Regulation of angiotensin-converting enzyme 2 and Mas receptor by Ang-(1–7) in heart and kidney of spontaneously hypertensive rats

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Abstract
Inter-regulation between components of the renin–angiotensin system is common, but little is known about the direct regulatory effects of Ang-(1–7) on expression of tissue ACE2 and the Mas receptor. Eighteen male spontaneously hypertensive rats (SHR) and 20 normotensive Wistar-Kyoto rats were randomly allocated to four groups of 9–10 rats each and received either 24 μg/kg per hour of Ang-(1–7) in saline or saline alone (5 ml/h) by infusion for 14 consecutive days. Tail-cuff systolic blood pressures were recorded and ACE2 and Mas expression was measured using quantitative real-time PCR (QRT-PCR) and Western blotting. Cardiac and renal ACE2 mRNA was decreased in SHR. Although having no effects on blood pressure, Ang-(1–7) down-regulated cardiac ACE2 mRNA in normotensive rats (1.80 ± 0.27 vs. 5.89 ± 0.62, \( p < 0.05 \)) but did not change renal ACE2. Ang(1–7) down-regulated cardiac Mas mRNA of Wistar rats only (2.50 ± 0.44 vs. 8.10 ± 1.33, \( p < 0.05 \)), and renal Mas mRNA of SHR receiving Ang-(1–7) was decreased (0.44 ± 0.09 vs. 1.00 ± 0.17, \( p < 0.05 \)). Results from Western blot tests were consistent with those from QRT-PCR tests. These results suggest organ-specific regulation of local ACE2 and Mas expression by continuous infusion of Ang-(1–7) which did not alter blood pressure of either SHR or Wistar rats.

Keywords
Angiotensin-converting enzyme 2, gene, Mas receptor, angiotensin-(1-7), regulation

Introduction
It has been well established that over-activation of the renin–angiotensin system (RAS) plays a detrimental role in several cardiovascular pathologies such as hypertension, endothelial dysfunction, progression of heart failure and chronic renal disease. However, it is only recently that some novel members of this system other than classical angiotensins and their synthesising enzymes and their receptors were found to be biologically active. Recent studies have found that angiotensin-(1–7) (Ang-(1–7)), the heptapeptide which can be formed from angiotensin II (Ang II) or angiotensin I (Ang I), is an important component of RAS with several biological effects that are opposite to those elicited by Ang II. For example, Ang-(1–7) showed anti-proliferation, vasodilator actions and anti-fibrosis effects in some studies.1,2 Angiotensin-converting enzyme 2 (ACE2) is now considered the most crucial rate-limiting forming enzyme of Ang-(1–7) in vivo. ACE2 was firstly cloned independently by Donoghue et al.3 and Turner et al.,4 and exhibited a high catalytic efficiency for the conversion of Ang II to Ang-(1–7).5,6 ACE2 is widely distributed in tissues but hardly at all in the circulation, and is most abundant in heart and kidney,7 which suggests the important effects of this enzyme on the local regulation of cardiac and renal functions. As for the specific receptor of Ang-(1–7), there have been a number of suggestions. However, in 2003 Santos et al. proved that the G protein-coupled receptor Mas was a special functional receptor for Ang-(1–7).7 Taken together, this axis of ACE2–Ang-(1–7)–Mas receptors may function as an important arm of the RAS which can antagonise the classical axis of ACE–Ang II–angiotensin receptors at corresponding levels.

It is well known that components of RAS can be inter-regulated. For example, some studies have revealed that Ang II is one of the most potent factors in the regulation of angiotensinogen and renin synthesis, and that Ang II infusion produces negative feedback regulation of pulmonary ACE activity.8 Ang-(1–7) has been found to have

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several cardiovascular effects, such as functioning as a vasodilator hormone and participating in the regulation of renal function. However little is known about the direct effects of Ang-(1–7) infusion on the regulation of tissue ACE2 and Mas gene expression, which are, respectively, the upstream synthetic enzyme and downstream receptor of these angiotensin polypeptides. Treatments using ACE inhibitors and Ang II receptor type 1 (AT1 receptor) blockade have been shown to increase plasma Ang-(1–7) of hypertensive and normotensive animals, and also to regulate the expression of ACE2 and Mas gene in some tissues. These regulatory effects may be the result of increased level of Ang-(1–7). It is at the present time not clear whether the increase of Ang-(1–7) can directly mediate the regulatory effects of certain drugs, i.e. ACE inhibitor (ACEI) or AT1 receptor blocker (ARB), on ACE2 and Mas expression. In other words, it is still unknown what the direct regulatory effects of Ang-(1–7) will be on the expression of other components of RAS. The focus of the present investigation is to study whether Ang-(1–7) itself influences expression of ACE2 and Mas in the heart and kidney of both the normotensive and hypertensive rats.

Methods

Animals and experimental protocol

Experiments were performed in 18 male 13-week-old spontaneously hypertensive rats (SHR) (276 ± 3 g body weight, Vital River Laboratory, Beijing, China) and 20 normotensive male Wistar-Kyoto rats (287 ± 7 g body weight, Animal Center of Sun Yat-sen University, Guangzhou, China). All the animals were allowed to accommodate to environmental conditions for 1 week. They were housed in individual cages on a 12-h light–dark cycle in a room with temperature (24 ± 2°C) and humidity control (50–60%), with ad libitum access to tap water and standard rodent chow. At 14 weeks of age, rats were randomly divided into four groups of 9–10 rats each. Each rat was anaesthetised with an intra-abdominal injection of sodium pentobarbital (40 mg/kg). An osmotic minipump (model 2ML2, Alzet osmotic pump, Alza, USA) implanted subcutaneously delivered via catheter (PE50, Alza, USA) either 24 μg/kg per hour of Ang-(1–7) (Bachem, Switzerland) in saline or saline alone (5 ml/h) into the jugular vein for 14 consecutive days. Tail-cuff systolic blood pressures (SBP) were measured (NIBP controller; AD instruments, Australia) before and after the treatment. At the end of the treatment period, rats were deeply anaesthetised and euthanised by cardiopulmonary excision. The heart and kidney were removed and rinsed in cold saline and then quick-frozen in liquid nitrogen and stored at -80°C. All the experimental procedures were performed in compliance with guidelines set out by the Animal Care and Use Committee of Sun Yat-Sen University.

Quantification of ACE2 and Mas mRNA

RNA was isolated from the heart and kidney using TRIzol reagent (Invitrogen Life Technologies, Australia) as directed by the manufacturer. cDNA was synthesised with a reverse transcriptase reaction carried out with the use of standard techniques (GeneAmp® PCR System 9700, Applied Biosystems Inc) with random hexamers, deoxyribonucleotides, RNase inhibitor and approximately 1 μg of total RNA. An aliquot of the resulting cDNA was used in the quantitative real-time PCR (QRT-PCR) experiments, as described below. QRT-PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye combined with a standard curve method on a 7500 Fast Real-Time PCR System (Applied Biosystems Inc), for determination of amounts of mRNA of ACE2 and Mas using the primers listed in table 1, and was carried out with GAPDH as the endogenous control. Amplification conditions (40 cycles) for measurements of ACE2 and Mas mRNAs were performed as follows: pre-denaturation at 93°C for 3 min; denaturation at 93°C for 30 s; annealing at 55°C for 45 s.

Western blot analysis

Protein extracted from heart and kidney was added to 2×SDS sample buffer and boiled for 5 min. SDS-PAGE was performed with a 10% gel and transferred to PVDF membranes (BioRad Electrophoresis/Transfer Apparatus). The membrane was blocked for 2 h in 5% fat-free milk in TBS

Table 1. Sequence of primers and probes used in RT-PCR

| Sequence Name | Forward Primer | Reverse Primer | Probe |
|---------------|----------------|----------------|-------|
| R-ACE2        | 5'-TCA GAG CTG GGA TGC AGA AA-3' | 5'-GGC TCA GTG AGC ATG GAG TTT-3' | 5'-FAM-AAG TTC TCT GTT TCT GGC C-TAMRA-3' |
| R-MAS1        | 5'-GAC CAG CCC ACA GTT ACC AGT T-3' | 5'-CCA GGG TTC CCC TCC TGA CT-3' | 5'-FAM-TCC CGG CTT TCT GGA TTA C-TAMRA-3' |
| R-GAPDH       | 5'-TGG TCT ACA TGT TCC AGT ATG ACT-3' | 5'-CCA TTT CAT GGT AGC GGG ATC T-3' | 5'-FAM-CCA CGG CAA GTT CAA CGG CAC AGT-TAMRA-3' |
containing 0.1% Tween-20 (TBST) at room temperature. The membrane was incubated in ACE2 or Mas polyclonal antibody (Santa Cruz) diluted 1:400 in 5% bovine serum albumin (BSA)/TBST at 4°C overnight, then washed (3 × 15 min in TBST), and incubated in rabbit anti-goat HRP antibody (1:8000, Golden Bridge Technology Inc, China) for 2 h and washed (3 × 15 min in TBST). Chemiluminescent HRP substrate (Millipore, USA) and blue film (Kodak Imaging) were used for detection of relevant proteins. The ACE2 band was visualised at an apparent molecular weight of about 90 kDa, and the Mas band was visualised at an apparent molecular weight of 40 kDa. GAPDH protein of heart and kidney was also measured as the endogenous control.

**Table 2. Main effects of treatments on systolic blood pressure**

| Systolic Blood Pressure (mmHg) | Control + Ang-(1–7) | Control + Vehicle | SHR + Ang-(1–7) | SHR + Vehicle |
|-------------------------------|---------------------|-------------------|-----------------|--------------|
| Before                        | 124 ± 4             | 128 ± 4           | 203 ± 5         | 198 ± 5      |
| After                         | 130 ± 4*            | 126 ± 3           | 193 ± 3*        | 200 ± 4      |

Data are means ± SE. *p values compare values before and after each treatment period. †p > 0.05 compared with data before treatment.

**Table 3. Real-time polymerase chain reaction (PCR) measures of ACE2 and Mas genes in heart and kidney**

| Gene of interest | Control + Vehicle | Control + Ang-(1–7) | SHR + Vehicle | SHR + Ang-(1–7) |
|------------------|-------------------|---------------------|--------------|-----------------|
| Cardiac ACE2     | 5.89 ± 0.62       | 1.80 ± 0.27*        | 1.01 ± 0.22* | 1.00 ± 0.09*    |
| Renal ACE2       | 1.64 ± 0.11       | 1.74 ± 0.26†        | 1.02 ± 0.13* | 1.10 ± 0.24*    |
| Cardiac Mas      | 8.10 ± 1.33       | 2.50 ± 0.44†‡       | 1.02 ± 0.32* | 0.74 ± 0.14*    |
| Renal Mas        | 1.00 ± 0.13       | 0.78 ± 0.12         | 1.00 ± 0.17  | 0.44 ± 0.09†    |

* < 0.05 compared with control + vehicle group; †p < 0.05 compared with SHR + vehicle group, ‡p < 0.05 compared with SHR + Ang-(1–7) group.

**Results**

Table 2 compares effects of the different treatment regimens on tail-cuff SBP of SHR and control Wistar rats. Ang-(1–7) had no significant effects on SBP of SHR or control rats (p > 0.05).

**Effects of Ang-(1–7) versus vehicle on local ACE2 and Mas mRNA expressions**

Figure 1 illustrates the effects of intravenous Ang-(1–7) or vehicle on ACE2 and Mas mRNA in heart and kidney. Cardiac and renal ACE2 mRNA were both significantly decreased in SHR compared with normotensive Wistar rats (relative gene expression results were, respectively, 1.01 ± 0.22 vs. 5.89 ± 0.62, p < 0.05 and 1.02 ± 0.13 vs. 1.64 ± 0.11, p < 0.05).

**Figure 1.** A: Real-time RT-PCR analysis of ACE2 mRNA in the heart of spontaneously hypertensive rats (SHR) showed a significant decrease compared with normotensive control rats. Treatment with Ang-(1–7) was associated with a decrease of cardiac ACE2 mRNA in control rats but not in SHR. B: ACE2 mRNA in the kidney of SHR showed a significant decrease compared with control rats. Ang-(1–7) treatments had no significant effects on renal ACE2 mRNA in control rats and SHR. Values are means ± SE, n = 6 in each group. *p < 0.05 compared with control + vehicle group.
The Ang-(1–7) treatment led to significant down-regulation of cardiac ACE2 mRNA in Wistar rats. However, Ang-(1–7) had no obvious effect on cardiac ACE2 mRNA of SHR (table 3 and figure 1A). Renal ACE2 mRNA was also decreased in SHR, though Ang-(1–7) did not change the expression of renal ACE2 mRNA in either normotensive Wistar rats or SHR (table 3 and figure 1B).

Ang-(1–7) treatment was associated with a significant decrease in cardiac Mas mRNA only of Wistar rats but not of SHR, and Mas mRNA in heart was less in SHR than in normotensive Wistar rats (relative gene expression: 1.02 ± 0.32 vs. 8.10 ± 1.33, p < 0.05) (table 3 and figure 2A). Renal Mas mRNA was not different between SHR and Wistar rats. Ang-(1–7) treatment given to normotensive Wistar rats did not induce significant change in renal Mas mRNA. However, the SHR which received Ang-(1–7) treatment showed decreased expression of Mas mRNA in kidney (table 3 and figure 2B).

Figure 2. A: Real-time RT-PCR analysis of Mas mRNA in the heart of spontaneously hypertensive rats (SHR) showed a significant decrease compared with normotensive control rats. Treatment with Ang-(1–7) was associated with decrease of cardiac Mas mRNA in control rats but not in SHR. B: Mas mRNA in the kidney of SHR was not different from that of control rats. Treatment with Ang-(1–7) was associated with a decrease of renal Mas mRNA in SHR but not in control rats. Values are means ± SE, n = 6 in each group. *p < 0.05 compared with control + vehicle group; ♦p < 0.05 compared with SHR + vehicle group.

Figure 3. A and B: Representative Western blot analysis of ACE2 and GAPDH in cardiac tissue (H-ACE2) and renal tissue (K-ACE2) of control rats and spontaneously hypertensive rats (SHR) treated with vehicle or Ang-(1–7). From left to right, lanes 1–4 are samples from the control + Ang-(1–7) group, control + vehicle group, SHR + Ang-(1–7) group and SHR + vehicle group, respectively. C and D: Densitometric analysis of bands corresponding to ACE2 immunoreactive proteins in cardiac and renal tissue of control rats and SHR treated with vehicle or Ang-(1–7). Values are means ± SE, n = 6 in each group. *p < 0.05 compared with control + vehicle group.
Results of Western blot were basically similar to those of RT-PCR. Figure 3 shows that infusion of Ang-(1–7) caused decreased expression of cardiac ACE2 (H-ACE2) in Wistar rats but did not change their renal ACE2 (K-ACE2). In addition, ACE2 expression in heart and kidney were both down-regulated in SHR, whereas treatment of Ang-(1–7) failed to further reduce ACE2 expression in these hypertensive rats.

Figure 4 illustrates the effects of Ang-(1–7) treatment or vehicle on Mas protein in heart and kidney. Cardiac Mas of control Wistar rats was decreased by Ang-(1–7) and was more abundant than that of SHR. Renal Mas was only affected by infusion of Ang-(1–7)(110,648),(888,939) in SHR.

Discussion

The main findings of the present study were that 14-day infusion of Ang-(1–7) was related to different regulation of ACE2 and Mas expressions in cardiac and renal tissues of SHR and normotensive Wistar rats, although it had no effect on blood pressure in these animals.

There have been many investigations using drugs which interfere with the RAS; ARB, and ACEI combined with ARB, for instance, have been showed to increase Ang-(1–7) level during treatments, and some of these drugs also altered local ACE2 expression in some local tissues of hypertensive rats and normotensive rats. Igase et al. found that SHR receiving olmesartan showed increased ACE2 mRNA expression in their aorta with increased plasma Ang-(1–7) level, and there was a research indicating that pregnant rats had up-regulated immunocytochemical distribution of Ang-(1–7) and ACE2 in kidneys. All these studies suggest linkage between ACE2 expression and Ang-(1–7), and Ang-(1–7) has been shown to directly downregulate another RAS component, the AT1 receptor, in cultured vascular smooth muscle cells. However, no previous report has examined the direct effects of Ang-(1–7) on the regulation of ACE2 and Mas expression in heart and kidney of SHR and normotensive Wistar rats. In the present investigation, Ang-(1–7) infusion was associated with decreased tissue ACE2 and Mas expression in the heart of Wistar rats but not SHR, and with decreased renal Mas expression only in the SHR, suggesting different organ-specific regulations of ACE2 and Mas expression by Ang-(1–7).

To maintain the steady rate of Ang-(1–7) treatment, we used osmotic minipumps to give the peptide to the tested animals. Fourteen days of infusion of Ang-(1–7) did not alter tail-cuff SBP in either SHR or Wistar rats. This is consistent with several previous studies which showed that Ang-(1–7) itself basically had no depressor effect. For example, neither the consecutive venous infusion of Ang-(1–7) for 4 h nor for 12 days in adult SHR altered the mean arterial pressure of these animals. This finding excluded the possibility that any regulation by Ang-(1–7) was through its influence on blood pressure.
ACE2 is the most crucial rate-limiting forming enzyme of Ang-(1–7) in vivo. Although the enzyme can hydrolyse several peptides, it hydrolyses AngII with the highest catalytic efficiency to form Ang-(1–7). Moreover, the Mas receptor, implicated as a site for Ang-(1–7) binding, was found to be an indispensable factor for functioning of Ang-(1–7). Rats whose Mas gene was knocked out showed few reactions and beneficial effects when given Ang-(1–7). Therefore, connected by Ang-(1–7), a functional axis of ACE2–Ang-(1–7)–Mas was formed. As we know from many past studies about other members of RAS, such as angiotensinogen, renin, Ang II and ACE, inter-regulations between components of RAS are quite common. However, little is known about direct feedback regulation of Ang-(1–7) to ACE2 expression and ‘feedforward’ regulation to Mas receptor in heart and kidney. Ferrario et al. found that lisinopril alone and in combination with losartan augmented plasma levels of Ang-(1–7) in adult Lewis normotensive rats but had no effects on renal ACE2 and Mas receptor mRNA expression. The same investigatory group also found that plasma levels of Ang-(1–7) as well as cardiac ACE2 mRNA expression were increased in Lewis rats given lisinopril and losartan. These indirect pieces of evidence suggested that Ang-(1–7) might have a role in the regulation of ACE2 and Mas receptor expression, which was proved by the present investigation.

Our study found that continuous infusion of Ang-(1–7) induced decreased cardiac ACE2 and cardiac Mas expression (both the mRNA and protein expression) in normotensive Wistar rats, but this down-regulation did not occur in the heart of SHR, suggesting that the regulatory effects of Ang-(1–7) may be different under diverse blood pressure backgrounds. This perhaps has something to do with the changed level of endogenous Ang-(1–7) in certain organs during hypertension. According to some previous investigations, concentration and excretion rate of urinary Ang-(1–7) were lower in untreated hypertensive patients, and women with preeclampsia and increased blood pressure (1–7) were lower in untreated hypertensive patients, and women with preeclampsia and increased blood pressure level induced varied responses.

The present investigation and previous studies all showed that ACE2 expression in the heart and kidney of SHR were significantly decreased compared with that of normotensive control animals. The precise regulating mechanism of this alteration of ACE2 expression is not known. However, since ACE2 is important in forming Ang-(1–7), which could antagonise some ‘bad’ effects of Ang II, these alterations could be harmful and participate in the progression of hypertension. High blood pressure itself, by directly changing local mechanical pressure and perfusion pressure, may be a factor affecting the expression of ACE2 and Mas, and the altered neuroendocrinological environment during hypertension may also play a role. These hypotheses need more research.

There is little information about regulation of Mas expression in the heart and kidney of hypertensive rats. We found that cardiac Mas expression of SHR was decreased but renal Mas expression of SHR remained unchanged compared with Wistar rats. But, the expression of Mas receptor in kidney of SHR was down-regulated by infusion of Ang-(1–7). Down-regulation of Mas expression during hypertension might be disadvantageous for cardiac and renal function. However, the balance between components of RAS is very delicate and subtle, therefore any final functional result from alteration of expressions of RAS members can only be proved by comprehensive studies.

In summary, 14-day infusion of Ang-(1–7) was related to decreased tissue ACE2 and Mas expression in the heart of normotensive Wistar rats but not SHR, and with decreased renal Mas expression only in SHR, suggesting organ-specific regulation of local ACE2 and Mas expression by Ang-(1–7) which did not alter tail-cuff SBP in either SHR or Wistar rats. Regulatory effects of Ang-(1–7) on ACE2 and Mas receptor may influence the overall local RAS and participate in the progression and prognosis of cardiovascular diseases.

**Funding**

This work was supported by the Department of Health of Guangdong Province, China (grant number B2008032).

**Conflict of interest**

The authors declare that they have no conflicts of interest.

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