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ORIGINAL ARTICLE

Role of angiotensin-converting enzyme 2/angiotensin-(1–7)/Mas axis in the hypotensive effect of azilsartan

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The possible counteracting effect of angiotensin (Ang)-converting enzyme (ACE)2/Ang-(1–7)/Mas axis against the ACE/Ang II/Ang II type 1 (AT_1) receptor axis in blood pressure control has been previously described. We examined the possibility that this pathway might be involved in the anti-hypertensive effect of a newly developed AT_1 receptor blocker (ARB), azilsartan, and compared azilsartan's effects with those of another ARB, olmesartan. Transgenic mice carrying the human renin and angiotensinogen genes (hRN/hANG-Tg) were given azilsartan or olmesartan. Systolic and diastolic blood pressure, as determined by radiotelemetry, were significantly higher in hRN/hANG-Tg mice than in wild-type (WT) mice. Treatment with azilsartan or olmesartan (1 or 5 mg kg^{-1} per day) significantly decreased systolic and diastolic blood pressure, and the blood pressure-lowering effect of azilsartan was more marked than that of olmesartan. The urinary Na concentration decreased in an age-dependent manner in hRN/hANG-Tg mice. Administration of azilsartan or olmesartan increased urinary Na concentration, and this effect was weaker with olmesartan than with azilsartan. Azilsartan decreased ENaC-α mRNA expression in the kidney and decreased the ratio of heart to body weight. Olmesartan had a similar but less-marked effect. ACE2 mRNA expression was lower in the kidneys and hearts of hRN/hANG-Tg mice than in WT mice. This decrease in ACE2 mRNA expression was attenuated by azilsartan, but not by olmesartan. These results suggest that the hypotensive and anti-hypertrophic effects of azilsartan may involve activation of the ACE2/Ang-(1–7)/Mas axis with AT_1 receptor blockade.
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INTRODUCTION

Recently, the counteracting effect of the angiotensin (Ang)-converting enzyme (ACE) 2/Ang-(1–7)/Mas axis against the ACE/Ang II/Ang II type 1 (AT_1) receptor axis has been highlighted in studies of blood pressure regulation and organ protection.[1,2] Administration of Ang-(1–7) attenuated the development of severe hypertension and end-organ damage in spontaneously hypertensive rats treated with L-NAME.[3] Giani *et al.*[4] reported that chronic administration of Ang-(1–7) normalized arterial pressure, reduced glycemia and triglyceridemia, improved proteinuria and ameliorated structural alterations in the kidneys of stroke-prone spontaneously hypertensive rat. To further evaluate the possible counter-regulatory effects of ACE2/Ang-(1–7)/Mas on the ACE/Ang II/AT_1 receptor in blood pressure control, we used transgenic mice carrying the human renin and angiotensinogen genes (hRN/hANG-Tg).[5]

We speculated that an increase in the Ang-(1–7) level during ACE inhibition and AT_1 receptor blockade might result in Mas receptor activation and contribute to the cardioprotective and renoprotective effects.[6] Moreover, it is possible that AT_1 receptor blockade directly activates the ACE2/Ang-(1–7)/Mas pathway.[7–10] We also demonstrated that in AT_1a receptor knockout mice, there was greater mRNA expression and immunostaining of ACE2 and Mas in the injured artery than in wild-type (WT) mice, with less neointimal formation.[11]

A newly developed AT_1 receptor blocker (ARB), azilsartan, has a high binding capacity for the AT_1 receptor and shows a marked hypotensive effect.[12,13] In a clinical study, azilsartan reduced blood pressure more effectively than olmesartan and valsartan in patients with stage 1 and 2 hypertension.[14] Therefore, we examined the possibility that the ACE2/Ang-(1–7)/Mas axis might be involved in the anti-hypertensive effect of azilsartan and compared its effects with those of olmesartan.

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MATERIALS AND METHODS

Animals

This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All animal studies were reviewed and approved by the Animal Studies Committee of Ehime University. Ten-week-old male C57BL/6 (Clea Japan, Tokyo, Japan) and human renin (hRN-Tg; C57BL/6 background) and human angiotensinogen (hANG-Tg; C57BL/6 background) double transgenic mice (hRN/hANG-Tg) were used in this study.[5] Mice were housed in a room where lighting was controlled (12 h on, 12 h off) and the temperature was kept at 24 °C. The mice were fed a standard diet (MF, Oriental Yeast, Tokyo, Japan) and water ad libitum.

Experiment 1

The blood pressure of the C57BL/6 and hRN/hANG-Tg mice was analyzed by radiotelemetry.[15] Briefly, mice were anesthetized with Nembutal in saline. A midline neck incision was made, and the left common carotid artery was isolated. The telemetry probe (PA-C10; Data Sciences International, New Brighton, MN, USA) was inserted into the left common carotid artery. Ten days after implantation, blood pressure was measured. Then, hRN/hANG-Tg mice were separated into two groups receiving either 1 mg kg^{-1} per day of azilsartan (provided by Takeda, Tokyo, Japan) or olmesartan (Olmesartan Acid, Toronto Research Chemicals, Toronto, Ontario, Canada) in chow for 2 weeks. Two weeks after the start of treatment, the mice were given 5 mg kg^{-1} per day of azilsartan or olmesartan for 2 weeks. Blood pressure was measured 2 weeks after the start of administration of each ARB.

Experiment 2

Ten-week-old hRN/hANG-Tg mice were given control chow or chow containing one of two different doses (1 or 5 mg kg^{-1} per day) of azilsartan or olmesartan for 4 weeks. Urine samples were obtained before the start of treatment, and 2 and 4 weeks later by using metabolic cages (3600M021, Tecniplast, Tokyo, Japan), and the urinary Na concentration was determined (DRI-CHEM 7000 V, FUJIFILM, Tokyo, Japan). Kidney and heart samples were obtained 4 weeks after the start of treatment.

Real-time reverse transcriptase-PCR

Kidney and heart samples were frozen in liquid nitrogen and stored at −80 °C until analysis. Total mRNA was extracted from heart and kidney samples with Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan). Quantitative real-time reverse transcriptase-PCR was performed with a SYBR green kit (MJ Research, Waltham, MA, USA). The PCR primers were as follows: 5′-TCAACCAGGCC CCCTGCAATCA-3′ (forward) and 5′-GCTCTGTGCGCAGTGTCAGGG-3′ (reverse) for ENaC-α and 5′-TGTGTCTGATGTCATTCCTAGAAGT-3′ (forward) and 5′-AGGCTGGTAAGGTGGCTCAAG-3′ (reverse) for ACE2.

Ang-(1–7) treatment

A telemetry transmitter was implanted in hRN/hANG-Tg mice. Ten days after implantation, blood pressure was measured and the hRN/hANG-Tg mice were given 5 mg kg^{-1} per day Ang-(1–7) (4332, Peptide Institute, Osaka, Japan) via an osmotic mini-pump (model 1004, Alzet, Cupertino, CA, USA). Blood pressure was measured 2 weeks after the onset of treatment.

Statistical analysis

All values are expressed as mean ± s.e.m. in the text and figures. Blood pressure response data were analyzed by two-way repeated measures analysis of variance with a Bonferroni multiple comparison post-test. All other data were evaluated by one-way analysis of variance followed by *post hoc* analysis for multiple comparisons.

RESULTS

Blood pressure

The systolic and diastolic blood pressure in hRN/hANG-Tg mice were significantly higher than in WT mice (Figure 1). Treatment with azilsartan or olmesartan (1 or 5 mg kg^{-1} per day) significantly decreased systolic and diastolic blood pressure, and the blood pressure-lowering effect of azilsartan was more marked than that of olmesartan (Figures 1a and b). Treatment with azilsartan or olmesartan at a dose of 10 mg kg^{-1} per day did not increase the blood pressure-lowering effect when compared with the effects of the 5 mg kg^{-1} per day dose (data not shown). There were no differences in heart rate between the two groups (Supplementary Figure 1).

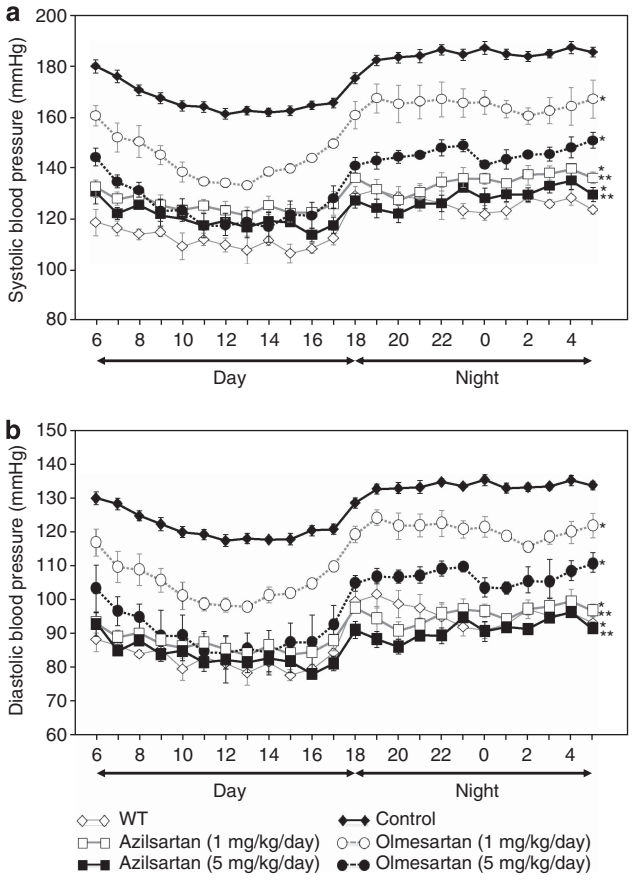

Figure 1 Effect of azilsartan or olmesartan on systolic (**a**) and diastolic (**b**) blood pressure in hRN/hANG-Tg mice. $n=5$ for each group. $^{*}P<0.01$ vs untreated hRN/hANG-Tg. $^{**}P<0.01$ vs the same dose in olmesartan-treated mice

Urinary Na concentration

The urinary Na concentration decreased in an age-dependent manner in hRN/hANG-Tg mice when compared with WT mice (Figure 2a). Administration of azilsartan or olmesartan increased the urinary Na concentration compared with that of untreated hRN/hANG-Tg mice 2 and 4 weeks after the start of treatment, and the effect of 1 mg kg^{-1} per day of olmesartan was weaker than the effect of the same dose of azilsartan (Figure 2a). The expression of ENaC-α mRNA in the kidneys of hRN/hANG-Tg mice did not differ from that seen in WT mice (Figure 2b). Administration of azilsartan significantly decreased ENaC-α mRNA expression compared with untreated hRN/hANG-Tg mice. In contrast, olmesartan treatment slightly decreased ENaC-α mRNA expression; however, this effect was not significant.

Cardiac hypertrophy

The ratio of heart to body weight in hRN/hANG-Tg mice was greater than in WT mice (Figure 3). Treatment with azilsartan at a dose of 1 or 5 mg kg^{-1} per day decreased the ratio of heart to body weight

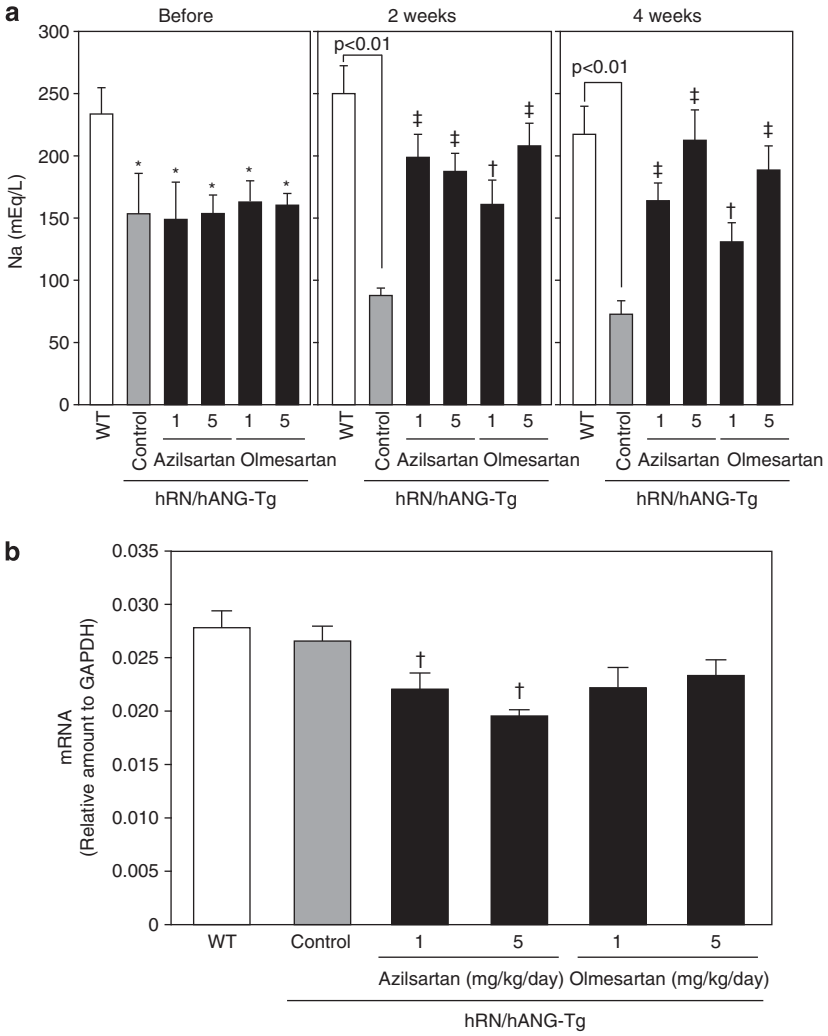

Figure 2 Effect of azilsartan or olmesartan on urinary Na excretion in hRN/hANG-Tg mice before and after 2 and 4 weeks of administration (**a**). Effect of azilsartan or olmesartan on expression of ENaC-α mRNA in the kidneys of hRN/hANG-Tg mice after 4 weeks of administration (**b**). $n=6$ for each group. $^{*}P<0.05$ vs WT mice. $^{\dagger}P<0.05$ vs untreated hRN/hANG-Tg mice. $^{\ddagger}P<0.01$ vs untreated hRN/hANG-Tg mice.

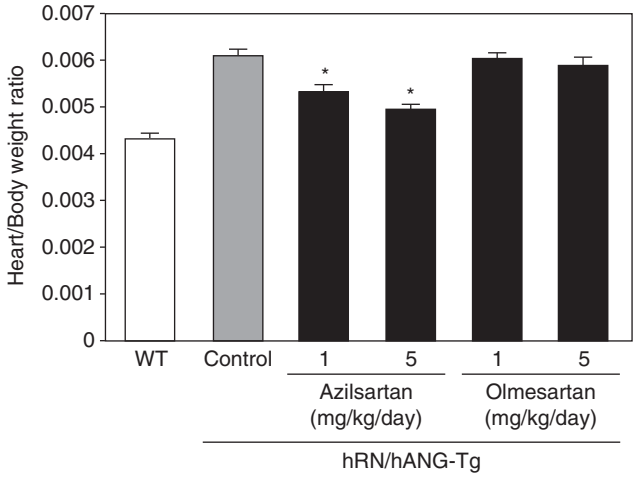

Figure 3 Effect of azilsartan or olmesartan on heart/body ratio in hRN/hANG-Tg mice after 4 weeks of administration. $n=6$ for each group. $^{*}P<0.05$ vs untreated hRN/hANG-Tg mice.

compared with the untreated hRN/hANG-Tg mice; however, the same dose of olmesartan did not decrease this ratio.

Expression of ACE2 mRNA

The expression of ACE2 mRNA in the kidneys and hearts of hRN/hANG-Tg mice was lower than in WT mice (Figures 4a and b). These decreases in ACE2 mRNA expression were attenuated by azilsartan; however, olmesartan did not have this effect.

Effect of Ang-(1–7) administration on blood pressure

Administration of 5 mg kg^{-1} per day Ang-(1–7) via an osmotic mini-pump significantly decreased systolic and diastolic blood pressure (Supplementary Figure 2).

DISCUSSION

Transgenic mice carrying the hRN/hANG-Tg had higher blood pressures and lower urinary Na concentration and expression of ACE2 mRNA in the kidney than did WT mice. Treatment with azilsartan significantly decreased systolic and diastolic blood pressure and increased urinary Na concentration and ACE2 mRNA expression

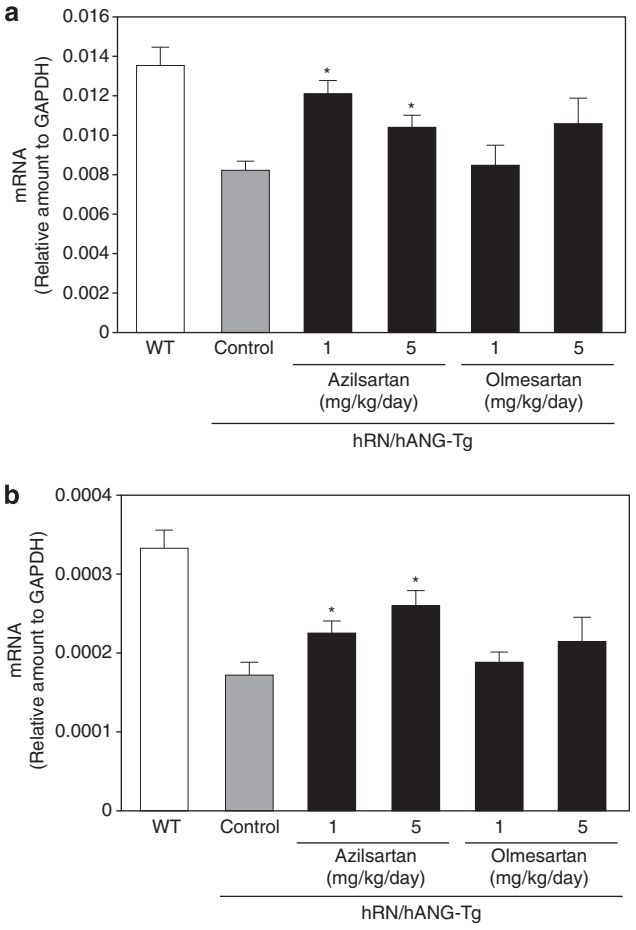

Figure 4 Effect of azilsartan or olmesartan on expression of ACE2 mRNA in the kidneys (**a**) and hearts (**b**) of hRN/hANG-Tg mice after 4 weeks of administration. $n=6$ for each group. $^*P<0.05$ vs untreated hRN/hANG-Tg mice.

in the kidney with a decrease in ENaC-α mRNA expression in the kidney. At each dose, these effects of azilsartan were more marked than the effects of olmesartan. These results suggest that the hypotensive effects of azilsartan may involve activation of the ACE2/Ang-(1–7)/Mas axis with AT_1 receptor blockade.

Activation of the Ang II/AT_1 receptor is pivotal in increasing blood pressure due to Na reabsorption in the kidney. Crowley *et al.*[16,17] reported that expression of Ang II in the kidney induces hypertension, possibly by promoting Na reabsorption. Moreover, Mamenko *et al.*[18] reported that Ang II activates ENaC and that this effect was additive to the effect of aldosterone.

Consistent with these results, we demonstrated that urinary Na concentration was lower in hRN/hANG-Tg mice than in WT mice, and that this decrease was attenuated by treatment with ARBs.

Accumulating evidence suggests that the ACE2/Ang-(1–7)/Mas pathway in the kidney has a role in blood pressure control. In the Goldblatt hypertension rat, an Ang-(1–7) antagonist or ACE2 inhibitor increased blood pressure and kidney damage.[19] It has been reported that the ACE2/ACE ratio is higher in the normal kidney than in the hypertensive nephropathy.[8] AT_1 receptor stimulation decreases the expression of ACE2 and increases that of ACE in the kidney. We observed that ACE2 mRNA expression in the kidneys of hRN/hANG-Tg mice was lower than in WT mice, and treatment with azilsartan increased ACE2 mRNA and decreased ENaC-α mRNA. However, these effects were not observed with olmesartan treatment at a dose of 1 or 5 mg kg^{-1} per day, suggesting that the anti-hypertensive effect of azilsartan in hRN/hANG-Tg mice might be partially due to changes in ACE2 and ENaC-α expression. ENaC has an important role in the control of blood pressure. Loss-of-function mutations in ENaC lead to type 1 pseudohypoaldosteronism and cause hypotension, whereas-gain-of function mutations lead to Liddle syndrome and cause hypertension;[20,21] this suggests that the decrease in the expression of ENaC-α caused by azilsartan could be partially responsible for its ability to lower blood pressure.

It has been reported that ARB treatment attenuates cardiac remodeling after myocardial infarction and increases plasma Ang-(1–7).[22,23] Ferrario *et al.*[24] also reported that blockage of RAS by an ACE inhibitor or ARB increases cardiac Ang-(1–7), ACE2 mRNA and ACE2 activity. In cardiac myocytes, ACE2 activity and mRNA expression are decreased by AngII treatment. However, this decrease is attenuated by cotreatment with the ARB losartan.[10] We have also reported that ACE2-deficient mice have cardiac hypertrophy compared with WT mice at the age of 10 weeks.[25] hRN/hANG-Tg mice demonstrated cardiac hypertrophy and reduced expression of ACE2 mRNA. This cardiac hypertrophy and ACE2 mRNA reduction were attenuated by treatment with azilsartan and were associated with an increase in ACE2; however, this effect was not observed with olmesartan, suggesting that this effect of azilsartan in hRN/hANG-Tg mice might be partially due to activation of the ACE2/Ang-(1–7)/Mas pathway. It is also possible that the blood pressure differences between mice treated with azilsartan or olmesartan could influence ENaC-α mRNA expression, cardiac hypertrophy and ACE2 mRNA expression.

In conclusion, our results demonstrate that azilsartan inhibits the blood pressure increase and cardiac hypertrophy with increased kidney and heart ACE2 that are seen in transgenic mice carrying both the hRN/hANG-Tg, suggesting that these effects of azilsartan might be in part due to activation of the ACE2/Ang-(1–7)/Mas axis. The same inhibitory effects of olmesartan were less marked in this mouse strain, although olmesartan is known to inhibit vascular remodeling, cardiac hypertrophy and renal damage, involving activation of the ACE2/Ang-(1–7)/Mas pathway.[7,9,11,26,27] Our previous report also demonstrated that treatment with olmesartan prevented a cuff-injury-induced decrease in ACE2 expression in femoral arteries.[11] Although the detailed mechanism is yet to be investigated, this apparent discrepancy might be because a different mouse model other than the cuff-injury model was used. Moreover, the antagonistic properties of azilsartan against AT_1 receptor blockade could be involved[12] because hRN/hANG-Tg mice overproduce Ang II associated with exaggerated AT_1 receptor activation. In contrast, Varagic *et al.*[28] recently reported that Ang-(1–7) does not mediate the long-term effects of olmesartan on blood pressure through Mas in addition to counterbalancing renin release in response to AT_1 receptor blockade. The Ang II-mediated ACE2 reduction is known to be regulated by the extracellular signal-regulated kinase (ERK) 1/ERK2/phosphatase pathway and/or the ERK/p38 mitogen-activated protein kinase pathway.[8,9] However, it is difficult to explain the different increases in ACE2 mRNA that were induced by azilsartan and olmesartan based only on their effects on the mitogen-activated protein kinase pathway. Further investigation will reveal the pathophysiological role of the ACE2/Ang-(1–7)/Mas axis in blood pressure control and contribute to the discussion of further possible drug effects of ARBs beyond their class effect.

CONFLICT OF INTEREST

MH received research support and lecturing fees from Takeda Pharmaceutical Company Ltd. The remaining authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Hypertension Research website (http://www.nature.com/hr)