Regulation of angiotensin-(1–7) and angiotensin II type 1 receptor by telmisartan and losartan in adriamycin-induced rat heart failure

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Aim: To investigate the possible effects of telmisartan and losartan on cardiac function in adriamycin (ADR)-induced heart failure in rats, and to explore the changes in plasma level of angiotensin-(1–7)[Ang-(1–7)] and myocardial expression of angiotensin II type 1/2 receptors (AT₁R / AT₂R) and Mas receptor caused by the two drugs.

Methods: Male Sprague-Dawley rats were randomly divided into 4 groups: the control group, ADR-treated heart failure group (ADR-HF), telmisartan plus ADR-treated group (Tel+ADR) and losartan plus ADR-treated group (Los+ADR). ADR was administrated (2.5 mg/kg, ip, 6 times in 2 weeks). The rats in the Tel+ADR and Los+ADR groups were treated orally with telmisartan (10 mg/kg daily po) and losartan (30 mg/kg daily), respectively, for 6 weeks. The plasma level of Ang-(1–7) was determined using ELISA. The mRNA and protein expression of myocardial Mas receptor, AT₁R and AT₂R were measured using RT-PCR and Western blotting, respectively.

Results: ADR significantly reduced the plasma level of Ang-(1–7) and the expression of myocardial Mas receptor and myocardial AT₂R, while significantly increased the expression of myocardial AT₁R. Treatment with telmisartan and losartan effectively increased the plasma level of Ang-(1–7) and suppressed myocardial AT₁R expression, but did not influence the expression of Mas receptor and AT₂R.

Conclusion: The protective effects of telmisartan and losartan in ADR-induced heart failure may be partially due to regulation of circulating Ang-(1–7) and myocardial AT₁R expression.

Keywords: angiotensin-(1–7); Mas; angiotensin I receptor; angiotensin II receptor; adriamycin; heart failure; telmisartan; losartan

Introduction
Adriamycin (ADR) is an anthracycline antibiotic commonly used in the treatment of a wide range of cancers, including hematological malignancies, many types of carcinomas, and soft tissue sarcomas. Importantly, the use of adriamycin can cause acute and chronic side effects. The chronic side effects are represented by the development of cardiomyopathy and ultimately, irreversible congestive heart failure[1,2]. Great effort has been expended in preventing or mitigating the cardiotoxic side effects of ADR[3–7]; however, the mechanisms underlying ADR-induced heart failure are not fully understood.

The renin-angiotensin system (RAS) is one of the key regulators of blood pressure and cardiovascular disease[8–10] and involves several enzymes and receptors: renin, angiotensin II (Ang II), the Ang II type 1 receptor (AT₁R), the Ang II type 2 receptor (AT₂R) and the Mas receptor. Many reports have demonstrated the critical roles that these factors have in heart failure[9]. Ang II is an oligopeptide that causes vasoconstriction, increased blood pressure, and release of aldosterone from the adrenal cortex, while AT₁R and AT₂R are the receptors for Ang II. AT₁R mediates the major cardiovascular effects of Ang II, including vasoconstriction, increased vasopressin secretion, cardiac hypertrophy, cardiac contractility and extracellular matrix formation[9]. Conversely, effects mediated by AT₂R include inhibition of cell growth, neuronal regeneration, cellular differentiation and possibly vasodilatation[9]. Angiotensin-(1–7) [Ang-(1–7)] is a peptide formed from either Ang I or Ang II[9]. Ang-(1–7) is considered to be an important peptide fragment of the RAS, and it plays crucial roles that are often opposite from those of Ang II[11]. Ang-(1–7) can induce vasodilatation, diuresis and natriuresis, anti-hypertrophy, anti-proliferation, anti-fibrosis and stimulate bradykinin and NO release via binding to the Mas receptor[12]. The Mas receptor is
an oncogene as well as a receptor for Ang-(1–7)[13]. In a previous work, Mas receptor-deficient mice showed higher blood pressure values, impaired endothelial function, decreased NO production and lower endothelial NO synthetase expression[14], indicating that the Ang-(1–7)/Mas receptor axis plays an important role in cardioprotective and antihypertensive effects. Importantly, Ang-(1–7) can prevent heart failure after myocardial ischemia[15].

Some recent studies have reported that angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs), two classes of drugs that target RAS, may prevent ADR-induced cardiotoxicity[16–19]. However, the underlying mechanisms are largely unclear. In the present study, we investigated the regulation of two ARBs (telmisartan and losartan) on plasma Ang-(1–7) levels and the mRNA and protein expression of the myocardial AT1R, AT2R and Mas receptors in ADR-induced heart failure.

Materials and methods

Animals
A total of 70 male Sprague-Dawley (SD) rats weighing (217±18) g were obtained from Slack Laboratory Animal Co Ltd in Shanghai (SCXK: 2008-0004, China). All experimental procedures were conducted according to the Institutional Animal Care guidelines and approved ethically by the Administrative Committee of Experimental Animals, Jiangsu Province, China.

Drugs
ADR, telmisartan and losartan were obtained from Hisun Pharmaceutical Co Ltd (Zhejiang, China), Novartis (Switzerland) and MSD Pharmaceutical Co Ltd (Hangzhou, China), respectively.

Experimental protocol
Rats were randomly divided into four groups: (1) the control group (n=10, intraperitoneally injected an equal volume of normal saline); (2) the ADR-treated heart failure group (ADR-HF, n=20, intraperitoneally injected ADR, a 2.5 mg/kg dose administered 6 times in 2 weeks, resulting in a cumulative dose of 15 mg/kg); (3) the ADR plus telmisartan group (Tel+ADR, n=20, intraperitoneally injected ADR as in the ADR-HF group and administered telmisartan 10 mg/kg per day orally for 6 weeks, for a cumulative dose of 420 mg/kg); and (4) the ADR plus losartan group (Los+ADR, n=20, intraperitoneally injected ADR as in the ADR-HF group and administered losartan 30 mg/kg per day orally for 6 weeks, for a cumulative dose of 1260 mg/kg). During the treatment period, body weights of the rats were measured every three days, and the doses of ADR, telmisartan or losartan were adjusted according to the change in body weight. The death of animals was recorded daily.

Echocardiography
Four weeks after the last injection, the cardiac function of the rats was evaluated by transthoracic echocardiography (Vivid 7, General Electric Co) with a 10-MHz linear-array transducer as reported previously[20]. Briefly, rats were weighed and anesthetized using 10% chloral hydrate intraperitoneally (30 mg/kg) and placed on a warm blanket. The cardiac long-axis and short-axis views were obtained in the 2-dimensional mode, and M-mode tracings were recorded. Left ventricular internal dimension systole (LVIDs), left ventricular internal dimension diastole (LVIDd) and left ventricular ejection fraction (LVEF) were recorded in the M-mode tracings by the same expert, and all measurements were performed by an observer blinded to the treatments. We defined heart failure in this rat model according to the cardiac function as assessed by echocardiography.

Plasma Ang-(1–7) assay
After echocardiography, the abdominal aorta was carefully isolated, and 5 ml of arterial blood was collected into tubes containing disodium EDTA. Then, the plasma was obtained and stored at -80°C until use. Plasma Ang-(1–7) was assayed using an ELISA kit (Catalog No: F1763, Xitang Biotechnology Co, Ltd, Shanghai, China) with a microplate reader (Clinibio-128C). The optical density at 450 nm was obtained within 30 min. The standard curve was constructed by plotting the mean absorbance obtained for each reference standard against its concentration[21]. Using the mean absorbance value for each sample, the corresponding concentration was determined from the standard curve.

RNA isolation and RT-PCR
After blood collection, deeply anesthetized rats were sacrificed. Hearts were isolated and rinsed in saline. The cardiac ventricles were separated from the atria, weighed, cut into two segments, frozen in liquid nitrogen and stored at -70°C. Total RNA in the aorta from the four groups of rats was extracted using Trizol reagent (Invitrogen, USA)[22]. The RNA concentration was determined by the absorbance at 260 nm. The mRNA expression of the myocardial Mas receptor, AT1R and AT2R was determined by RT-PCR (TaKaRa Biotechnology Co Ltd, Dalian, China). The reaction conditions of RT-PCR were as follows: 30°C for 10 min, 47°C for 30 min, 99°C for 5 min and 5°C for 5 min. After reverse transcription, the cDNA was denaturated at 94°C for 2 min and subjected to 35 cycles of PCR at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min (Mas receptor) and 35 cycles of PCR at 94°C for 30 s, 59°C for 30 s and 72°C for 1 min (AT1R, AT2R, and β-actin), followed by extension at 72°C for 7 min. The selected primers designed for Mas receptor, AT1R, AT2R, and β-actin were as follows: forward: 5’-ACTGCGGAGGCTACATCAGC-3’, reverse: 5’-GGTGGAAGGCGGCGGTCATCATC-3’; forward: 5’-ACTGCCGGCGGTCACTCACTC-3’, reverse: 5’-GGTGGAAGGCGGCGGTCATCATC-3’; and forward: 5’- GCCCGGCCTGATTTATGC-3′, reverse: 5’-GGAAAGGGAAACAGGACGACG-3′ for Mas receptor (263 bp); forward: 5’-GCCCGGCCTGATTTATGC-3′, reverse: 5’-GGAAAGGGAAACAGGACGACG-3′ for AT1R; forward: 5’- GCCCGGCCTGATTTATGC-3′, reverse: 5’-GGAAAGGGAAACAGGACGACG-3′ for AT2R; forward: 5’-GCCCGGCCTGATTTATGC-3′, reverse: 5’-GGAAAGGGAAACAGGACGACG-3′ for β-actin.
Western blotting analysis

Western blotting analysis was performed as described previously. Protein (20 μg) extracted from the heart tissues from the four groups was subjected to 10% polyacrylamide gel electrophoresis and then transferred onto PVDF membranes. The membranes were probed using primary antibodies against the Mas receptor, AT1R and AT2R (all from Santa Cruz Biotechnology, USA) overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, USA) overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, USA) overnight at 4°C. Antibody binding was visualized using the ECL system (Yuehua Medical Instrument Co Ltd, Guangdong, China), and the amount of Mas receptor, AT1R and AT2R were expressed relative to that of GAPDH.

Statistical analysis

Results are presented as the mean±standard deviation (SD). Comparisons between two groups were assessed by Student’s t-test, and comparisons of three or more groups were performed using one-way ANOVA followed by LSD post-hoc using SPSS 11.5 software (Chicago, IL, USA). Statistical significance was set at *P*<0.05.

Results

Heart failure model

Body weights were comparable among the four groups at the start of the study. However, after treatment, the weight gain was less in the ADR-HF group than in controls (304.4±19.8 g vs 408.6±12.8 g, *P*<0.01, Table 1, Figure 1). The body weights of the Tel+ADR and Los+ADR groups were higher than that of ADR-HF group (*P*<0.01, Table 1, Figure 1), although these were still lower than that of the control (*P*<0.01, Table 1, Figure 1). No significant differences between the weights of the Tel+ADR and Los+ADR groups were observed (*P*>0.05, Table 1, Figure 1). At the end of the treatment (4 weeks after the last injection), the numbers of rats in each group were as follows:

Table 1. Weight and cardiac functional parameters at the end of the study (week 6) in four groups. Data are expressed as mean±SD. *P*<0.05, *P*<0.01 vs controls. *P*<0.05, *P*<0.01 vs adriamycin-induced heart failure rats.

|                  | Controls (n=10) | ADR-HF (n=9) | Tel+ADR (n=12) | Los+ADR (n=13) |
|------------------|-----------------|--------------|----------------|----------------|
| Weight (g)       | 408.6±12.8      | 304.4±19.8   | 434.0±32.16    | 348.5±31.52    |
| LVIDd (mm)       | 3.64±0.33       | 4.53±0.41    | 3.99±0.52      | 3.79±0.41      |
| LVIDd (mm)       | 5.79±0.48       | 6.81±0.30    | 6.08±0.33      | 6.14±0.44      |
| LVEF (%)         | 81.5±1.4        | 63.8±4.4     | 72.5±1.8       | 73.9±2.7       |

Control, *n=10*; ADR-HF, *n=9*; Tel+ADR, *n=12*; and Los+ADR, *n=13*.

Plasma levels of Ang(1–7)

The plasma level of Ang-(1–7) in the ADR-HF group was lower than that in the control group (4.27±2.79 ng/mL vs 10.26±2.39 ng/mL, *P*<0.01, Figure 3). Ang-(1–7) plasma levels of the Tel+ADR and Los+ADR groups were higher than that in ADR-HF group (7.16±2.13 ng/mL vs 4.27±2.79 ng/mL; 7.08±1.49 ng/mL vs 4.27±2.79 ng/mL, both *P*<0.05, Figure 3) but were still lower than that in control group (*P*<0.05, Figure 3). There was no difference in plasma levels of Ang-(1–7) between the Tel+ADR and Los+ADR groups (*P*>0.05, Figure 3).

Expression of the Mas receptor, AT1R and AT2R in the heart

The expression levels of the Mas receptor, AT1R and AT2R in cardiomyocytes were examined by RT-PCR analysis (Figure 4) and Western blotting (Figure 5). The mRNA and protein expression of the Mas receptor and AT1R were reduced in the ADR-HF group compared with the control group. Conversely, mRNA and protein expression of AT2R were increased. Treatment with telmisartan and losartan significantly suppressed the upregulation of AT1R but did not change the expression of
In this study, we showed that ADR decreased plasma Ang-(1–7) levels and myocardial Mas receptor and AT2R mRNA and protein expression, whereas it upregulated the myocardial mRNA and protein expression of AT1R. Two kinds of ARBs, telmisartan and losartan, attenuated the ADR-induced reduction of plasma Ang-(1–7) and suppressed the ADR-induced enhancement of myocardial AT1R expression. Telmisartan and losartan did not change the expression of the Mas receptor and AT2R.

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**Discussion**

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Ang-(1–7) is produced from Ang I and Ang II. Under physi-
ological conditions, plasma concentrations of Ang-(1–7) are similar to the plasma levels of Ang II[25]. Conversely, under some pathological conditions, e.g., untreated essential hypertensive subjects, the urinary concentrations of Ang-(1–7) is lower than in normotensive controls, but chronic treatment with ARBs increases plasma levels of Ang-(1–7) 5- to 25-fold in both rats and humans[26]. Moreover, previous reports have suggested that Ang-(1–7) is cardioprotective in myocardial ischemia/reperfusion and heart remodeling after heart infarction or heart failure[27, 28]. In our study, the plasma Ang-(1–7) level was significantly lowered by ADR treatment. Surprisingly, treatment with either telmisartan or losartan resulted in increases in plasma Ang-(1–7) compared with the untreated ADR-HF group (both \( P<0.05 \)). Based on the current results, we postulate that endogenous Ang-(1–7) could be a positive physiological protector for the heart, possibly mediating the beneficial effects of ARBs for ADR-induced heart failure.

AT1R and AT2R both belong to the family of G protein-coupled receptors, but they share only a 32%–34% identity at the amino acid level[9]. AT1R is widely distributed in the adrenal glands, kidneys, blood vessels, heart, brain, liver, bronchial tissue and other tissues[9]. After stimulation with Ang II, AT1R can cause vasoconstriction, Na/H2O reabsorption, inflammatory response, hypertrophy, hyperplasia, cell proliferation and so on[9]. It has been reported that the increase in oxidative stress coexists with AT1R upregulation in models of hypertension[29]. The data presented in this study showed that in the ADR-HF group, expression of the AT1R was upregulated compared with the control group (both \( P<0.05 \)). Based on the current results, we postulate that endogenous Ang-(1–7) could be a positive physiological protector for the heart, possibly mediating the beneficial effects of ARBs for ADR-induced heart failure.

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In summary, our results confirmed that ARBs are beneficial for ADR-induced heart failure and demonstrated that the influences of ARBs on circulating Ang-(1–7) levels and AT1R expression in cardiomyocytes may contribute to the cardioprotection of ARBs in this model. Our data may add new evidence to this research field and provide new mechanisms for applications of ARBs to reduce cardiotoxicity in ADR-treated patients.

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Author contribution
Xin-zheng LU, Ke-jiang CAO and Jun-HUANG designed research; Wen-na ZONG, Xiao-hui YANG, Xiu-mei CHEN, Hong-juan HUANG, Hong-jian ZHENG and Xiao-yi QIN performed research; Yong-hong YONG contributed new analytical tools and reagents; Wen-na ZONG analyzed data; Wen-na ZONG and Xin-zheng LU wrote the paper.

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