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Alteration of Cardiac ACE2/Mas Expression and Cardiac Remodelling in Rats with Aortic Constriction

Yanling Zhang[1], Bing Li[1], Bingxiang Wang[1], Jingjun Zhang[1], Junyan Wu[1], and Trefor Morgan[2]

[1]*Department of Physiology, Institute of Neurobiology, Taishan Medical University, Taian 271000 Shandong, People's Republic of China*
and
[2]*Department of Physiology, University of Melbourne, Parkville, Victoria, Australia*

Abstract

The recent discovery of the new components of the renin-angiotensin system (RAS) suggests the importance of the maintenance of cardiovascular structure and functions. To assess the role of the angiotensin-converting enzyme 2 (ACE2)-Mas receptor axis in the regulation of cardiac structure and function, the present work investigated the expression of ACE2 and Mas receptor in the heart in the cardiac remodeling that occurs in aortic constricted rats. Partial abdominal aortic ligation was carried out in Sprague-Dawley rats. Angiotensin AT1 receptor blockade and ACE inhibition were achieved by losartan and enalapril treatment, respectively. Results showed that aortic constriction increased left ventricular hypertrophy, fibrosis, mean arterial pressure (MAP), plasma renin activity (PRA) and cardiac ACE levels, but decreased the expression of cardiac ACE2 and Mas receptor. Losartan treatment significantly decreased MAP, left ventricle hypertrophy (LVH), fibrosis, and increased cardiac ACE2 and Mas expression. Enalapril also improved the cardiac parameters with a rise in cardiac ACE2, but did not change the Mas level. In conclusion, aortic constriction results in cardiac hypertrophy, fibrosis and a rise of cardiac ACE expression. Both AT1 receptor blocker and ACE inhibitor play a cardioprotective role in aortic constriction. However, AT1 receptor blocker particularly promotes cardiac ACE2 and Mas receptor levels. ACE inhibitor is associated with the inhibition of ACE and normalization of cardiac ACE2 activity.

Key Words: ACE2, aortic constriction, fibrosis, hypertension, hypertrophy

Introduction

The renin-angiotensin system (RAS) is involved in the pathological mechanisms of target organ damage and in the induction of hypertension. Angiotensin II (Ang II) is the principal vasoactive substance of the RAS and causes cardiac, arterial and renal lesions. The levels of Ang II in the circulation are controlled by the opposing action of two enzymes, angiotensin-converting enzyme (ACE) and angiotensin-converting enzyme 2 (ACE2) (8, 19, 22, 29). ACE primarily catalyzes the conversion of Ang I to the 8-amino acid peptide, Ang II (16). Interestingly, ACE2 catalyzes Ang II to Ang (1-7), which results in the reduction of Ang II formation. The role of ACE in the regulation of blood pressure and cardiovascular function is well established. Several inhibitors of ACE are widely used for antihypertensive therapies (15, 27). Less is known about the role of ACE2 in cardiac structure and function (17, 20, 24).

To date, the altered expression of ACE2 associated with cardiac, vascular and renal dysfunctions

Corresponding author: Yanling Zhang, Ph.D., Department of Physiology, Taishan Medical University, 2 Yingsheng East Road, Taian 271000, Shandong, PRC. Fax: +86-5386237212, E-mail: lzhang98@hotmail.com
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has been studied (11, 13, 25, 26, 28). ACE2 may play an important role by reducing concentration of Ang II and raising levels of Ang (1-7) which limits the vasoconstrictor action (2, 14). Therefore, manipulation of ACE2 activity has potential therapeutic use. Overexpression of ACE2 by local delivery of lentivirus in the hearts in rats reduced high blood pressure and cardiac hypertrophy (10). Conversely, transgenic overexpression of ACE2 in murine myocardium led to ventricular fibrillation and arrest (12). Although hypertension is one cause of cardiac hypertrophy, heart structural injury may occur independently of blood pressure (1) and the mechanisms of such cardiac damage remain unclear (7, 31). The rat model of aortic constriction has been useful for this issue, since it is associated with hypertension, cardiac remodeling, characterized by hypertrophy and impaired cardiac functions (21, 32, 34).

The present study used a partial aortic constricted rat model to examine whether the imbalance between ACE2 and ACE levels in the heart plays a role in the cardiac remodeling during aortic constriction. We have also investigated the effects of AT1 angiotensin type 1 receptor blocker and ACE inhibitor on the expression of cardiac ACE, ACE2 and Mas, and on cardiac remodeling.

Materials and Methods

Animals

All of the animal procedures were performed according to the guidelines of the National Research Council, and were approved by the Taishan Medical University Ethics Committee. Male Sprague-Dawley rats weighing 180-200 g were housed in a temperature-controlled animal facility (12-hour light/dark cycle) with *ad libitum* access to rat chow and tap water.

Experimental Protocols

Forty male Sprague-Dawley rats were randomized to the abdominal aortic constriction (30 rats) and sham-operated (n = 10) groups, as described previously (32). Briefly, animals were anesthetized with sodium pentobarbitone (60 mg/kg, intraperitoneally). The abdominal aorta was exposed at a site slightly above the renal arteries and a 21-gauge blunt needle was then placed along the isolated aorta segment. The aorta and needle were tightened with 1.0 silk suture. The needle was then removed, leaving the vessel constricted to the diameter of the needle. Sham-operated animals underwent the same procedure without ligation of the aorta (n = 10). Following aortic ligation, animals were randomly allocated to vehicle (Veh, n = 10), AT1 receptor blocker losartan (Los, n = 10) 30 mg/kg/d or ACE inhibitor enalapril (Ena, n = 10) 20 mg/kg/d by daily oral gavage.

Blood pressure was measured by the tail-cuff method using photoelectric volume oscillometry (ALC-NIBP Analysis System, Aoer Biotech, Shanghai, PRC) as described previously (32).

At 4 weeks of treatment, the animals were sacrificed by an overdose of sodium pentobarbitone. Blood for plasma and the heart were collected. The left ventricle (LV) was dissected and snap frozen in isopentane/dry ice for immunohistochemistry studies. The remainder of the LV was preserved at -80°C for RT-PCR and western blot analysis.

Renin Assays

Plasma renin activity (PRA) was measured by angiotensin I (Ang I) generation (ng/plasma, ml/incubation time, h; ng/ml/h) using a commercial kit (Puer Weiye Biotech, Beijing, PRC) based on methods described previously (33).

Measurement of Collagen Fraction

Left ventricle paraffin sections were stained with 0.1% Sirius Red staining for collagen I and III. A total of 8 fields without vessels were randomly selected from each section. The collagen area and total area of each field were measured, and myocardial collagen volume fraction (CVF) was calculated as collagen area/total area. The CVFs from 8 fields were averaged and used as the final CVF of this sample.

Measurement of ACE, ACE2 and Mas Receptor mRNAs

Total RNA was extracted from the heart using TRIzol (Invitrogen, Grand Island, NY, USA). RT-PCR was carried out using PCR Reagent Kit (TaKaRa, Tokyo, Japan). Template cDNA was prepared using reverse transcriptase. The primers used in this study were as follows: ACE forward 5'- TAACTCGAGTGCCGAGGTG-3' and reverse 5'-CCAGCAGGTG GCAGTCTT-3'; ACE2 forward 5'-CTTCAGCACTCTCAGCAGACA-3' and reverse 5'-CAACTTCCTCCTCACATAGGC-3'; Mas forward 5'-ACTGTCGGGCGGTC ATCATC-3' and reverse 5'-GGTGGAGAAAAGCAAGGAGA-3'. Densitometric analysis of PCR was performed using the Digital Imaging System (Bio-Rad, Hercules, CA, USA). The use of equal amounts of mRNA in the RT-PCR assays was confirmed by analysing the expression levels of house-keeping gene β-actin. The results were normalized to β-actin mRNA quantified from the same samples. All groups were compared with the sham-operated group, which was arbitrarily expressed as 1.0.

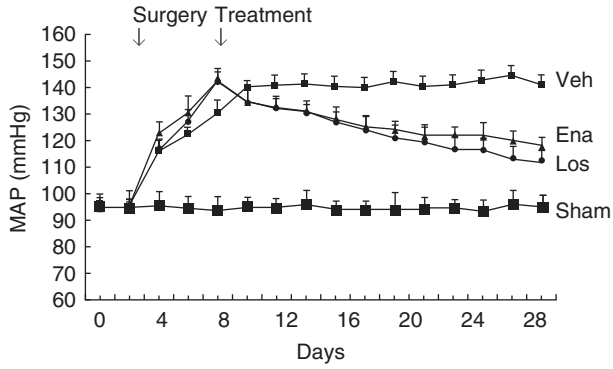

Fig. 1. The MAP in sham-operated (Sham), vehicle (Veh), losartan (Los)- and enalapril (Ena)-treated rats. Repeated measures analysis in SPSS showed that the treatment significantly affected the MAP ($F = 320.2$, $P < 0.01$). *Post hoc* tests by Student-Newman-Keuls showed that the MAP was elevated in the aortic ligated rats compared to sham ($P < 0.01$), and was decreased with losartan ($P < 0.01$) and enalapril ($P < 0.01$) compared to the Vehicle rats, n = 10 each group.

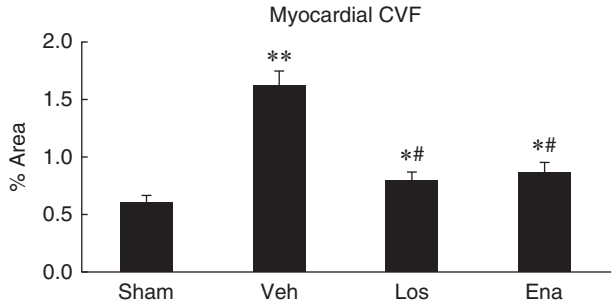

Fig. 2. Collagen volume fraction (CVF) in sham, vehicle (Veh), losartan (Los) and enalapril (Ena) groups. $*P < 0.05$ *vs.* Sham, $^{\#}P < 0.01$ *vs.* Veh rats, n = 10 each group.

Western Blot Analysis

Protein was extracted from heart tissue and subjected to western blot analysis. Thirty µg protein was loaded per lane, separated in a 10% SDS-PAGE and transferred to PVDF membranes. The membranes were probed using primary antibodies (Santa Cruz Biotech Inc., Dallas, TX, USA) against ACE, ACE2 and Mas receptor respectively, followed by horseradish peroxidase-conjugated secondary antibody for 2 h. Chemiluminescence was detected with an ECL kit (Pierce, Boston, MA, USA) and subsequent exposition to Hyperfilm (Amersham, Piscataway, NJ, USA). The amount of ACE, ACE2 and Mas was expressed relative to that of GAPDH.

ACE2 Immunohistochemistry

Sections of unfixed hearts were embedded in PolyFreeze Tissue Freezing Medium (Polysciences, Warrington, PA, USA) and snap frozen in isopentane/dry ice. Cryostat sections, 7 µm thick, were cut and fixed in cold 4% paraformaldehyde for 30 min at 4°C. The sections were incubated with the dilution of primary antibody against ACE2 (Santa Cruz) at dilution of 1:200 in PBS solution. After washing with PBS, they were incubated with peroxidase-conjugated anti-rabbit IgG (goat polyclonal, 1:5,000) for 1 h at 4°C. The color was developed with 0.05% 3, 3'- diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2. Myocyte ACE2 staining was assessed by measuring the area of stained tissue within a given field. Twenty fields were analyzed for each animal. The immunostaining area of ACE2 was obtained in a given area and expressed as a percentage of the entire image (9).

Statistical Analysis

All data are expressed as the mean ± SEM. The mean arterial pressure (MAP) data was analyzed by Repeated Measures in SPSS followed by Student-Newman-Keuls *post hoc* tests. Statistical significance of ACE, ACE2, Mas and PRA data was determined with one-way ANOVA in SPSS followed by LSD *post hoc* tests. Differences were considered statistically significant at values of $P < 0.05$.

Results

Aortic constriction increased MAP ($P < 0.01$, Fig. 1) and PRA ($P < 0.01$, Table 1). The CVF in the left ventricle was also increased ($P < 0.05$, Fig. 2). Aortic constriction resulted in the increased heart ($P < 0.01$), left and right ventricle ($P < 0.01$) and atrial weight ($P < 0.01$, Table 1). Losartan and enalapril decreased blood pressure ($P < 0.01$, Fig. 1) with a rise in PRA ($P < 0.01$, Table 1). The two reagents reduced whole heart, left and right ventricle weight ($P < 0.01$, Table 1). The CVFs in both groups were dramatically lower than those in the vehicle-treated group ($P < 0.01$, Fig. 2).

Aortic constriction increased cardiac ACE mRNA levels ($P < 0.05$, Fig. 3, A and B). After treatment of losartan, cardiac ACE mRNA levels did not differ from the values in the vehicle animals. However, enalapril significantly decreased cardiac ACE mRNA levels compared to the vehicle rats ($P < 0.05$). Similarly, aortic constriction increased the ACE protein level in rats ($P < 0.05$, Fig. 3, C and D). Enalapril significantly decreased ACE level ($P < 0.05$), but losartan did not affect cardiac ACE protein level compared with the vehicle animals.

Aortic constriction decreased cardiac ACE2 mRNA in the vehicle rats by 45% compared with the controls ($P < 0.05$, Fig. 4, A and B). Losartan and

Table 1. Body and heart weight and PRA

	Sham	Veh	Los	Ena
Body Weight (g)	253 ± 6	236 ± 5*	241 ± 4*,†	243 ± 5*,†
Cardiac Parameters				
HW (g)	0.935 ± 0.067	0.976 ± 0.045*	0.856 ± 0.061*,†	0.823 ± 0.045*,†
LV (g)	0.715 ± 0.035	0.768 ± 0.023*	0.683 ± 0.026*,†	0.695 ± 0.021*,†
RV (g)	0.172 ± 0.006	0.185 ± 0.01*	0.152 ± 0.005*	0.149 ± 0.006*,†
AW (g)	0.033 ± 0.001	0.040 ± 0.002*	0.037 ± 0.001	0.036 ± 0.002
HW/BW g/(100 g)	0.369 ± 0.006	0.413 ± 0.004*	0.355 ± 0.005*,†	0.338 ± 0.003*,†
LV/BW g/(100 g)	0.282 ± 0.005	0.325 ± 0.006*	0.283 ± 0.004*,†	0.286 ± 0.003*,†
RV/BW g/(100 g)	0.068 ± 0.002	0.078 ± 0.004*	0.063 ± 0.001*,†	0.061 ± 0.002*,†
AW/BW g/(100 g)	0.013 ± 0.001	0.016 ± 0.001*	0.015 ± 0.002*,†	0.014 ± 0.003
PRA (ng/ml/h)	0.59 ± 0.03	1.04 ± 0.04*	1.59 ± 0.03*,†	2.19 ± 0.17*,†

*$P < 0.01$, *vs*. sham; †$P < 0.01$, *vs*. Veh. Abbreviations: BW, body weight; HW, heart weight; LV, left ventricle weight; RV, right ventricle weight; AW, atrial weight; PRA, plasma renin activity.

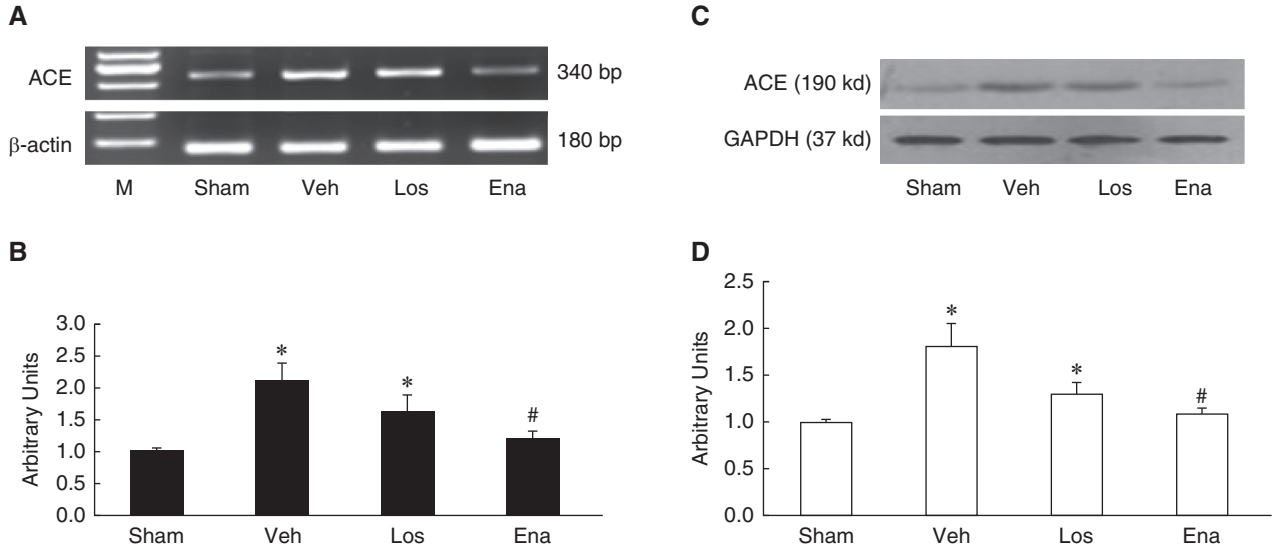

Fig. 3. Relative cardiac ACE mRNA (A and B) and cardiac ACE protein levels (C and D) in sham, vehicle (Veh), losartan (Los) or enalapril (Ena) treated rats. *$P < 0.05$ *vs*. sham, #$P < 0.05$ *vs*. Vet, n = 10 each group.

enalapril significantly increased ACE2 mRNA levels ($P < 0.05$) compared with the controls and ($P < 0.01$) compared to the vehicle rats.

Aortic constriction decreased the levels of cardiac ACE2 protein ($P < 0.05$). Both losartan and enalapril significantly increased ACE2 protein levels compared with the controls ($P < 0.05$) and with the vehicle rats ($P < 0.01$, Fig. 4, C and D).

Immunohistochemistry for ACE2 is shown in Fig. 5A-5E. In control rats, ACE2 expression occurred in cardiomyocytes (Fig. 5A). Aortic constriction decreased ACE2 immunostaining (Fig. 5B, $P < 0.05$). Losartan and enalapril significantly increased ACE2 levels (Fig. 5, C, D and E, $P < 0.05$) compared with that in the vehicle rats (Fig. 5B).

Aortic constriction decreased cardiac Mas mRNA levels (Fig. 6, A and B). Losartan treatment increased Mas mRNA levels ($P < 0.01$). However, enalapril had no effect on Mas mRNA levels. There was a significant difference in Mas mRNA levels between the losartan- and enalapril-treated groups ($P < 0.01$).

Western blot analysis showed that losartan significantly increased Mas protein level ($P < 0.01$), but enalapril did not appear to have such an effect on the Mas. The cardiac Mas protein levels were altered in the similar pattern as its gene expression in the treatment groups (Fig. 6, C and D). These data suggests that losartan promotes the expression of cardiac Mas,

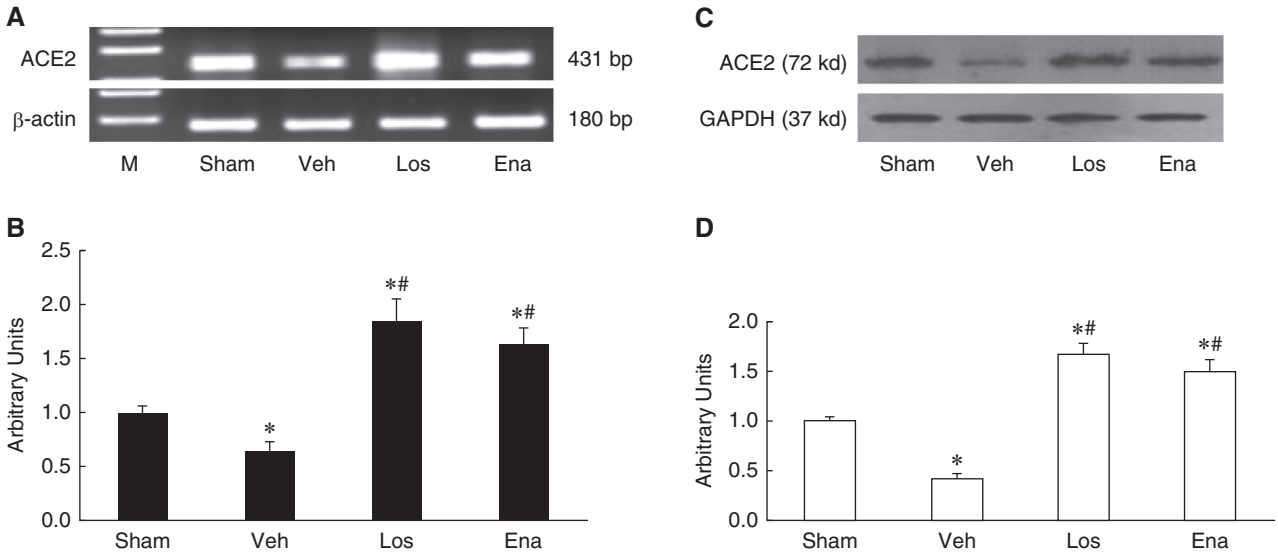

Fig. 4. Cardiac ACE2 mRNA (A and B) and ACE2 protein levels (C and D) in sham, vehicle (Veh), losartan (Los)- or enalapril (Ena)-treated rats. $^{*}P < 0.05$ *vs.* sham, $^{\#}P < 0.01$ *vs.* Veh, n = 10 each group.

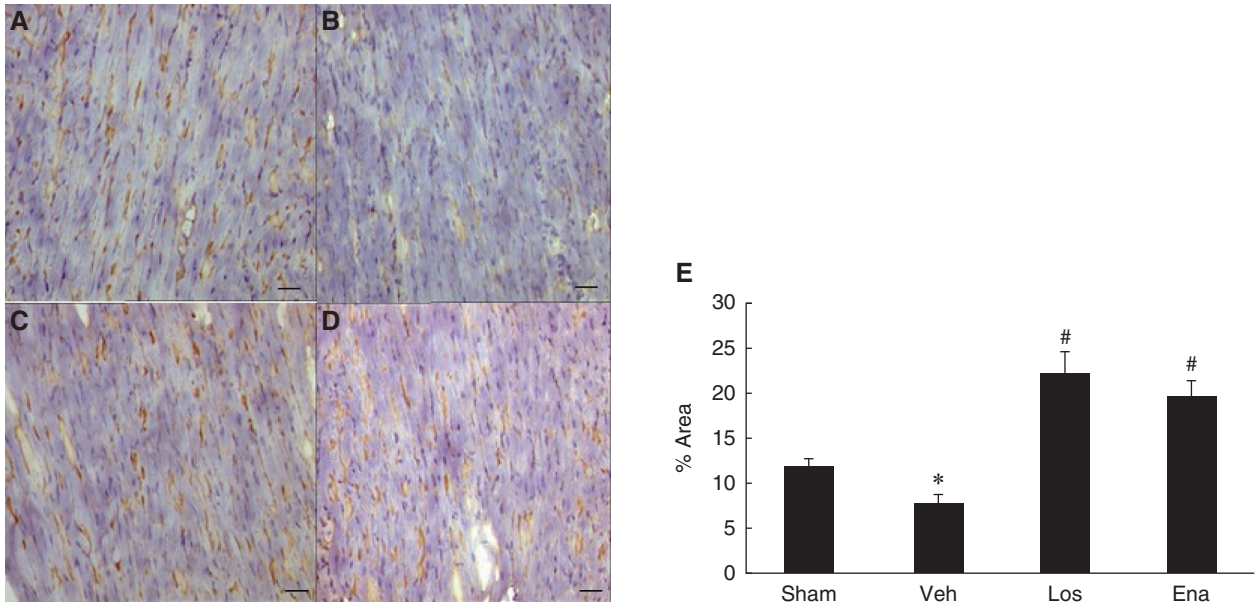

Fig. 5. Light microscopic images of ACE2 immunohistochemical labeling (brown staining) of cardiomyocytes in left ventricle of sham (A), vehicle (Veh, B), losartan (Los, C) and enalapril (Ena, D) rats. Scale bar = 100 μm; magnification, 40×. Data are expressed as means ± SEM (Fig. 5E). $^{*}P < 0.05$ *vs.* sham, $^{\#}P < 0.05$ *vs.* Veh, n = 10 each group.

but enalapril does not.

Discussion

In this study in SD rats, we found that abdominal aortic constriction of a duration of 4 weeks led to cardiac hypertrophy and fibrosis, and that these structural injuries of the heart were associated with decreased levels of ACE2 and increased levels of ACE. We rigorously examined the comparative effect of treatment with an AT1 receptor blocker and ACE inhibitor on the expression of cardiac ACE, ACE2 and Mas. In the previous study, we demonstrated that blockade of AT1 receptors significantly increased renal ACE2 mRNA levels (34), and we now report that AT1 receptor blocker increases the levels of cardiac ACE2 and Mas mRNA and protein. Furthermore, ACE inhibitor significantly decreased the level of the cardiac ACE. Unlike AT1 receptor blocker, ACE inhibitor did not have an effect on the expression of cardiac

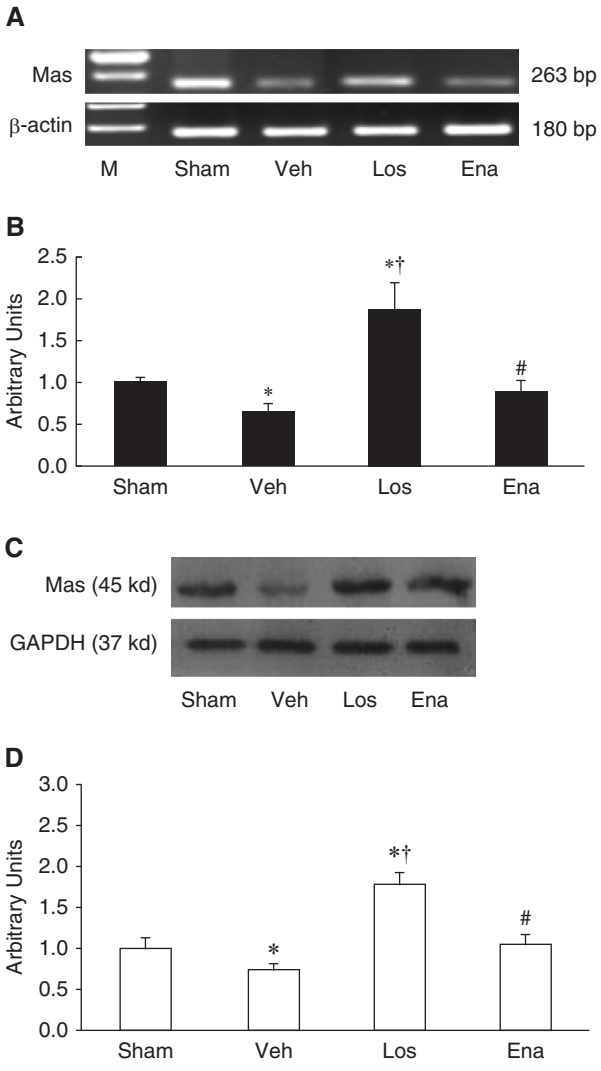

Fig. 6. Cardiac Mas mRNA (A and B) and Mas protein levels (C and D) in sham, vehicle (Veh), losartan (Los)- or enalapril (Ena)-treated rats. $^{*}P < 0.05$ *vs.* sham, $^{\dagger}P < 0.01$ *vs.* Veh, $^{\#}P < 0.01$ *vs.* Los rats, n = 10 each group.

Mas receptor.

The rat model of aortic constriction is often used for assessing the effect of renal ischaemia on the heart, and results in hypertension and cardiac remodeling (5, 6, 32, 35). While we did not measure cardiac function directly, the cardiac remodeling in rats we observed was associated with the imbalance of ACE2 and ACE levels. The role of ACE2 is under intensive investigation, and the concept that ACE2 is associated with cardiac functions is supported by a number of studies (4, 30). Our data showed that the high level of ACE2 mRNA was accompanied by an increased protein level, implying that a rise in transcription and translation of ACE2 could result in an increase of ACE2 activity in the heart. In this study, the expression of cardiac ACE2 increased with the Mas level after AT1 receptor blocker treatment, indicating that the ACE2/Ang-(1-7)/Mas axis in the RAS is specifically activated. This result demonstrates that the increased ACE2 upregulates the Mas expression which is involved in the reduction and degradation of Ang II. However, enalapril did not show such action on the cardiac Mas. Although cardiac Ang (1-7) was not measured in this study, it showed that AT1 receptor blocker significantly increased cardiac ACE2 in a protective role, and indirectly reflected the beneficial effects of Ang (1-7) on cardiac remodeling.

Indeed, beneficial effects in treatment of losartan and enalapril have been reported for controlling blood pressure and cardiopathy (3, 18, 23), but little was known about the potential mechanism for the cardiovascular benefits of the two agents, particularly on the cardiac ACE2/Ang (1-7)/Mas axis in hypertension. Our data show that the effects of ACE inhibitor are at least, in part, attributable to the elevation of ratio of cardiac ACE2/ACE since it significantly reduces the level of ACE. In contrast, losartan mainly increased the expression levels of cardiac ACE2 and Mas.

Our data showed that the increase in cardiac ACE2 and Mas levels induced by losartan was probably not directly caused by a hemodynamic effect of the drug, since treatment of enalapril also decreases blood pressure, but without affecting Mas expression. The involvement of AT2 receptors on ACE2 expression was not considered because blockade of AT2 receptor, PD123319, had no effect (18). Combined with findings of others, we suggest that AT1 receptor is an important factor modulating the activity of the cardiac ACE2/Ang (1-7)/Mas axis in this animal model.

These data showed aortic constriction induced hypertension, cardiac hypertrophy and fibrosis, and AT1 receptor blocker improved these parameters. The results demonstrated that the increased levels of cardiac ACE2 and Mas induced by AT1 receptor blocker may protect the heart against the effects of the activated ACE and AT1 receptor, and hypertension induced by aortic constriction in this animal model. Although ACE inhibitor has the similar effects of AT1 receptor blocker on cardiac structure, this study suggests that the main favorable effect of ACE inhibition is to reduce the production of Ang II in the heart. Further work should address the mechanisms of cardiac remodeling observed in this study using AT1 receptor blockers and infusion of Ang (1-7). Ang (1-7) signalling and its interaction with AT1 receptor signalling need to be elucidated.

Acknowledgments

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