The Antioxidant Edaravone Attenuates Pressure Overload–Induced Left Ventricular Hypertrophy

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Abstract—The free radical scavenger 3-methyl-1-phenyl-2-pyrazolin-5-one (edaravone) is used to treat patients with ischemic brain damage. We and others reported previously that in vitro and in vivo reactive oxygen species (ROS) act as second messengers to develop cardiac hypertrophy. In this study, we used an in vivo murine model of pressure overload–induced cardiac hypertrophy to examine the effects of edaravone on left ventricular hypertrophy. The animals were subjected to the transverse thoracic aorta constriction, and edaravone (10 mg/kg) was infused intraperitoneally twice daily. Seven days after the operation, we observed a significant increase in ROS production in hearts, which was eliminated by the treatment with edaravone. Pressure-overloaded hearts showed a significant increase in left ventricular weight/body weight ratio and the expression level of atrial natriuretic factor mRNA, which were attenuated by edaravone. It also reduced perivascular and intermuscular fibrosis and inhibited pressure overload–induced activation of apoptosis signal-regulating kinase 1 (ASK1) and its downstream kinases of c-Jun N-terminal protein kinase and p38 mitogen-activated protein kinase. Edaravone attenuated the hypertrophic response even when the treatment was started after the onset of cardiac hypertrophic response. These findings indicate that edaravone significantly attenuates pressure overload–induced cardiac hypertrophy mediated through its antioxidative function and subsequent inhibition of ASK1 signaling pathway. (Hypertension. 2005;45:921-926.)

Key Words: hypertrophy ■ oxidative stress ■ antioxidants ■ signal transduction ■ protein kinases ■ stress, mechanical

Cardiac hypertrophy occurs in response to various extracellular stimuli to the heart. The hypertrophic response is compensatory to decrease wall stress and to maintain the structure of cardiac muscle and cardiac function. However, sustained excessive workloads cause a breakdown of the compensatory mechanism and may lead to heart failure. Epidemiological studies suggest that an increase in left ventricular mass is an independent risk factor for cardiac morbidity and mortality. Therefore, a better understanding of the mechanisms of cardiac hypertrophy may lead to the development of a novel therapy to prevent cardiac hypertrophy and subsequent heart failure.

Reactive oxygen species (ROS) have been shown to act as intracellular signaling molecules in stress response in a variety of cell types, leading to apoptosis, proliferation, and transformation. In cardiomyocytes, ROS have been found to be involved in cardiac hypertrophy in vitro and to mediate the hypertrophy induced by several stimuli, such as mechanical stretch, α1-adrenergic receptor stimulation, endothelin-1, phenylephrine, angiotensin II, and tumor necrosis factor-α (TNF-α). Similar results have been obtained with in vivo study.

Apoptosis signal-regulating kinase 1 (ASK1) is an ROS-sensitive mitogen-activated protein (MAP) kinase kinase kinase that activates the c-Jun N-terminal protein kinase (JNK) and p38 MAP kinase. ASK1 is associated with thioredoxin as an inactive form in nonstressed cells. ROS induce the dissociation of thioredoxin from ASK1, leading to the activation of ASK1. Recently, ASK1 has been reported to be involved in cardiac hypertrophy in vitro and in vivo.

In the study presented here, we investigated the effects of the antioxidant 3-methyl-1-phenyl-2-pyrazolin-5-one (edaravone) on cardiac hypertrophy in an in vivo model of pressure overload–induced hypertrophy. Edaravone is known as a free radical scavenger clinically used in Japan. Edaravone is reported to prevent lipid peroxidation by scavenging free radicals produced during brain ischemia, which offers protection against ischemic and postischemic brain damage. Pressure overload–induced hypertrophy was introduced with the transverse thoracic aorta constriction (TAC) technique. This model is widely used in the study of mice to identify molecular mechanisms for cardiac hypertrophy. We used this model to examine whether edaravone significantly attenuates...
cardiac hypertrophy and fibrosis induced by pressure overload.

Materials and Methods

Animal Experiments
This study was performed under the supervision of the animal research committee and in accordance with the guidelines for animal experiments of Osaka University and the Japanese government animal protection and management law (No. 105). Ten-week-old male mice (C57BL/6J) were anesthetized and subjected to TAC as described previously.18 Mice were kept separate throughout all procedures. Seven days after TAC, mice were anesthetized and weighed, and results of simultaneous measurements of right and left carotid artery pressure were recorded as reported previously.19 After the measurements, hearts were excised, weighed, and frozen in liquid nitrogen for analysis.

Edaravone Treatment
Edaravone was synthesized at the Research Center of Mitsubishi Chemical Industries Ltd. Mice were injected with edaravone (3 mg/kg or 10 mg/kg IP) or an equal volume of its vehicle (saline) as a control twice daily until they were euthanized. The intraperitoneal injections of edaravone were started 3 days preoperatively or 2 days after TAC.

RNA Dot Blot Analysis
Total RNA was extracted from the ventricular apexes using TRIzol reagent (Life Technologies). Quantitative assessment of atrial natriuretic factor (ANF) was performed by means of RNA dot blot analysis as described previously.19 Radiolabeled RNA dots were quantified with Scion Image software, and the value of each dot was normalized to the GAPDH signal.

Lipid Peroxidation
Shortly after euthanizing mice, hearts were homogenized with the aid of a Teflon homogenizer in 20 mmol/L phosphate buffer, pH 7.4, with 5 mmol/L butylated hydroxytoluene on ice. The level of malonaldehyde (MDA) in left ventricles (LVs) was determined with a BIOXYTECH LPO-586 kit (Oxis International Inc.) according to manufacturer instructions.

Measurement of Isoprostanes
Urinary content of isoprostanes, namely 8-epi-prostaglandin F2α (8-epi-PGF2α), was quantitated by using enzyme immunoassay kit according to manufacturer instruction (Cayman Chemical). Urine samples 7 days after TAC were collected for 24 hours. All data were normalized to urinary creatinine content.

In Vitro ASK1 Kinase Assay and Western Blots
The activity of ASK1 was measured with an immune complex kinase assay as described previously.13 Total protein homogenates (50 μg per lane) were subjected to Western blot analysis using the antibodies against mouse p38 (N-20) and INK1 (FL) obtained from Santa Cruz Biotecnoy and phospho-p38 and phospho-JNK from Cell Signaling Technology.

Histological Analysis
Heart samples were arrested in diastole and immediately fixed with buffered 3.7% formalin, embedded in paraffin, and sectioned into 3-μm thickness. Hematoxylin-eosin or Mallory–Azan staining was performed on serial sections. The cross-sectional area of cardiomyocytes was measured by tracing their outline in each of the sections.

Statistical Analysis
Results are shown as mean±SEM. A 1-way ANOVA with the Bonferroni’s post hoc test or repeated-measures ANOVA was used for multiple comparisons. A value of \( P<0.05 \) was considered statistically significant.

Results

Effect of Edaravone on TAC-Induced Cardiac Hypertrophy
Mice were assessed 7 days postoperatively for hemodynamic functions and hypertrophic responses. TAC induced a remarkable increase in heart size compared with the size in sham-operated mice, and this increase was attenuated by treatment with edaravone (Figure 1A). As shown in the Table, heart weight and LV weight significantly increased in the vehicle-treated TAC-operated (control-TAC) group. Treatment with edaravone (edaravone-treated TAC-operated group [edaravone-TAC]) reduced the increase in the ratio of LV weight to body weight (B). * \( P<0.05 \) vs control-TAC group; † \( P<0.05 \) vs vehicle-treated or edaravone-treated sham-operated groups.

To evaluate the effects of edaravone on cardiac hypertrophy, we investigated the histological phenotype of TAC-operated mice. Hematoxylin-eosin showed that the mean cross-sectional area of cardiomyocytes was significantly larger in the control-TAC group than that in the vehicle-treated sham-operated (control-sham) group (Figure 2A and 2B). Pressure overload is associated with marked changes in cardiac fetal gene expression, and ANF is used as a marker of cardiac hypertrophy.18 We examined the expression level of ANF mRNA by means of RNA dot blot analysis. The level was elevated in the hearts of control-TAC mice compared
with that in the hearts of control-sham mice, and treatment with edaravone reduced this increase in hearts (Figure 2C), but the treatment had no effect on histological phenotypes or ANF mRNA expression in sham-operated mice.

TAC induced significant perivascular and intermuscular fibrosis, as demonstrated by Mallory–Azan staining. Edaravone treatment diminished the extent of fibrosis (Figure 2A), and the area of fibrosis was reduced from 9.5 ± 1.3% to 6.0 ± 0.3% by edaravone treatment (Figure 2B).

Reduction of Oxidative Stress in Heart
To estimate the antioxidant effect of edaravone, we measured the concentration of lipid peroxide in hearts, estimated in terms of MDA. MDA is used as a well-established indicator of oxidative stress in cells and tissues. The MDA concentration had significantly increased in the LV of control-TAC mice compared with that in the LV of control-sham mice (Figure 3A). Treatment with edaravone inhibited the increase in MDA level induced by TAC. Next, to investigate whether edaravone affects oxidative stress of whole body, we measured urinary level of 8-epi-PGF₂α 7 days after TAC, which has been reported to correlate with systemic level of oxidative stress. The 8-epi-PGF₂α level 7 days after TAC had not increased by TAC, and also had not decreased by the treatment of edaravone (Figure 3B). These results indicate that oxidative stress of whole body had not increased in response to pressure overload to heart.

Effect of Edaravone on Activity of ASK1 and Its Downstream

<table>
<thead>
<tr>
<th>Variables</th>
<th>Sham (n=4)</th>
<th>Edaravone (n=4)</th>
<th>Control (n=7)</th>
<th>Edaravone (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>24.6 ± 0.52</td>
<td>24.0 ± 0.52</td>
<td>25.5 ± 0.96</td>
<td>24.6 ± 0.53</td>
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<tr>
<td>HW (mg)</td>
<td>117.4 ± 6.9</td>
<td>109.7 ± 6.5</td>
<td>164.3 ± 8.8*</td>
<td>141.1 ± 2.0†</td>
</tr>
<tr>
<td>LVW (mg)</td>
<td>86.6 ± 5.4</td>
<td>83.2 ± 5.9</td>
<td>127.1 ± 7.1*</td>
<td>107.2 ± 6.4†</td>
</tr>
<tr>
<td>RW (mg)</td>
<td>16.4 ± 1.2</td>
<td>13.5 ± 2.0</td>
<td>15.8 ± 0.8</td>
<td>15.8 ± 0.8</td>
</tr>
<tr>
<td>AW (mg)</td>
<td>15.8 ± 2.7</td>
<td>11.3 ± 1.4</td>
<td>16.8 ± 2.8</td>
<td>13.8 ± 1.0</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>81.5 ± 3.5</td>
<td>82.2 ± 4.8</td>
<td>142.0 ± 11.9*</td>
<td>145.7 ± 14.4†</td>
</tr>
<tr>
<td>PG (mm Hg)</td>
<td></td>
<td></td>
<td>63.4 ± 4.8</td>
<td>53.7 ± 6.3</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>4.76 ± 0.21</td>
<td>4.57 ± 0.21</td>
<td>6.44 ± 0.23*</td>
<td>5.58 ± 0.13†‡</td>
</tr>
<tr>
<td>LVW/BW (mg/g)</td>
<td>3.51 ± 0.16</td>
<td>3.46 ± 0.20</td>
<td>4.97 ± 0.15*</td>
<td>4.26 ± 0.11†‡</td>
</tr>
</tbody>
</table>

Data are expressed as mean value ± SEM. *P < 0.05 vs control-sham group; †P < 0.05 vs edaravone-sham group; ‡P < 0.05 vs control-TAC group.

BW indicates body weight; HW, heart weight; LVW, LV weight; RW, right ventricular weight; AW, atrium weight; SBP, systolic blood pressure, proximal for TAC; PG, pressure gradient, calculated as the difference between right and left carotid artery systolic pressure.

Table 1. Effect of Edaravone (10 mg/kg) on Characteristics of TAC-Operated Mice
6 (MKK6) as a substrate. As shown in Figure 4A, an increase in ASK1 activity was detected in the hearts of the control-TAC group compared with that in the hearts of the control-sham group (4.3-fold increase). Edaravone treatment markedly attenuated this TAC-induced ASK1 activation. The phosphorylation level of JNK and p38 increased in control-TAC mice but was reduced in edaravone-TAC mice (Figure 4B and 4C). Edaravone had no effect on the phosphorylation levels of JNK and p38 in sham-operated mice. The total protein levels of JNK and p38 showed no significant differences among any of the groups.

**Effect of Edaravone on the Established Cardiac Hypertrophy**

We investigated whether edaravone can effectively attenuate hypertrophic responses on established cardiac hypertrophy. We found that LVW/BW had significantly increased 2 days after TAC (Figure 5A). Then we started the treatment of edaravone 2 days after TAC and evaluated the extent of hypertrophy 7 days after TAC. LVW/BW 7 days after TAC in edaravone-TAC had significantly decreased compared with that in control-TAC, indicating that edaravone can attenuate the hypertrophic response even after hypertrophy was established (Figure 5B).

**Discussion**

In this study, we demonstrated that administration of the antioxidant edaravone resulted in the suppression of cardiac hypertrophy induced by pressure overload. In vitro studies have indicated that ROS are involved in cardiac hypertrophy induced by G-protein–coupled receptor (GPCR) agonists such as angiotensin II and cytokines such as TNF-α.9 In an in vivo murine model of pressure overload, we were able to show that pressure overload–induced oxidative stress in hearts and the resultant cardiac hypertrophy were attenuated by treatment with the antioxidant mercaptopropionyl glycine.11 However, for clinical use, an antioxidant with high accessibility to tissue or relatively slow clearance is needed. Edaravone was proven to have an inhibitory effect on a water-soluble and a lipid-soluble peroxyl radical–induced peroxidation system and to have sufficient accessibility to tissue, including hearts, so that it can effectively scavenge ROS in heart.17 Edaravone has already been used in Japan as an antioxidant in the treatment of patients with ischemic brain damage. It was found in animal experiments that the dose for intraperitoneal injection of edaravone needed to obtain a comparable serum concentration with intravenous injection of the drug for maximum efficacy without causing any side effects is 10 × more than that with intravenous injection. This dose of intraperitoneally administered edaravone (10 mg/kg) was proven effective for reducing the MDA contents of hearts effectively after TAC. In addition, we demonstrated that edaravone effectively attenuated hypertrophic responses in established hypertrophied heart (Figure 5). This finding is very important in the clinical setting because the patient would already have hypertrophy at the initiation of treatment. Together, edaravone may be a potential new therapeutic agent for the prevention and treatment of cardiac hypertrophy.

Edaravone attenuated pressure overload–induced cardiac hypertrophy without lowering systemic systolic blood pres-
showed that hypertrophic responses were attenuated in ASK1−/− mice infused with angiotensin II. On the other hand, we have shown previously that hypertrophic responses induced by TAC did not differ between ASK1−/− and control mice. This apparent discrepancy might be explained by the difference in activation pattern of downstream kinases of ASK1 such as p38 and JNK. In the former study, activations of p38 and JNK were attenuated, whereas JNK activation, but not p38, was diminished in the latter study. JNK and p38 activation appears to be necessary for ASK1-mediated cardiac hypertrophy. This hypothesis was supported by the report presented here that edaravone produced a marked inhibition in the activation levels of ASK1, JNK, and p38 to attenuate TAC-induced hypertrophy. However, it is curious that TAC activated p38 in an ASK1-independent manner in ASK1−/− mouse hearts but mainly in an ROS-dependent and probably ASK1-dependent manner in wild-type mice. This might reflect a compensatory response to ASK1 ablation. Another possibility is that another ROS-sensitive signaling pathway than ASK1 may be involved in TAC-induced cardiac hypertrophy.

In conclusion, the antioxidant edaravone was shown to significantly attenuate pressure overload–induced LV hypertrophy mediated through its antioxidant function and subsequent ASK1 inhibition. In addition, this study suggests the potential therapeutic value of long-term edaravone treatment for preventing the pathogenesis of cardiac hypertrophy.

Acknowledgments

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