previous reports suggest that the effects of Ang-(1-7) in the cardiovascular system are antagonistic against AT1 receptor-mediated function.5,6,13–17,22 The reason why Mas expression in the femoral artery was higher than aorta and heart is not clear. However, from the results in previous reports, it might be possible that the activation of Mas by circulating or tissue-specific Ang-(1-7) may have protective effects against the inflammation or injury of peripheral vessels, like thromboangiitis obliterans and atherosclerosis obliterans. To prove the possibility of the changes in Ang-(1-7) and the interplay with the AT1 signaling, it is important to measure Ang-(1-7) levels in the tissue. However, we could not detect Ang-(1-7) in femoral artery, probably because of the small amount of protein obtained.

In the present study, administration of Ang-(1-7) started with cuff placement reduced the neointimal area of the injured artery. In our study, Ang-(1-7) was still able to cause a reduction in neointimal formation despite of a marked reduction of ACE2 and Mas (Figure 4). One possibility might be that Ang-(1-7) was effective at the initial period of injury where the Mas level was not yet severely decreased. It may also be possible that Ang-(1-7) has an indirect action on vascular remodeling. These possibilities remained to be clarified. In contrast, [D-Ala(7)]-Ang-(1-7), a Mas antagonist, enhanced neointimal formation (Figure 4). Moreover, the inhibitory effect of Ang-(1-7) on neointimal formation was reversed by a Mas antagonist (Figure 4), suggesting the involvement of the ACE2-Ang-(1-7)-Mas axis in vascular remodeling after cuff placement. In the present study, [D-Ala(7)]-Ang-(1-7) reversed the inhibitory effect of olmesartan only partially (Figure 7D). To explain the incomplete effect of the Mas antagonist, it may be possible that the involvement of the ACE2-Ang-(1-7)-Mas axis in injured artery is only partial because of the low levels of ACE2 and Mas expression and their decrease after injury. Therefore, the reversal by the Mas antagonist was incomplete. Another possibility may be that the inhibitory effect of olmesartan on neointimal formation includes the blockade of AT1 receptor stimulation plus the activation of the ACE2-Ang-(1-7) pathway, which antagonizes AT1 receptor-mediated signaling. The Mas antagonist could reverse the latter part of olmesartan’s action, but the former action remained. These possibilities should be clarified in future study.

The physiological and pathological effects of Ang II are mainly mediated through AT1 receptors, composed of the
ACE-Ang II-AT1 receptor axis.24 Previous studies indicated that stimulation of the ACE2-Ang-(1-7)-Mas axis showed antagonistic actions against the ACE-Ang II-AT1 receptor axis.2,14,16–18 Because 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 by ACE2.

AT1 receptor blockade by ARBs appeared to alter the balance between the ACE2-Ang-(1-7)-Mas axis and the

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**Figure 7.** Effect of olmesartan on expression of angiotensin-converting enzyme (ACE), ACE2, and Mas and neointimal formation in injured artery after cuff placement in wild-type (WT) mice. Cuff placement was performed and paraffin-embedded sections were prepared as in Figure 3. Olmesartan and an Mas antagonist were administered IP using an osmotic minipump at a dose of 1.0 mg/kg per day and 0.5 mg/kg per day, respectively. Levels of mRNA were determined by quantitative RT-PCR. Neointimal area was measured using computer-imaging software after elastica van Gieson staining. A, Expression of ACE, ACE2, and Mas in injured arteries treated with olmesartan. B, Expression of ACE, ACE2, and Mas in femoral artery without cuff placement. Results of control mice were taken from Figure 2. Values are mean±SEM of 4 to 5 pooled arteries, n=4 for control and n=5 for olmesartan. *P<0.05 vs control. C, Representative immunostaining of ACE2 and Mas in injured arteries treated with olmesartan. D, Effect of olmesartan on neointimal formation after cuff placement. Values are mean±SEM in each group, n=6 for control, n=5 for olmesartan, and n=4 for olmesartan plus Mas antagonist. *P<0.05 vs control. †P<0.05 vs olmesartan alone.
ACE-Ang II-AT₁ receptor axis to improve vascular remodeling. A previous report suggested that AT₁ receptor stimulation regulated ACE2 and Ang-(1-7) expression in the aorta of spontaneously hypertensive rats. In addition, 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. It has been reported that Ang II 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 AT₂ receptor blockade increased it.

Thus, blockade of the AT₁ receptor in the present study seemed to increase the activity of the ACE2-Ang-(1-7)-Mas axis. ACE2 expression in the injured artery was higher in AT₁aKO mice or after treatment with olmesartan (Figures 6 and 7). On the other hand, Mas expression in the injured artery was higher in AT₂aKO mice, whereas it was not changed by olmesartan. Such a difference of Mas expression between AT₁aKO mice and olmesartan-treated mice might be because of the relatively short-term treatment with olmesartan or the possible involvement of AT₂b receptor function, which was preserved in AT₁aKO mice. However, the latter possibility might not occur in our case, because treatment with olmesartan did not significantly alter the expression of Mas, as well as ACE2, in AT₂aKO mice (Figure S4). Because the inhibition of neointimal formation by olmesartan was weakened by administration of [D-Ala(7)]-Ang-(1-7), the results suggest that the ACE2-Ang-(1-7)-Mas axis was involved at least in part in the inhibitory effect of olmesartan on vascular remodeling after cuff placement.

In our previous study, it was shown that Ang II type 2 (AT₂) receptor stimulation was involved in the effects of ARB on cardiovascular remodeling. The counteractions of AT₂ receptor stimulation against the AT₁ receptor seemed to be similar to the biological effects of the ACE2-Ang-(1-7)-Mas axis. In the present study, we have not examined the involvement of AT₂ receptor function in the action of ACE2-Ang-(1-7)-Mas axis. However, because it is reported that AT₂ receptor expression in the femoral artery was increased after cuff placement, it might be possible that there is an interaction between the ACE2-Ang-(1-7)-Mas axis and AT₂ receptor-mediated signaling.

**Disclosures**

None.

**References**

15. Iwai et al. ACE2-Ang-(1-7)-Mas Axis in Vascular Remodeling

**Perspectives**

In summary, our results indicated a reduction of ACE2 and Mas expression in the injured artery after cuff placement, suggesting downregulation of the ACE2-Ang-(1-7)-Mas axis in vascular remodeling. Activation of the ACE2-Ang-(1-7)-Mas axis appeared to show counterregulation against vascular remodeling. In addition, blockade of the AT₁ receptor may enhance the activity of the ACE2-Ang-(1-7)-Mas axis, and this mechanism may be involved in the beneficial effects of ARBs.

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**Novelty and Significance**

What Is New?

- Mouse femoral artery expressed a relatively higher level of Mas than heart or aorta. In injured artery, expression of Mas and ACE2 was decreased. Remodeling in injured artery was exaggerated by a Mas antagonist but ameliorated by Ang-(1-7). Vascular remodeling after injury and reduction of ACE2 expression were suppressed in AT1a receptor null mice or by treatment with an ARB, olmesartan.

What Is Relevant?

- We showed a possible involvement of the ACE2-Ang-(1-7)-Mas axis in the inhibitory action on vascular remodeling. This observation suggests a new mechanism of action of ARB.

Summary

Our results suggest a possible role of the ACE2-Ang-(1-7)-Mas axis in the regulation of vascular remodeling.
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POSSIBLE INVOLVEMENT OF ACE2 AND MAS ACTIVATION IN INHIBITORY EFFECTS OF AT1 RECEPTOR BLOCKADE ON VASCULAR REMODELING

Masaru Iwai, Hirotomo Nakaoka, Izumi Senba, Harumi Kanno, Tomozo Moritani, Masatsugu Horiuchi

Department of Molecular Cardiovascular Biology and Pharmacology, Ehime University Graduate School of Medicine, Tohon, Ehime, Japan

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

Correspondence to:

Masatsugu Horiuchi, MD, PhD
Department of Molecular Cardiovascular Biology and Pharmacology
Ehime University Graduate School of Medicine
Shitsukawa, Tohon, Ehime 791-0295, Japan
Tel: +81-89-960-5249
Fax: +81-89-960-5251
E-mail: horiuchi@m.ehime-u.ac.jp
Expanded Methods

Quantitative Reverse-Transcription Polymerase Chain Reaction

Pooled samples of 7-8 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 at 7 days after cuff placement. Quantitative real-time reverse-transcription polymerase chain reaction (PCR) was performed using Premix Ex Taq (Takara Bio, Inc., Shiga, Japan). The PCR primers for ACE2 were 5’-TGTGTCTGTGAGAGCCCTAGAGGAC-3’ (forward) and 5’-AGGCTGGAAGGTGGCTCAAG-3’ (reverse); for Mas, 5’-GCTTGGAGGCTATCTTCGACTC-3’ (forward) and 5’-GAGGCTTCCAACACTCAGTCAGTC-3’ (reverse); for ACE, 5’-AGAGTACCAACCAGATCCTGCTAGAC-3’ (forward) and 5’-TCCAGCTCTTTCCATGGCCCATG-3’ (reverse); for tumor necrosis factor (TNF)-α, 5’-CGAGTGACGACCTGTAGCC-3’ (forward) and 5’-GGTGAGGAGCACGTAGTCG-3’ (reverse) and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5’-TGCGACTTCAAAGCAGCAGCTC-3’ (forward) and 5’-ATGTAGGCCATGACGTCAC-3’ (reverse).
Figure S1. Immunostaining of ACE2 and Mas in heart, aorta and femoral artery in wild type mice. Tissue samples were taken from wild type (C57BL/6J) mice. Formalin-fixed, paraffin-embedded sections were prepared and immunohistochemical staining was performed using anti-ACE2 and anti-Mas antibodies as described in “Methods”. Representative staining are shown.
Figure S2. Expression of ACE, ACE2 and Mas in injured femoral artery in WT mice treated with Ang-(1-7) and a Mas antagonist, [D-Ala(7)]-Ang-(1-7). Cuff placement was performed and artery samples were taken at 7 days. Total RNA was purified and mRNA level was assayed with quantitative real-time RT-PCR as in Figure 2. Results of WT mice were taken from Figure 2. Values are mean ± SEM of 3 pooled arteries in each group.
Figure S3. Neointimal formation in AT\textsubscript{1a}KO mice after cuff placement. Cuff placement was performed and paraffin-embedded sections were prepared from injured artery at 14 days after cuff placement. Neointimal area was measured using computer-imaging software after elastica van Gieson staining. Values are mean ± SEM in each group (n= 6 for WT and 5 for AT\textsubscript{1a}KO). *p<0.05 vs. Control.
Figure S4. Effect of olmesartan on expression of ACE, ACE2 and Mas in injured artery after cuff placement in AT1aKO mice. Cuff placement was performed and paraffin-embedded sections were prepared as in Figure 3. Olmesartan was administered intraperitoneally using an osmotic minipump at a dose of 1.0 mg/kg/day. Levels of mRNA were determined by quantitative RT-PCR as in Figure 2. Values are mean ± SEM of 3 pooled arteries in each group.