Angiotensin II Increases HMGB1 Expression in the Myocardium Through AT1 and AT2 Receptors When Under Pressure Overload

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Summary
High-mobility group box 1 (HMGB1) is increased in the myocardium under pressure overload (PO) and is involved in PO-induced cardiac remodeling. The mechanisms of the upregulation of cardiac HMGB1 expression have not been fully elucidated. In the present study, a mouse transverse aortic constriction (TAC) model was used, and an angiotensin II (Ang II) type 1 (AT1) receptor inhibitor (losartan) or Ang II type 2 (AT2) receptor inhibitor (PD123319) was administrated to mice for 14 days. Cardiac myocytes were cultured and treated with Ang II for 5 minutes to 48 hours conditionally with the blockage of the AT1 or AT2 receptor. TAC-induced cardiac hypertrophy was observed at 14 days after the operation, which was partially reversed by losartan, but not by PD123319. Similarly, the upregulated HMGB1 expression levels observed in both the serum and myocardium induced by TAC were reduced by losartan. Elevated cardiac HMGB1 protein levels, but not mRNA or serum levels, were significantly decreased by PD123319. Furthermore, HMGB1 expression levels in culture media and cardiac myocytes were increased following Ang II treatment in vitro, positively associated with the duration of treatment. Similarly, Ang II-induced upregulation of HMGB1 in vitro was inhibited by both losartan and PD 123319. These results suggest that upregulation of HMGB1 in serum and myocardium under PO, which are partially derived from cardiac myocytes, may be induced by Ang II via the AT1 and AT2 receptors. Additionally, amelioration of PO-induced cardiac hypertrophy following losartan treatment may be associated with the reduction of HMGB1 expression through the AT1 receptor.

Key words: Transverse aortic constriction, Cardiac hypertrophy

Cardiac hypertrophy is primarily caused by hypertension.1) The heart initiates a remodeling process, with the aim to maintain normal cardiac function, as a compensatory mechanism when under pressure overload (PO), whereas consistent cardiac hypertrophy leads to a decrease in cardiac function and eventually to heart failure.2,3) The pathologic mechanisms of action behind cardiac remodeling under PO are complicated,4-8) but the underlying molecular mechanisms of action have not yet been fully defined.

A number of studies have indicated that inflammation is also implicated in cardiac remodeling and cardiomyocyte hypertrophy.9-11) High-mobility group box 1 (HMGB1), which was only identified as a non-histone DNA-binding nuclear protein expressed in numerous cell types in the past few decades, has also been demonstrated to play a critical role in various inflammatory diseases as a crucial proinflammatory cytokine.12-14) Consistent with a previous study,15) our study found that HMGB1 was upregulated in the myocardium under PO, aggravating cardiac hypertrophy and accelerating the deterioration of cardiac function.16) Moreover, HMGB1 exacerbates mechanical stress-induced cardiac myocyte hypertrophy in vitro,17) suggesting that HMGB1 plays an obligatory role in hypertensive cardiac remodeling. However, the mechanisms of action behind the upregulation of cardiac HMGB1 expression levels when under PO remain unclear.

Angiotensin II (Ang II) not only promotes the expression of proteins associated with cardiac hypertrophy through binding with its receptors but also acts as “a proinflammatory cytokine” exerting proinflammatory effects by inducing the release of various cytokines from both inflammatory and non-inflammatory cells.18) For example, Ang II induces the translocation and acetylation of HMGB1.19,20) Its proinflammatory effects are likely mediated by the Ang II type 1 (AT1) receptor, and the func-
stimulation with lipopolysaccharide.24) It has been reported that AT1 receptor antagonists reduce the expression levels of HMGB1 in cerebral thromboembolism or septic shock animals and provide protection for organs.22,23) Furthermore, in vitro, the expression levels of HMGB1 are reduced by inhibiting AT1 receptor activation following stimulation with lipopolysaccharide.24)

The aforementioned findings, along with the fact that Ang II is increased under PO2 and that HMGB1 expression is enhanced by mechanical stress in cultured cardiac myocytes,25) led to the hypothesis that Ang II may regulate expression levels of HMGB1 in hearts under PO through AT1 and AT2 receptor signaling. The present study explored this hypothesis by blocking Ang II receptors in transverse aortic constriction (TAC)-induced hypertrophic mice in vivo and in cardiac myocytes treated with Ang II in vitro.

Methods

Animals: Wild-type (WT) male C57BL/6J mice (8-10-week old, 22-24 g) were purchased from the Shanghai Branch of National Rodent Laboratory Animal Resources (Shanghai, China). Mice were bred and kept at 24°C ± 2°C under a 12 hour light-dark cycle with water and standard laboratory mouse chow available ad libitum. All experimental procedures in the mice were performed in accordance with the guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and were approved by the Animal Care and Use Committee of Zhongshan Hospital, Fudan University (Shanghai, China).

TAC and treatment with Ang II receptor inhibitors: PO was imposed on the hearts of mice using TAC as previously published.25) In brief, after being anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg), the mice were endotracheally intubated and mechanically ventilated (type 7025; Harvard Apparatus). Subsequently, thoracotomy to the second rib was performed on the partial left-side chest wall, and a chest retractor was used to retract the sternum. The aortic arch was isolated, and a blunt 27-gauge needle was placed over it. The blunt 27-gauge needle and the aorta, between the left common carotid artery and the innominate artery, were ligated using a 7-0 nylon string, and the needle was quickly removed to produce a constriction of ~0.4 mm in diameter. Sham-operated mice were treated with the same interventions but without the constriction of the aorta.

Before the TAC operation, the WT mice were randomly divided into four groups: Sham, TAC + normal saline (NS), TAC + losartan (an AT1 receptor inhibitor; TargetMol, Wellesley Hills, USA), and TAC + PD123319 (an AT2 receptor inhibitor; TargetMol, Wellesley Hills, USA). The mice were treated with equivalent volumes of the two freshly prepared inhibitor solutions or saline once daily by oral gavage and subcutaneous injection starting from 3 days before the TAC or sham surgeries to the end of the experiments. At 14 days after the TAC or sham surgeries, the mice in all groups were sacrificed. The dosages for losartan and PD123319 administration were 13.4 and 15 mg/kg/day, respectively.

Echocardiography measurement: Transthoracic echocardiography was carried out at 14 days after the TAC or sham surgeries in mice as described previously.25) Echocardiography was performed on mice sedated with isoflurane vaporized in oxygen, and echocardiographic analyses were used to determine the cardiac parameters in regard to the left ventricle (LV) structure or systolic function, including LV wall thickness, LV dimensions, and LV volume, with a guided M-mode. LV ejection fraction was calculated as previously described.26) All measurements were averaged over 3-5 consecutive cardiac cycles and were measured by a blinded experienced technician.

Histological analysis: Mice were sacrificed after 14 days of TAC or sham surgeries. The procedures were described previously.25) Hearts were excised from the mice, rinsed in NS, and weighed after wiping with a gauze. Subsequently, hearts were fixed in 10% formalin, dehydrated through increasing concentrations of ethanol, embedded in paraffin, and sectioned in the short axis at the papillary muscle level. Hematoxylin and eosin staining was performed on 5-μm thick slices of heart tissue following a standard protocol. Immunohistochemistry for HMGB1 was also performed on paraffin sections. The slices of the ventricles were incubated with primary antibodies targeting HMGB1 (Abcam, Cambridge, UK) overnight prior to incubation with the secondary antibodies (Invitrogen, Carlsbad, USA) on the subsequent day. For negative controls, primary antibody incubation was omitted from the procedure.

Cell culture and treatment: Neonatal rat cardiac myocytes were isolated and cultured as previously described.27) Primary cultures of the ventricular myocytes were acquired from 1- or 2-day-old Sprague-Dawley rat pups. After a series of digestion and centrifugation of the heart tissue and removal of the non-myocyte cells, the enriched primary cardiac myocytes were cultivated in low glucose DMEM supplemented with 10% (v/v) FBS, 20 mM HEPES (pH 7.2), and 100 U/mL each penicillin/streptomycin at 37°C in humidified air with 5% CO2. On the basis of the groupings, cells were treated with control (vehicle) and Ang II (10-6 mol/L; TargetMol) for 5 minutes to 48 hours or pretreated with losartan (10-5 mol/L; TargetMol) or PD 123319 (10-8 mol/L; TargetMol) in culture media for 30 minutes, and then, Ang II (10-6 mol/L; TargetMol) was administered to the culture media for 48 hours.

ELISAs: Serum samples were obtained when the mice were sacrificed on day 14 after the TAC or sham operations, and the levels of HMGB1 were measured using ELISAs with an immunoassay kit (Shino-Test Corporation, Tokyo, Japan). Similarly, the concentrations of HMGB1 and interleukin-6 (IL-6) in the supernatants were measured using commercially available ELISA Kits from Shino-Test Corporation and R&D systems (Minneapolis, USA), respectively. All of the procedures followed the manufacturer’s instructions.

RNA extraction and real-time quantitative PCR (RT-qPCR): As described previously,28) total RNA was prepared from LV tissue or cultured cardiac myocytes using TRIzol reagent® (Invitrogen, Carlsbad, USA), and RT-
qPCR was performed using RT-qPCR kits (TaKaRa, Tokyo, Japan) following the manufacturer’s protocol. A total of 4 μg of total RNA was reverse-transcribed to cDNA using AMV reverse transcriptase and random 9 primers as the first-strand primer. qPCR using equivalent amounts of the synthesized cDNA was performed with a 40-cycle two-step PCR using sequence-specific primer pairs with the ABI 7900 Fast RT-PCR System (PE Applied Biosystems, Foster City, USA). The expression levels of each gene were standardized to that of β-actin. Specific primers for HMG1, IL-6, and β-actin are presented in Table I.

**Protein extraction and western blot analysis:** The procedures were described previously. Total protein was extracted from homogenized LV tissues and cultured cardiac myocytes using RIPA lysis buffer (Beyotime, Nanjing, China) with a protease and phosphatase inhibitor cocktail following the manufacturer’s instruction. Equal amounts of proteins were electrophoresed on a 12% polyacrylamide gel and subsequently transferred to PVDF membranes. The blots were incubated with primary antibodies targeting HMGB1 (Abcam, Cambridge, UK) or IL-6 (Abcam, Cambridge, UK) at 4°C overnight, followed by incubation with horse radish peroxidase-conjugated rabbit antibodies targeting HMGB1 (Abcam, Cambridge, UK) or IL-6 (Abcam, Cambridge, UK) or β-actin antibodies (Kangchen Biotechnology, Shanghai, China) were used as the internal control. The antigen-antibody complexes were detected using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL, USA), and visualized densitometry was performed using LAS-300 Image software (Fujifilm, Kanagawa, Japan).

**Statistical analysis:** Data are expressed as the mean ± SEM. Statistical comparisons were performed using one-way ANOVAs, followed by post hoc LSD tests using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was defined as statistically significant.

### Results

**Effects of losartan and PD123319 on TAC-induced cardiac hypertrophy:** PO-induced cardiac hypertrophy models in mice were successfully established using TAC, which were verified by the elevation of peak velocity at the banding site. The effects of blocking Ang II receptors on cardiac hypertrophy were then examined. Echocardiographic assessments revealed that the thicknesses of both the LV posterior wall and interventricular septal wall at 14 days post TAC were significantly increased compared with those in the sham-operated mice both at the end-diastolic phase and end-systolic phase (P < 0.01; Table II). The increased thickness induced by PO was found to be significantly decreased following treatment with losartan but not from PD123319 treatment (P < 0.05; Table II). Similarly, an enlarged heart size and increased heart weight to body weight ratio were observed after 14 days of PO, which was also reduced by losartan but not PD123319 treatment (Figure 1).

**Effects of losartan and PD123319 treatment on the increased HMGB1 expression induced by TAC:** To examine the roles of the AT1 and AT2 receptors on the expression levels of HMGB1 under PO, losartan and PD123319 were used to treat mice with TAC. At 14 days after TAC, there was no significant difference in the location of HMGB1 as compared with the sham-operated mice, with HMG1 being predominantly expressed in the nuclei in both cases, as observed by immunohistochemical staining (Figure 2A). As shown in Figure 2B-E, there was a sig-

| Gene       | Forward (F) | Reverse (R) primers                  |
|------------|-------------|--------------------------------------|
| HMGB1      | 5’-GCTGACAAAGGCTGTATGAA-3’ | 5’-CCTTGTATTGGGCGGTA-3’ |
|            | 5’-CTGAGAGAAGATCCATCAG-3’ | 5’-AGTGTATAAGCAGCTGTTTG-3’ |
| IL-6       | 5’-CGATGCCCTGAGGCTCCCTT-3’ | 5’-TGGATGCCACAGGATTCCA-3’ |
| β-actin    | 5’-CGATGCCCTGAGGCTCCCTT-3’ | 5’-TGGATGCCACAGGATTCCA-3’ |

HMGB1 indicates high-mobility group box-1; and IL-6, interleukin-6.

### Table II. Echocardiography Measurement in Mice After TAC at 14 Days

| Parameters        | Sham       | TAC + NS  | TAC + Losartan | TAC + PD123319 |
|-------------------|------------|----------|----------------|----------------|
| PVb (mm/second)   | 733 ± 81   | 4233 ± 95** | 4110 ± 87**  | 4119 ± 69**    |
| HR (beats/minute) | 512 ± 26   | 520 ± 8   | 516 ± 9       | 524 ± 10       |
| LVEF (%)          | 70 ± 1     | 71 ± 2    | 71 ± 2        | 71 ± 3         |
| End-diastolic     |            |          |                |                |
| LVFPW (mm)        | 0.67 ± 0.02| 0.82 ± 0.03**| 0.73 ± 0.02#  | 0.77 ± 0.03*   |
| IVS (mm)          | 0.78 ± 0.02| 1.02 ± 0.02**| 0.91 ± 0.02**#| 0.98 ± 0.04**  |
| LVID (mm)         | 3.50 ± 0.09| 3.43 ± 0.17| 3.34 ± 0.25   | 3.43 ± 0.14    |
| LVV (μL)          | 54 ± 1     | 53 ± 6    | 52 ± 9        | 53 ± 1         |
| End-systolic      |            |          |                |                |
| LVFPW (mm)        | 0.77 ± 0.04| 1.18 ± 0.05**| 1.02 ± 0.02**#| 1.12 ± 0.06**  |
| IVS (mm)          | 0.96 ± 0.04| 1.20 ± 0.01**| 1.13 ± 0.02**#| 1.18 ± 0.02**  |
| LVID (mm)         | 2.28 ± 0.05| 2.16 ± 0.16| 2.15 ± 0.11   | 2.21 ± 0.08    |
| LVV (μL)          | 17 ± 1     | 16 ± 3    | 15 ± 3        | 16 ± 2         |

*P < 0.05, **P < 0.01 versus Sham, P < 0.05, **P < 0.01 versus TAC + NS (n = 4–6 per group). Values are expressed as mean ± SEM. HR indicates heart rate; IVS, interventricular septal wall thickness; LVEF, left ventricular ejection fraction; LVID, left ventricular internal dimension; LVFPW, left ventricle posterior wall thickness; LVV, left ventricular volume; NS, normal saline; PVb, peak velocity at banding site; and TAC, transverse aortic constriction.
Figure 1. Effects of losartan and PD123319 on TAC-induced cardiac hypertrophy at 14 days. A: Representative pictures of whole hearts at 14 days after TAC in each treatment group (grid size, 1 mm). B: Histological sections from hearts were stained with hematoxylin and eosin (scale bars, 2 mm). C: Ratios of HW to BW. Data are presented as the mean ± SEM. **P < 0.01 versus Sham, *P < 0.05 versus TAC + NS; n = 4–6 per group. BW indicates body weight; HW, heart weight; NS, normal saline; and TAC, transverse aortic constriction.

Significant elevation of HMGB1 expression levels in both the serum and myocardium observed at 14 days after TAC. Furthermore, the upregulated serum HMGB1 and cardiac HMGB1 expression levels induced by TAC could be markedly suppressed by losartan treatment, and elevated cardiac HMGB1 protein expression levels, but not cardiac mRNA or serum expression levels, were significantly neutralized by PD123319 treatment (Figure 2B-E, P < 0.05).

Ang II enhances the expression levels of HMGB1 and IL-6 in cardiac myocytes: Cultured cardiac myocytes were treated with Ang II for 5 minutes to 48 hours, and the results showed that Ang II facilitated the secretion of HMGB1 in a time-dependent manner. Specifically, HMGB1 levels in the supernatant were increased after 5 minutes of Ang II treatment and peaked at 48 hours (Figure 3A, P < 0.01). In order to compare with the HMGB1 expression pattern, IL-6 expression levels were simultaneously detected following Ang II stimulation. As shown in Figure 3B, supernatant IL-6 levels were also increased at 5 minutes under Ang II treatment and peaked at 48 hours.
Effects of losartan and PD123319 on HMGB1 expression induced by TAC at 14 days. A: Immunohistochemical staining of HMGB1 in the myocardium at 14 days (scale bars, 50 μm). Blue, counterstaining of nuclei; brown, HMGB1-positive cells. Images are representative heart sections. B: Serum levels of HMGB1 in the four groups at 14 days. C: Real-time quantitative PCR analyses of HMGB1 mRNA expression levels in the myocardium at 14 days. D: Western blotting analysis of HMGB1 expression in myocardial tissue at 14 days. Representative blots from four independent experiments are shown. E: Quantitative analysis of four independent experiments. Relative expression levels of HMGB1 were normalized to β-actin. Data are presented as the mean ± SEM. *P < 0.05, **P < 0.01 versus Sham, #P < 0.05, ##P < 0.01 versus TAC + NS; n = 4–6 per group. HMGB1 indicates high-mobility group box-1; NS, normal saline; and TAC, transverse aortic constriction.

Discussion

HMGB1 is upregulated and activated in the myocardium under PO and is involved in PO-induced cardiac remodeling, as well as mechanical stress-induced cardiac myocyte hypertrophy. However, the mechanisms of action behind the upregulation of cardiac HMGB1 expression levels when under PO have not been fully elucidated. The present study investigated the potential mechanisms of action behind the upregulation of HMGB1 in the myocardium under PO through in vivo and in vitro studies.

In agreement with previous studies, the present study showed that cardiac hypertrophy could be largely prevented through the treatment with an AT1 receptor inhibitor when under PO. In regard to the AT2 receptor, Castoldi et al. discovered that cardiac remodeling could be hindered by its activation, whereas Carneiro-Ramos et al. paradoxically found that the blockage of the AT2 receptor could suppress myocardial hypertrophy. However, as shown in the present study, cardiac hypertrophy in-
Ang II enhances the expression levels of HMGB1 and IL-6 in cardiac myocytes. A, B: ELISAs were conducted to measure the expression levels of HMGB1 and IL-6 in the supernatant after cardiac myocytes were treated with Ang II for 5 minutes to 48 hours. C, D: real-time quantitative PCR analysis of HMGB1 and IL-6 mRNA expression levels in cultured cardiac myocytes stimulated with Ang II for 5 minutes to 48 hours. Relative expression levels of intracellular HMGB1 and IL-6 were normalized to β-actin expression levels. Data are presented as the mean ± SEM. *P < 0.05, **P < 0.01 versus the Con. Experimental data were obtained ≥ 3 independent experiments. Ang II indicates angiotensin II; Con, control; HMGB1, high-mobility group box-1; and IL-6, interleukin-6.

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duced by TAC could be partially mitigated by antagonizing the AT2 receptor, although this was not found to be statistically significant, indicating that the AT2 receptor may not play a major role in the pathogenesis behind PO-induced cardiac hypertrophy.

It is widely accepted that Ang II is increased under PO following the activation of the renin-angiotensin system (RAS).25 As a critical effector of the RAS, Ang II also plays a crucial role in inflammation.26,27 It is well established that Ang II exerts proinflammatory effects through the AT1 receptor,25 although the role of the AT2 receptor in regard to a pro- or anti-inflammatory action is unclear.26,27 In the present study, the serum levels and cardiac expression levels of HMGB1 were elevated following TAC-induced cardiac hypertrophy. Furthermore, the increase in HMGB1 levels in the serum and myocardium under PO could be dramatically neutralized by losartan treatment. By blocking the AT2 receptor, the serum and transcriptional levels of HMGB1 were slightly but not significantly decreased under PO, whereas the protein levels of myocardial HMGB1 were significantly alleviated, suggesting that the AT1 receptor may be of more importance than the AT2 receptor for regulating the expression and activity of HMGB1 under PO in vivo.

Given that cardiac myocytes are the main cellular component of heart tissue,29 in vitro experiments were subsequently performed to determine whether HMGB1 and IL-6 expression can be induced by Ang II treatment in cultured cardiac myocytes. Ang II apparently increased the intracellular and extracellular expression levels of HMGB1, indicating that the upregulation of HMGB1 under PO in vivo may partially derived from cardiomyocytes. Moreover, the aforementioned elevation of HMGB1 and IL-6 expression levels was reduced by both losartan and PD123319. The varied role of the AT2 receptor in mediating the expression levels of HMGB1 between in vivo and in vitro studies may be accounted for by several reasons. Firstly, there are other types of cells that are implicated in the expression and secretion of HMGB1 under PO beyond cardiac myocytes. Moreover, the aforementioned elevation of HMGB1 and IL-6 expression levels was reduced by both losartan and PD123319. The varied role of the AT2 receptor in mediating the expression levels of HMGB1 between in vivo and in vitro studies may be accounted for by several reasons. Firstly, there are other types of cells that are implicated in the expression and secretion of HMGB1 under PO beyond cardiac myocytes. Secondly, mechanical stress under PO in vivo activates the AT1 receptor in an Ang II-independent manner,30 which may enhance the expression of HMGB1. Furthermore, it is well established that HMGB1 is a late mediator of inflammation.30 Similarly, in the present study, HMGB1 levels elevated sharply in the culture media between 12 and 48 hours, which ap-
Figure 4. Reduction of HMGB1 and IL-6 expression by losartan and PD123319 treatment. A, B: Neonatal rat cardiac myocytes were stimulated with Ang II (10^{-6} mol/L) for 48 hours in the absence or presence of losartan (10^{-5} mol/L) or PD 123319 (10^{-5} mol/L). The supernatants were collected, and the released HMGB1 and IL-6 were measured using ELISAs.

C, D: mRNA expression levels of HMGB1 and IL-6 of the neonatal rat cardiac myocytes were detected using real-time quantitative PCR at 48 hours. Data are presented as the mean ± SEM. *P < 0.05, **P < 0.01 versus the Con; #P < 0.05, ##P < 0.01 versus Ang II. Experimental data were obtained from ≥ 3 independent experiments. Ang II indicates angiotensin II; Con, control; HMGB1, high-mobility group box-1; and IL-6, interleukin-6.

It has been reported that Ang II induces cells to produce reactive oxygen species (ROS) by binding to the AT1 receptor, facilitating the expression of inflammatory mediators. A previous study found that decreasing intracellular ROS levels may weaken the expression and activation of HMGB1 in cardiac myocytes under oxidative stress. As such, it was hypothesized that Ang II may regulate the expression and release of HMGB1 under PO by modulating intracellular ROS levels through binding with the AT1 and AT2 receptors. However, further studies are needed to elucidate the underlying signaling pathways.

In addition to mechanical stress, inflammation is attributed to the progression of cardiac hypertrophy induced by PO. Moreover, not only inflammatory cells but also non-inflammatory cells play a critical role in the development of inflammation. As the principal cell type within the myocardium, cardiac myocytes produce inflammatory effects under a variety of stimuli, secreting cytokines such as IL-6 and TNF-α. The present study found that Ang II induced the expression and release of IL-6 in cultured cardiac myocytes through the AT1 and AT2 receptors, indicating that cardiac myocytes are involved in the inflammatory response under PO.

In conclusion, the present study revealed that the upregulation of HMGB1 in serum and myocardium under PO, which are partially derived from cardiac myocytes, may be induced by Ang II through the AT1 and AT2 receptors. Furthermore, amelioration of PO-induced cardiac hypertrophy by losartan treatment may be associated with the decrease in HMGB1 expression levels through interactions with the AT1 receptor.

Disclosure

Conflicts of interest: The authors confirm that there are no competing interests.
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