ORIGINAL ARTICLE

Hemodynamic effects of vasorelaxant compounds in mice lacking one, two or all three angiotensin II receptors

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The renin–angiotensin system (RAS) has a vital role in regulating the cardiovascular system. The primary effector of the RAS is the octapeptide angiotensin (Ang) II, a potent regulator of blood pressure and water homeostasis. Ang II mediates its functions through the stimulation of two distinct receptors, AT₁ (two subtypes in rodents (AT_{1a} and AT_{1b})) and AT₂. It was shown that in addition to Ang II, shorter fragments of Ang are also biologically active. Ang-(1–7) came into focus because it opposes many of the detrimental effects of Ang II. However, it is still controversial whether Ang II receptors are involved in Ang-(1–7)-mediated signaling. To characterize the impacts of Ang II receptors on Ang-(1–7)-stimulated vascular relaxation, the effects of acute infusion of the three vasorelaxant compounds, that is, Ang-(1–7), bradykinin (BK) and acetylcholine (ACh), on heart rate (HR) and mean arterial pressure (MAP) were investigated in mice deficient for one, two or all three Ang II receptors. Ang-(1–7) and BK reduced MAP in wild-type, AT_{1a}/AT_{1b}-deficient and AT₂-deficient mice. Although the change in absolute MAP values in the hypotensive triple knockouts (KO) could not be further reduced by both peptides, the percent change in MAP was comparable between the triple KO and wild-type mice. Both peptides did not alter the HR in all four genotypes. ACh significantly reduced absolute MAP values in all four genotypes with a similar percentage of reduction. In contrast to Ang-(1–7) and BK, ACh significantly reduced HR without genotypic differences. Our results generate proof that Ang-(1–7)-induced effects on MAP are mediated by a receptor that is independent of AT₁ and AT₂.

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INTRODUCTION

The renin–angiotensin system is essentially involved in the regulation of blood pressure, electrolyte homeostasis and the intake of water and sodium.^{1,2} Angiotensin (Ang) II, the main acting peptide of the renin–angiotensin system, stimulates its two known receptors, the Ang II receptors type 1 (AT₁) and type 2 (AT₂). In mice and rats, the AT₁ exists in two distinct subtypes (AT_{1a} and AT_{1b}), whereas it appears that other mammalian species do not have AT₁ subtypes. AT_{1a} and AT_{1b} exhibit similar ligand binding and signal transduction properties, and cannot be distinguished pharmacologically, but differ in their tissue distribution.³ In addition to Ang II, shorter Ang metabolites, such as Ang IV^{4,5} and Ang-(1–7),^{6–8} demonstrate potent biological activity. The heptapeptide Ang-(1–7) is an Ang metabolite of special interest because it can often counteract the detrimental effects of Ang II, for example, its vasoconstrictive properties.⁷ In addition to its vascular effects, it was shown that Ang-(1–7) also has beneficial effects on cardiac function.^{9–11} Moreover, it opposes the growth-stimulating

effects of Ang II in vascular smooth muscle cells¹² and cardiomyocytes.¹³ Ang-(1–7) also demonstrates effects on renal function by participating in the control of water and electrolyte homeostasis.¹⁴ However, research involving Ang-(1–7) is not restricted to cardiovascular and renal function. In recent years, investigations have demonstrated Ang-(1–7)-dependent effects on wound healing,¹⁵ mechanisms involved in learning and memory,¹⁶ cancer^{17–19} and early progenitor cells.²⁰

Previously, we have shown that the receptor Mas is associated with Ang-(1–7)-stimulated intracellular signaling.²¹ However, whether Ang II receptors are involved in Ang-(1–7)-stimulated effects is still under debate because some of the stimulated pathways are also affected by co-treatment with AT₁ blockers^{22,23} or AT₂ blockers.^{24,25} To further characterize the impact of Ang II receptor subtypes on Ang-(1–7)-mediated vascular relaxation, we investigated the cardiovascular response to acute Ang-(1–7) infusion in recently generated mice lacking one, two or all three Ang II receptor subtypes.^{26,27}

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METHODS

Animals

Males (aged ~7 months) lacking either both AT₁ subtypes (AT_{1a}^{-/-}/AT_{1b}^{-/-}/AT₂^{+/+}; double KO), AT₂ (AT_{1a}^{+/+}/AT_{1b}^{+/+}/AT₂^{-/-}) or all three receptor subtypes (AT_{1a}^{-/-}/AT_{1b}^{-/-}/AT₂^{-/-}; triple KO) of the recently described Ang II receptor-deficient mice^{26,27} and their wild-type controls (AT_{1a}^{+/+}/AT_{1b}^{+/+}/AT₂^{+/+}; WT) were used in this study. Experiments on adult mice were approved by the local authorities and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996), the regulations of the Animal Care Committee of the Erasmus MC and the Federal Law on the Use of Experimental Animals in Germany.

Figure 1 Baseline mean arterial pressure (MAP) of mice lacking both AT₁ subtypes (AT_{1a}^{-/-}/AT_{1b}^{-/-}/AT₂^{+/+}), AT₂ (AT_{1a}^{+/+}/AT_{1b}^{+/+}/AT₂^{-/-}) or all three Ang II receptors (AT_{1a}^{-/-}/AT_{1b}^{-/-}/AT₂^{-/-}) and their wild-type controls (AT_{1a}^{+/+}/AT_{1b}^{+/+}/AT₂^{+/+}). **P*<0.05; ****P*<0.001 vs. wild-type; *n*≥7.

Figure 2 (a) Changes in mean arterial pressure (MAP) of mice lacking both AT₁ subtypes (AT_{1a}^{-/-}/AT_{1b}^{-/-}/AT₂^{+/+}), AT₂ (AT_{1a}^{+/+}/AT_{1b}^{+/+}/AT₂^{-/-}) or all three Ang II receptors (AT_{1a}^{-/-}/AT_{1b}^{-/-}/AT₂^{-/-}) and their wild-type controls (AT_{1a}^{+/+}/AT_{1b}^{+/+}/AT₂^{+/+}) induced by a bolus injection of Ang-(1-7). **P*<0.05; ****P*<0.001 vs. saline; *n*≥7. (b) Relative changes in MAP in all four genotypes induced by a bolus injection of Ang-(1-7). **P*<0.05 vs. wild-type; *n*≥7. (c) Changes in heart rate (HR) induced by a bolus injection of Ang-(1-7).

PCR (genotyping)

Genotyping was performed using the PCR with specific primers for each genotype as described previously.²⁶

Blood pressure measurement

The hemodynamic response was recorded as described previously.²⁶ Briefly, mice of the four selected genotypes were weighed and anesthetized with pentobarbital (60 mg kg⁻¹; i.p.). After the left carotid artery and right jugular vein were cannulated with a PE-10 (0.011"ID×0.025"OD) catheter, heart rate and mean arterial pressure (MAP) were continuously monitored with a disposable pressure transducer (TSE Systems GmbH, Bad Homburg, Germany). After resting for 5–15 min for stabilization, hemodynamic baseline values were determined using Invasive Blood Pressure Monitoring System recording software (TSE Systems GmbH). Following the stabilization period, the hemodynamic response to Ang-(1-7) (0.5 µg ml⁻¹; 100 µl bolus injection) was evaluated. After a recovery period of ~10 min, the vascular response to 1.5 µg ml⁻¹ bradykinin (BK; 100 µl) was recorded. Finally, after an additional recovery period, the protocol was repeated for 0.1 µg ml⁻¹ acetylcholine (ACh; 100 µl).

Statistics

The results were represented as the mean ± s.e.m. For determination of intergroup differences, one-way analysis of variance with the Bonferroni *post hoc* test was used. To compare the differences before and after injection, the Student's *t*-test was used (Graph Pad Prism 3.01; Graph Pad Software, San Diego, CA, USA). A value of *P*<0.05 was considered to be significant.

RESULTS

Under baseline conditions, double KO and triple KO mice had a significantly reduced basal MAP compared with WT mice (Figure 1), while AT₂-deficient mice showed significantly increased blood pressure. To elucidate the effects of the three Ang II receptors on Ang-(1-7)-induced changes in blood pressure and HR, we administered a single bolus injection of the heptapeptide. The acute infusion of Ang-(1-7) led to a significant reduction of MAP in WT mice (Figure 2a). In double KO mice and in mice deficient for AT₂, the infusion of Ang-(1-7) also led to a significant drop in MAP, while the Ang-(1-7) infusion could not significantly reduce absolute MAP values in triple KO mice. However, the percentage MAP changes were not different between the hypotensive triple KO mice and mice from the other three genotypes (Figure 2b). Notably, there was a

Figure 3 (a) Changes in mean arterial pressure (MAP) of mice lacking both AT_{1a} subtypes ($AT_{1a}^{-/-}/AT_{1b}^{-/-}/AT_2^{+/+}$), AT_2 ($AT_{1a}^{+/+}/AT_{1b}^{+/+}/AT_2^{-/-}$) or all three Ang II receptors ($AT_{1a}^{-/-}/AT_{1b}^{-/-}/AT_2^{-/-}$) and their wild-type controls ($AT_{1a}^{+/+}/AT_{1b}^{+/+}/AT_2^{+/+}$) induced by a bolus injection of bradykinin (BK). * $P < 0.05$; *** $P < 0.001$ vs. saline; $n \geq 7$. (b) Relative changes in MAP in all four genotypes induced by a bolus injection of BK. * $P < 0.05$ vs. wild-type; $n \geq 7$. (c) Changes in heart rate (HR) induced by a bolus injection of BK.

Figure 4 (a) Changes in mean arterial pressure (MAP) of mice lacking both AT_{1a} subtypes ($AT_{1a}^{-/-}/AT_{1b}^{-/-}/AT_2^{+/+}$), AT_2 ($AT_{1a}^{+/+}/AT_{1b}^{+/+}/AT_2^{-/-}$) or all three Ang II receptors ($AT_{1a}^{-/-}/AT_{1b}^{-/-}/AT_2^{-/-}$) and their wild-type controls ($AT_{1a}^{+/+}/AT_{1b}^{+/+}/AT_2^{+/+}$) induced by a bolus injection of acetylcholine (ACh). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. saline; $n \geq 7$. (b) Relative changes in MAP in all four genotypes induced by a bolus injection of ACh. $n \geq 7$. (c) Changes in heart rate (HR) induced by a bolus injection of ACh.

significantly stronger response to Ang-(1–7) in the hypertensive AT_2 -deficient mice. The infusion of Ang-(1–7) had no impact on HR in mice from all four examined genotypes (Figure 2c).

To elucidate whether the lack of all three Ang II receptors led to a general impairment in vasorelaxant properties, especially in triple KO mice, we infused two other vasoactive compounds. First, we administered BK as a renin–angiotensin system-independent peptide. BK mediates its vasorelaxant effects by stimulation of its own receptor, the BK receptor type 2 (B_2). BK infusion led to a significant drop in MAP in double KO, AT_2 -deficient and WT mice (Figure 3a). Similar to Ang-(1–7), there was no further significant drop in absolute MAP in triple KO mice; in addition, a similar relative change in MAP was observed in triple KO mice (Figure 3b) but a more pronounced change in AT_2 -deficient mice compared with that in WT mice. Furthermore, similar to Ang-(1–7), the acute infusion of BK had no impact on HR (Figure 3c) in mice from all four genotypes.

Second, we used the non-peptide vasodilator ACh. Similar to Ang-(1–7) and BK, ACh infusion induced significant blood pressure changes in double KO, AT_2 -deficient and WT mice (Figure 4a). In contrast to the two peptides, ACh injection also induced a significant drop of absolute MAP in triple KO mice. Notably, even when MAP changes were expressed as percentages, ACh reduced MAP in triple KO mice without significant differences compared with mice from the other three genotypes (Figure 4b). In contrast to Ang-(1–7) and BK, a bolus injection of ACh reduced HR in mice from all genotypes tested without statistical differences among the groups (Figure 4c).

DISCUSSION

In 1988, it was shown that the Ang II metabolite Ang-(1–7) mediates biological effects.²⁸ In pharmacological approaches using the specific Ang-(1–7) antagonist A779,^{21,29,30} and in genetically modified animals lacking the G protein-coupled receptor Mas,^{21,31} it has been

demonstrated that Ang-(1-7)-stimulated signaling is associated with the receptor Mas. However, there is an ongoing controversy concerning the extent to which the known Ang II receptors are involved in mediating Ang-(1-7) effects. In cell culture, it was shown that Ang-(1-7) can inhibit the activity of the distal tubule Na^+/K^+ ATPase in a dose-dependent manner via AT_1 .²² Oudot *et al.* have demonstrated AT_1 -dependent vasoconstriction and nicotinamide adenine dinucleotide phosphate oxidase activation by the heptapeptide.³² In addition to these AT_1 -mediated effects, other publications have demonstrated AT_2 -mediated effects of Ang-(1-7), for example, on the Na^+ -ATPase of the inner³³ and outer cortex.³⁴ Furthermore, Walters *et al.*³⁵ have demonstrated that vasorelaxant effects of Ang-(1-7) can be mediated by AT_2 .

Because most investigations postulating effects of AT_1 or AT_2 on Ang-(1-7)-stimulated signaling have utilized pharmacological approaches, we used an experimental setting with genetic deficiencies in one, two or all three murine Ang II receptors to arrive at a more definite conclusion regarding the vascular effects of Ang-(1-7). In this study, we therefore elucidated the impact of the three known Ang II receptors on Ang-(1-7)-induced vasorelaxant effects. We investigated the acute effects of Ang-(1-7) injections in mice deficient in either both AT_1 subtypes, in AT_2 or in all three receptors. Using these $\text{AT}_{1a}/\text{AT}_{1b}$ double-deficient or AT_2 -deficient mice, we observed Ang-(1-7)-stimulated vasorelaxant effects with no differences in the blood pressure-lowering effects of the heptapeptide in WT mice. These results strongly contradict the conclusion that the three Ang II receptors are involved in the actions of Ang-(1-7). However, it has been shown that the different Ang II receptors can influence each other.³⁶⁻⁴⁰ To exclude the possibility that the lack of one of the receptor subtypes alters the properties of the remaining receptors, we also investigated the effects of Ang-(1-7) in mice lacking all three Ang II receptor types. Although Ang-(1-7) was not able to reduce absolute MAP in the hypotensive triple KO mice, the percent changes induced by Ang-(1-7) were comparable with that in WT mice. Because injections of the non-renin-angiotensin system-associated peptide BK were also unable to significantly reduce absolute MAP in triple KO mice but did induce similar percent changes in WT and triple KO mice, our findings demonstrate that mild vasorelaxant compounds cannot induce a massive drop in MAP due to the already attenuated blood pressure in this animal model. However, the lack of all three Ang II receptors did not lead to a general vascular dysfunction; a bolus injection of ACh, a stronger endothelium-dependent vasorelaxant, also lowered absolute MAP values in triple KO mice.

Interestingly, we identified a stronger response to Ang-(1-7) and BK in AT_2 -deficient mice. These findings have two possible explanations. First, the AT_2 -deficient mice exhibit a generally altered responsiveness to vasorelaxant compounds. Because the effects of ACh in AT_2 -deficient mice were unchanged (Figure 4b), this general change seems unlikely. Second, because AT_2 effects can be blocked by the specific B_2 antagonist icatibant, other studies have suggested that there might be interactions between AT_2 and the BK/B_2 system.^{41,42} Nevertheless, further experiments are needed to arrive at a final conclusion.

The discrepancy in the importance of AT_1 and AT_2 for vasorelaxant effects of Ang-(1-7) obtained in approaches using receptor blockers and in our approach using animals genetically deficient for the receptors may be due to ectopic effects of the compounds used in pharmacological interventions. It is well accepted that some AT_1 blockers also function as agonists of the peroxisome proliferator-activated receptor- γ ^{43,44} or as cyclooxygenase inhibitors.⁴⁵ The most widely used AT_2 blocker, PD123319, was described originally as an AT_2 agonist.⁴⁶ In view of these Ang II receptor-independent effects of the

receptor blockers, we consider our KO model to be superior and more accurate than the previously described pharmacological approaches. Taken together, our findings generate strong evidence that both Ang II receptors, AT_1 and AT_2 , are not involved in mediating Ang-(1-7)-stimulated vascular effects.

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