MG132, a proteasome inhibitor, attenuates pressure-overload-induced cardiac hypertrophy in rats by modulation of mitogen-activated protein kinase signals

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Proteasome inhibitors are involved in cell cycle control, growth and inflammatory signaling, and transcriptional regulation of mitotic cells. A recent study has suggested that specific proteasome inhibitor MG132 may suppress cardiomyocyte hypertrophy in vitro. However, the underlying molecular mechanisms are not clear. In this study, we investigated the effects of long-term MG132 treatment on cardiac hypertrophy and the related molecular mechanisms in vivo. MG132 (0.1 mg/kg/day) was intraperitoneally injected to rats with abdominal aortic banding (AAB) for 8 weeks. Results showed that treatment with MG132 significantly attenuated left ventricular (LV) myocyte area, LV weight/body weight, and lung weight/body weight ratios, decreased LV diastolic diameter and wall thickness, and increased fractional shortening in AAB rats. AAB induced the phosphorylation of ERK1/2, JNK1, and p38 in cardiac myocytes. The elevated phosphorylation levels of ERK1/2 and JNK1 in AAB rats were significantly reversed by MG132 treatment. In conclusion, our results suggested that long-term treatment with MG132 attenuates pressure-overload-induced cardiac hypertrophy and improves cardiac function in AAB rats through regulation of ERK1/2 and JNK1 signaling pathways.

Keywords MG132; cardiac hypertrophy; MAPK; signal transduction; abdominal aortic banding

Introduction

Cardiac hypertrophy is a key compensatory mechanism in response to pressure or volume overload that involves alterations in the regulation of signaling transduction pathways and transcription factors [1]. Hypertrophic signals are further integrated within the cardiomyocytes and result in enhanced protein synthesis, altered cell cycle regulation, and hypertrophic gene expression [2]. Thus, cardiac hypertrophy appears to be a specialized form of cellular growth that requires mechanisms normally involved in proliferation control and cell cycle regulation [2]. The ubiquitin–proteasome system is the major pathway for intracellular protein degradation in mitotic cells [3], controlling the level of many key proteins involved in cell cycle control, cellular mass, growth signaling, and transcriptional regulation [4–6]. Previous evidences indicated an association of proteasome dysfunction with the pathogenesis of heart disease, such as ischemia–reperfusion injury [7], doxorubicin cardiotoxicity [8], heart failure [9], and hypertrophic cardiomyopathy [10,11]. It has been reported that pharmacological inhibition of the proteasome was associated with alterations in protein expression profile of cardiomyocyte, and modulated cardiovascular disease progression [12,13]. MG132, a specific proteasome inhibitor, has been shown to suppress proliferation and inflammation [14], and possess proapoptotic [15,16] and antineoplastic [17,18] activities.

Recently, another report has shown that MG132 inhibits isoproterenol-induced hypertrophy in cultured cardiomyocytes [4]. However, there has been no similar study about the effects of MG132 on hypertrophic cardiomyocytes in vivo. Thus, the aim of the present study was to investigate whether long-term treatment with MG132 could attenuate left ventricular (LV) hypertrophy induced by pressure overload in rats and to elucidate the underlying mechanisms.

Materials and Methods

Animal models of cardiac hypertrophy
Male Sprague–Dawley rats (8 weeks old, 180 – 230 g) were used to establish pressure-overload model as described previously [19]. All animals were separated into four groups (10 rats per group): (i) vehicle-treated sham...
group; (ii) MG132-treated sham group; (iii) vehicle-treated abdominal aortic banding (AAB) group; and (iv) MG132-treated AAB group. Under intraperitoneal pentobarbital (50 mg/kg) anesthesia, AAB was created using a 5-0 suture tied twice around the abdominal aorta in which a 21-gauge needle was inserted. The needle was then retracted yielding a 70–80% constriction with an outer aortic diameter of ~0.8 mm. In the sham surgery rats, the same surgery was performed as described above except the aorta was constricted. At Day 3 after the surgery, MG132-treated rats were intraperitoneally injected with 0.1 mg/kg/day of MG132 (Z-Leu-Leu-Leu-CHO, ALEXIS, San Diego, USA) for 8 weeks. All control animals were injected with a corresponding volume of vehicle only (0.1% DMSO). Animals and all surgeries involved in the experiments were approved by the Animal Care and Use Committee of Sun Yat-Sen University (Guangzhou, China).

Echocardiographic and hemodynamic measurements
Eight weeks after the surgery, each rat was weighed, and transthoracic echocardiography was performed on each rat. Rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg), and left atrial diameter, LV end-diastolic diameter, fractional shortening, and septal and posterior wall thickness were measured or analyzed by an echocardiographic system (ATL-HDI5000; Philips Medical System, Bothell, USA) equipped with a 10-MHz imaging transducer. All measurements were averaged for 10 consecutive cardiac cycles.

Then, a catheter was introduced through the right carotid artery into the LV for hemodynamic measurements in all animals. Heart rate, LV systolic pressure, and LV end-diastolic pressure were measured with a commercially available analog-to-digital converter and BIOPAC knowledge analysis software (Goleta, USA).

Neurohormonal factors
Angiotensin II (Ang II) concentration and renin activity levels were determined using commercially available kits (Ambion, Austin, USA). Eight weeks after surgery, 5 ml of blood collected from the right carotid artery was used to measure the concentration of Ang II and renin activity by radioimmunoassay (Northern Biot Co., Beijing, China) according to the manufacturer’s instructions.

Histological analysis and cardiomyocyte size measurements
Rats were sacrificed at 8 weeks after AAB. Hearts were arrested in diastole with KCl (30 mM). Hearts and lungs were dissected and weighed in all cases. LV samples were frozen in liquid nitrogen and then stored at −80°C or fixed with 10% formalin. Fixed hearts were embedded in paraffin and sectioned to 4-μm thickness. Samples were stained with hematoxylin–eosin for overall morphology. Myocyte size from each group was evaluated by measuring the cross-sectional area of cells using the Image-Pro Plus system. Mean myocyte area was calculated by measuring 100 cells from sections stained with hematoxylin–eosin.

Western blot analysis
Heart tissue was lysed in RIPA buffer. Total protein concentration was determined using the bicinchoninic acid protein assay (Pierce, Rockford, USA). Fifty micrograms of total proteins were electrophoresed by 10% SDS–polyacrylamide gel and then transferred onto a PVDF membrane. The membrane was blocked for 2 h at room temperature in blocking solutions, incubated overnight at 4°C with anti-atrial natriuretic peptide (anti-ANP, 1:400 dilution; Santa Cruz, Santa Cruz, USA), anti-B-type natriuretic peptide (anti-BNP, 1:400 dilution; Santa Cruz), anti-p-ERK1/2 (1:800 dilution; Cell Signal Technology, Beverly, USA), anti-ERK1/2 (1:1000 dilution; Cell Signal Technology), anti-p-JNK1 (1:1000 dilution; Cell Signal Technology), anti-JNK1 (1:1000 dilution; Cell Signal Technology), anti-p-p38 (1:800 dilution; Cell Signal Technology), anti-p38 (1:600 dilution; Cell Signal Technology), and anti-GAPDH (1:40,000 dilution; Kangcheng Inc., Shanghai, China) primary antibodies. The membrane was then washed with TBS-T (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (Boshide Inc., Wuhan, China) at 37°C for 1 h. The immune complex was detected with enhanced chemiluminescence (Millipore, Billerica, USA) system, exposed to X-ray film, and analyzed using NIH Image software. GAPDH was used as a control.

Statistical analysis
Data are expressed as the mean ± SEM. Differences among groups were tested by one-way ANOVA. Comparisons between two groups were performed by unpaired Student’s t-test. A value of P < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 16.0 statistics software.

Results
Mortality after surgery
In our study, all animals in the sham group survived, and the mortality in AAB rats 8 weeks after operation was 25%. Of all 40 rats, the final surviving number was 10 in the vehicle-treated sham group, 10 in the MG132-treated sham group, 7 in the vehicle-treated AAB group, and 8 in the MG132-treated AAB group.
**Morphometric characterization**

As shown in Table 1, the LV weight/body weight (LVW/BW) and lung weight/BW ratios of AAB rats were significantly increased compared with vehicle-treated sham rats ($P < 0.01$ and $< 0.05$, respectively). Compared with vehicle-treated AAB rats, the LVW/BW and lung weight/BW ratios in MG132-treated AAB rats were markedly decreased ($P < 0.05$, respectively).

**Hemodynamic measurements**

After surgery, an increased LV systolic pressure was observed in AAB rats ($P < 0.01$ vs. sham group). The LV systolic pressure gradient was similar in vehicle- or MG132-treated AAB rats ($P > 0.05$). The LV end-diastolic pressure was higher in vehicle-treated AAB rats compared with vehicle-treated sham rats ($P < 0.01$). MG132 treatment significantly prevented the increase in LV end-diastolic pressure in AAB rats ($P < 0.01$) (Table 1).

**Echocardiographic data**

As shown in Table 2, we observed an enlargement of the LV end-diastolic diameter and left atrial diameter as well as an increase in the septal and posterior wall thickness in the vehicle-treated AAB group when compared with the vehicle-treated sham rats ($P < 0.05$ and $< 0.01$, respectively). Fractional shortening was significantly lower in the vehicle-treated AAB group compared with the vehicle-treated sham group ($P < 0.01$). In the MG132-treated AAB group, LV end-diastolic diameter, left atrial diameter, septal and posterior wall thickness, and fractional shortening were significantly improved compared with the vehicle-treated AAB group ($P < 0.05$ and $< 0.01$, respectively).

**Measurements of neurohormonal factors**

Ang II and renin are established markers of cardiac hypertrophy. Therefore, we measured them in blood samples from the animals. The plasma Ang II concentration and renin activity were significantly increased in the vehicle-treated AAB group compared with the vehicle-treated sham group ($P < 0.01$, respectively). MG132 treatment markedly prevented these alterations induced by AAB ($P < 0.01$, respectively) (Table 2).

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**Table 1 Effects of AAB and MG132 on body weight, organ weights, and hemodynamic measurements**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham (Vehicle $n = 10$)</th>
<th>AAB (MG132 $n = 10$)</th>
<th>Vehicle (MG132 $n = 7$)</th>
<th>AAB (MG132 $n = 8$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (b.p.m.)</td>
<td>382 ± 16</td>
<td>396 ± 18</td>
<td>395 ± 15</td>
<td>411 ± 18</td>
</tr>
<tr>
<td>LVW/BW (mg/g)</td>
<td>2.13 ± 0.10</td>
<td>1.99 ± 0.07</td>
<td>2.64 ± 0.12**</td>
<td>2.35 ± 0.12a</td>
</tr>
<tr>
<td>Lung wt/BW (mg/g)</td>
<td>5.44 ± 0.22</td>
<td>5.39 ± 0.18</td>
<td>6.26 ± 0.21*</td>
<td>5.64 ± 0.15a</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td>103.8 ± 2.9</td>
<td>99.4 ± 3.5</td>
<td>160.1 ± 3.7**</td>
<td>156.9 ± 2.5</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>9.32 ± 0.56</td>
<td>8.82 ± 0.63</td>
<td>23.47 ± 1.07**</td>
<td>16.85 ± 0.8500</td>
</tr>
</tbody>
</table>

HR, heart rate; LVW, left ventricular weight; BW, body weight; lung wt, lung weight; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure. Data are expressed as the mean ± SEM. $^*P < 0.05$ and $^{**}P < 0.01$ vs. vehicle-treated sham group, and $^{##}P < 0.05$ and $^{###}P < 0.01$ vs. vehicle-treated AAB group.

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**Table 2 Echocardiographic assessment and neurohormonal factors**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham (Vehicle $n = 10$)</th>
<th>AAB (MG132 $n = 10$)</th>
<th>Vehicle (MG132 $n = 7$)</th>
<th>AAB (MG132 $n = 8$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSd (mm)</td>
<td>1.91 ± 0.05</td>
<td>1.89 ± 0.06</td>
<td>2.14 ± 0.05*</td>
<td>1.97 ± 0.04a</td>
</tr>
<tr>
<td>PWd (mm)</td>
<td>1.86 ± 0.04</td>
<td>1.84 ± 0.05</td>
<td>2.16 ± 0.08**</td>
<td>1.97 ± 0.04a</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>4.97 ± 0.14</td>
<td>4.87 ± 0.13</td>
<td>5.97 ± 0.15**</td>
<td>5.23 ± 0.1800</td>
</tr>
<tr>
<td>LA (mm)</td>
<td>3.21 ± 0.12</td>
<td>3.01 ± 0.13</td>
<td>4.09 ± 0.11**</td>
<td>3.65 ± 0.13a</td>
</tr>
<tr>
<td>FS (%)</td>
<td>54.88 ± 1.84</td>
<td>55.79 ± 2.15</td>
<td>44.94 ± 1.72**</td>
<td>51.08 ± 2.00a</td>
</tr>
<tr>
<td>Ang II (pg/ml)</td>
<td>325 ± 36</td>
<td>362 ± 22</td>
<td>1954 ± 88**</td>
<td>887 ± 4000</td>
</tr>
<tr>
<td>Renin activity (ng/ml/h)</td>
<td>1.84 ± 0.08</td>
<td>1.72 ± 0.08</td>
<td>6.70 ± 0.27**</td>
<td>3.62 ± 0.1400</td>
</tr>
</tbody>
</table>

IVSd, end-diastolic interventricular septal thickness; PWd, end-diastolic posterior wall thickness; LVDd, left ventricular end-diastolic diameter; LA, left atrium; FS, fractional shortening; Ang II, angiotensin II. Data are expressed as the mean ± SEM. $^*P < 0.05$ and $^{**}P < 0.01$ vs. vehicle-treated sham group, and $^{##}P < 0.05$ and $^{###}P < 0.01$ vs. vehicle-treated AAB group.
Histological analysis
The cross-sectional area of LV myocytes was measured in the different groups. Compared with the vehicle-treated sham group, the mean myocyte area of the vehicle-treated AAB group was significantly increased \((P < 0.01)\). Treatment of AAB rats with MG132 markedly reduced the mean myocyte area compared with the AAB rats without MG132 treatment \((P < 0.01)\) (Fig. 1).

Effects of MG132 on the expression of ANP and BNP protein
Cardiac hypertrophy is characterized by induction of ‘fetal’ gene expression. As shown in Fig. 2, ANP and BNP protein levels were significantly higher in the vehicle-treated AAB group compared with those in the vehicle-treated sham group \((P < 0.01, \text{respectively})\). MG132 treatment reversed the AAB-induced increases in ANP and BNP protein expression \((P < 0.01, \text{respectively})\).

Effects of MG132 on mitogen-activated protein kinase signaling pathways
Previous studies demonstrated that mitogen-activated protein kinases (MAPKs) are involved in the regulation of cardiac hypertrophy. To determine whether AAB and MG132 treatment affect the activity of MAPKs, we measured changes in the phosphorylation levels of ERK1/2, JNK1, and p38. As shown in Fig. 3, the phosphorylation of ERK1/2, JNK1, and p38 were significantly enhanced in the vehicle-treated AAB group \((P < 0.01 \text{ vs. vehicle-treated sham, respectively})\). Long-term injection of MG132 had different impacts on them during the process of hypertrophic growth. Specifically, MG132 treatment resulted in decreased ERK1/2 and JNK1 phosphorylation in AAB-induced cardiac hypertrophy \((P < 0.01, \text{respectively})\), whereas no significant difference was shown in the phosphorylation of p38 between vehicle- and MG132-treated AAB groups \((P > 0.05)\). The observed effects of MG132 on AAB-mediated MAPK activation were not due to the changes in total ERK1/2, JNK1, and p38 protein levels.

Discussion
Although much has been learned about the protective properties of MG132 on the heart, the role of MG132 treatment on cardiac hypertrophy \(\text{in vivo}\) has not been established. The present study for the first time elucidates the effects of MG132 on cardiac hypertrophy \(\text{in vivo}\) and, based on the
data reported here, supports the notion that MG132 could be an effective preventive and therapeutic agent against cardiac hypertrophy. The cardioprotection of MG132 is caused, at least in part, by direct or indirect interruption of the activation of MAPK signaling transduction pathways.

There is a dynamic state of continual degradation and resynthesis of proteins in cardiomyocytes [20,21]. The proteasome plays a key role in heart muscle homeostasis by affecting the level of specific proteins and adapting the expression of signaling proteins [4]. Proteasome dysfunction is observed in patients with heart disease [20]. Although proteasome inhibitor MG132 has been shown to protect cardiomyocytes against oxidative stress [22,23] and effectively block artery restenosis [14], its role in the process of cardiac hypertrophy has been also the subject of speculation. In the present study, we used a rat model of AAB to determine the effects of MG132 treatment on cardiac hypertrophy and function. We showed that long-term treatment with MG132 significantly attenuated pressure-overload-induced cardiac hypertrophy in AAB rats, which is evidenced by decreased LVW/BW ratio, septal and posterior wall thickness, myocyte area, as well as ANP and BNP protein levels. Cardiac function was markedly improved by MG132 treatment as determined by increased LV fractional shortening, attenuated LV end-diastolic pressure, and decreased lung weight/BW ratio. On the basis of morphological and molecular evidence, we suggested that chronic treatment with MG132 significantly reduces cardiac hypertrophy and improves cardiac function in the AAB rats. In the present study, we also found that MG132 has no effect on blood pressure. Therefore, we suggested that the inhibitory effect of MG132 on cardiac hypertrophy is independent of blood pressure.

To understand the molecular determinants of the hypertrophic response, recent investigation has focused on characterizing intracellular signal transduction pathways in the heart. One of the major systems participating in the transduction of signal from the cell membrane to nuclear and other intracellular targets are MAPK signaling pathways. Involvement of all three classical MAPK pathways has been implicated in the mechanisms of cardiac hypertrophy [24]. Numerous pathological mediators of cardiac hypertrophy have been shown to activate different MAPK pathways [25,26]. Considerable evidences pointed to the key role of ERK1/2 and JNK1 MAPKs in contributing to the hypertrophic growth [27–29]. Pharmacological interventions related to these signaling pathways have been expected to become promising therapeutic options in treating cardiac hypertrophy [30]. Our results demonstrated that MG132 treatment markedly decreased the activity of ERK1/2 and JNK1 in AAB-induced cardiac hypertrophy. As inhibition of ERK1/2 and JNK1 activation inhibits the development of cardiomyocyte hypertrophy in vitro and in vivo, it is well conceivable that MG132-mediated down-regulation of these central signaling pathways contributes to suppression of hypertrophic growth of cardiomyocytes. However, the precise molecular mechanisms by which MG132 inhibits ERK1/2 and JNK1 activation remain unclear at this point and need further investigations. Because proteasome is considered to play a critical role in intracellular protein degradation, including inhibitor of hypertrophy, we speculated that MG132 might block degradation of upstream phosphatase that is dephosphorylating ERK1/2, and JNK1 by inhibiting proteasome. In this study, we found that MG132 did not affect the activity of p38 in vivo. Unexpectedly, recent evidence demonstrated that MG132 is capable of activating p38 in cultured cardiomyocytes [23]. Since the modulation of signaling transduction pathways in vivo is more complicated than that in vitro. Therefore, we speculated that the differences in experimental models might explain the discrepancy regarding the effect of MG132 on activation of p38.

In summary, the present work for the first time demonstrated that long-term treatment with MG132 attenuates
cardiac hypertrophy in vivo. We concluded that MG132 can mediate cardioprotection and enhance cardiac function against hypertrophy through interfering with ERK1/2 and JNK1 signaling transduction pathways, which are associated with cardiac hypertrophy. Combined with previous evidence of antihypertrophic activity of MG132 in vitro, it is tempting to suggest that MG132 may be used as a pharmacological inhibitor of cardiac hypertrophy. However, further investigations in vivo in the human system are required before such recommendations can be made.

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