Doxorubicin is an effective antineoplastic drug and is frequently used in the treatment of hematologic and solid tumors including leukemia, breast cancer, and sarcomas; however, clinical benefit is limited by its cardiotoxicity. Doxorubicin-induced cardiomyopathy is characterized by irreversible left ventricular dysfunction and congestive heart failure with poor prognosis.1,2 Recent studies suggest that the inhibition of mitochondrial biogenesis is responsible for the pathogenesis of doxorubicin-induced cardiomyopathy. Endothelin-1 is a vasoconstrictive peptide produced from big endothelin-1 by endothelin-converting enzyme-1 (ECE-1) and a multifunctional peptide. Although plasma endothelin-1 levels are elevated in patients treated with doxorubicin, the effect of ECE-1 inhibition on doxorubicin-induced cardiomyopathy is not understood. Cardiomyopathy was induced by a single IP injection of doxorubicin (15 mg/kg). Five days after treatment, cardiac function, histological change, and mitochondrial biogenesis were assessed. Echocardiography revealed that cardiac systolic function was significantly deteriorated in doxorubicin-treated wild-type (ECE-1+/+) mice compared with ECE-1 heterozygous knockout (ECE-1+/-) mice. In histological analysis, cardiomyocyte size in ECE-1+/- mice was larger, and cardiomyocyte damage was less. In ECE-1+/- mice, tissue adenosine triphosphate content and mitochondrial superoxide dismutase were decreased, and reactive oxygen species generation was increased compared with ECE-1+/- mice. Cardiac mitochondrial deoxyribonucleic acid copy number and expressions of key regulators for mitochondrial biogenesis were decreased in ECE-1+/- mice. Cardiac cGMP content and serum atrial natriuretic peptide concentration were increased in ECE-1+/- mice. In conclusion, the inhibition of ECE-1 attenuated doxorubicin-induced cardiomyopathy by inhibiting the impairment of cardiac mitochondrial biogenesis. This was mainly induced by decreased endothelin-1 levels and an enhanced atrial natriuretic peptide-cGMP pathway. Thus, the inhibition of ECE-1 may be a new therapeutic strategy for doxorubicin-induced cardiomyopathy. (Hypertension. 2010;55:738-746.)

Key Words: cardiomyopathy ■ doxorubicin ■ endothelin-converting enzyme-1 ■ mitochondria ■ endothelin-1 ■ atrial natriuretic peptide

Attenuation of Doxorubicin-Induced Cardiomyopathy by Endothelin-Converting Enzyme-1 Ablation Through Prevention of Mitochondrial Biogenesis Impairment

Kazuya Miyagawa, Noriaki Emoto, Bambang Widyantoro, Kazuhiko Nakayama, Keiko Yagi, Yoshiyuki Rikitake, Takashi Suzuki, Ken-ichi Hirata

Abstract—Doxorubicin is an effective antineoplastic drug; however, its clinical benefit is limited by its cardiotoxicity. The inhibition of mitochondrial biogenesis is responsible for the pathogenesis of doxorubicin-induced cardiomyopathy. Endothelin-1 is a vasoconstrictive peptide produced from big endothelin-1 by endothelin-converting enzyme-1 (ECE-1) and a multifunctional peptide. Although plasma endothelin-1 levels are elevated in patients treated with doxorubicin, the effect of ECE-1 inhibition on doxorubicin-induced cardiomyopathy is not understood. Cardiomyopathy was induced by a single IP injection of doxorubicin (15 mg/kg). Five days after treatment, cardiac function, histological change, and mitochondrial biogenesis were assessed. Echocardiography revealed that cardiac systolic function was significantly deteriorated in doxorubicin-treated wild-type (ECE-1+/+) mice compared with ECE-1 heterozygous knockout (ECE-1+/-) mice. In histological analysis, cardiomyocyte size in ECE-1+/- mice was larger, and cardiomyocyte damage was less. In ECE-1+/- mice, tissue adenosine triphosphate content and mitochondrial superoxide dismutase were decreased, and reactive oxygen species generation was increased compared with ECE-1+/- mice. Cardiac mitochondrial deoxyribonucleic acid copy number and expressions of key regulators for mitochondrial biogenesis were decreased in ECE-1+/- mice. Cardiac cGMP content and serum atrial natriuretic peptide concentration were increased in ECE-1+/- mice. In conclusion, the inhibition of ECE-1 attenuated doxorubicin-induced cardiomyopathy by inhibiting the impairment of cardiac mitochondrial biogenesis. This was mainly induced by decreased endothelin-1 levels and an enhanced atrial natriuretic peptide-cGMP pathway. Thus, the inhibition of ECE-1 may be a new therapeutic strategy for doxorubicin-induced cardiomyopathy.
mice are embryonically lethal or die within 30 minutes after birth with craniofacial and cardiovascular abnormality caused by lack of ET-1. In chronic heart failure, ECE-1 is the major ET-1–forming enzyme, and the inhibition of ECE-1 results in the improvement of heart failure. Thus, ECE-1 is the key regulator of the endothelin system.

It has been shown that plasma ET-1 levels are elevated in patients treated with doxorubicin. In addition, previous studies have demonstrated that ET-1 inhibited mitochondrial biogenesis in cardiomyocytes. These findings suggest that ET-1 induces mitochondrial dysfunction and is involved in the development of doxorubicin-induced cardiomyopathy.

We hypothesized that the inhibition of ECE-1 would induce a cardioprotective effect against doxorubicin by decreasing ET-1 production. Furthermore, ECE-1 is known to cleave not only ET-1 but also cardioprotective peptides, such as atrial natriuretic peptide (ANP), and inactive these peptides. Decreased ET-1 production and increased ANP levels by ECE-1 inhibition can induce a cardioprotective effect synergistically. Unlike the effect of endothelin receptor blockade, the effect of ECE-1 inhibition on cardiovascular disease has not been fully investigated. We believe that ECE-1 inhibition can be a more effective therapeutic strategy. To test this hypothesis, we investigated the effect of ECE-1 inhibition on cardiovascular function and myocardial cGMP levels in cardiomyopathic and control mice.

Methods
An expanded Methods section is available in the online Data Supplement (available at http://hyper.ahajournals.org).

Animals
Mice heterozygous deficient for ECE-1 were generated by homologous recombination, as described previously. We backcrossed the founder mice with 129/SvEv mice ≥8 times. We used 8- to 10-week-old male ECE-1+/− mice and their wild-type littermates (ECE-1+/+ mice). Cardiomyopathy was induced by a single IP injection of doxorubicin (Sigma) at a dose of 15 mg/kg. Mice were assigned to 4 groups: (1) ECE-1+/− mice; (2) ECE-1+/− mice with injection of normal saline; (3) ECE-1+/− mice with doxorubicin; and (4) ECE-1+/− mice with doxorubicin. Five days after doxorubicin injection, analyses were performed. All of the procedures were conducted according to the Kobe University Graduate School of Medicine Guidelines for Animal Experiments.

Treatment of Endothelin Receptor Antagonists
129/SvEv mice were randomly assigned to receive either BQ123 (Enzo Life Science) plus BQ788 (Phoenix Pharmaceuticals; each 0.25 mg/kg per hour) or vehicle (sterile saline) via osmotic minipumps (DURECT Corporation). Osmotic minipumps were implanted 2 days before the induction of cardiomyopathy.

Gene Expression Analysis
Real-time RT-PCR was performed on an ABI PRISM 7500 system (Applied Biosystems) with a One-Step SYBR PrimeScript RT-PCR kit (Takara) and Superscript III Platinum One-Step qRT-PCR System (Invitrogen). β-Actin was used as an internal control.

ECE-1 Enzyme Assay
Membrane fractions from the heart were prepared as described previously. Membrane fractions were incubated with 0.1 μmol/L of human big ET-1 (PEPTIDE INSTITUTE) in 0.1 mol/L of sodium phosphate buffer (pH 6.8) and 0.5 mol/L of NaCl at 37°C for 30 minutes. Enzyme reactions were terminated by adding 5 mmol/L of EDTA, and mature ET-1 productions were measured by QuantiGlo ET-1 immunoassay (R&D Systems).

Tissue and Serum Levels of ET-1 and ANP
Myocardial tissue samples were homogenized and centrifuged at 15 000g for 30 minutes at 4°C. The supernatants were applied through Sep-Pak C-18 cartridges (Waters). These samples were dried, lyophilized, and resuspended in assay buffer. The samples and serum were applied on QuantiGlo ET-1 immunoassay and Fluorescent ANP immunoassay kits (Phoenix Pharmaceuticals) and analyzed.

Tissue Adenosine Triphosphate Content
Tissue adenosine triphosphate (ATP) content was measured with a tissue ATP measurement kit (TOYO INK) according to the manufacturer’s instructions.

Lipid Peroxidation
Cardiac lipid peroxidation was measured with a colorimetric assay kit (Oxford Biomedical Research) according to the manufacturer’s instructions. Values were normalized to the relative protein contents.

Mitochondrial DNA Copy Number
Cardiac mitochondrial DNA copy number was quantified with real-time quantitative PCR as described previously. Mitochondria-encoded cytochrome b was collected for genomic DNA-encoded GAPDH.

Western Blot Analysis
Protein samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were incubated with antibodies to each target. The membranes were developed with an ECL Plus Western Blot Detection System (GE Healthcare), and proteins were quantified on digitized images from the middynamic range and normalized to actin or heat shock protein 70 (HSP70).

Myocardial cGMP Levels
Myocardial cGMP levels were measured by cGMP enzyme immunoassay kit (Cayman Chemical Company) according to the manufacturer’s instructions.

Statistical Analysis
Data are presented as mean±SEM. Statistical significance was tested by 1-way ANOVA followed by a Fisher protected least significant difference test. Cardiomyopathy scores were analyzed by the Mann–Whitney test. Differences were considered statistically significant at P<0.05.

Results
Physiological Data in Doxorubicin-Induced Cardiomyopathy
The results of physiological studies are shown in Figure 1. Heart rate and mean blood pressure were not different between groups. Echocardiography revealed that cardiac systolic function was significantly deteriorated in doxorubicin-treated ECE-1+/− mice compared with ECE-1+/− mice. In the sham-treated animals, decreased ECE-1 had no effect on cardiac function. The survival of doxorubicin-treated ECE-1+/− mice had a tendency to be
improved compared with that of ECE-1+/+ mice (data not shown). These results suggest that decreased ECE-1 expression attenuates doxorubicin-induced impairment of cardiac function.

Histological Analysis
Doxorubicin-induced cardiotoxicity was analyzed with hematoxylin-eosin staining of cardiac sections by light microscopy (Figure 2A). There was no injury in the hearts of sham-treated animals. Doxorubicin-treated hearts had myofibrillar loss and cytoplasmic vacuolation of cardiomyocytes. The analysis of morphological grade for anthracycline-induced cardiotoxicity revealed that the cardiac injury of ECE-1−/− mice was significantly less compared with that of ECE-1+/+ mice (Table), and cardiomyocyte size was significantly larger (Figure 2B). Electron microscopic examination demonstrated severe myofibrillar loss and vacuolar degeneration in doxorubicin-treated ECE-1+/+ mice compared with ECE-1−/− mice (Figure 2C). These results indicate that doxorubicin-induced cardiotoxicity is inhibited by decreased ECE-1.

Endothelin System in Doxorubicin-Induced Cardiomyopathy
In ECE-1−/− mice, the expression of ECE-1 mRNA was less than half that of ECE-1+/+ mice (Figure 3A). ECE-1 protein level and enzyme activity were also decreased in ECE-1−/− mice (Figure 3B and 3C). These were not affected by doxorubicin. The expression of preproET-1 mRNA was increased by doxorubicin treatment; however, there were no differences between ECE-1+/+ groups and ECE-1−/− groups (Figure 3D). In sham-treated animals, tissue and serum ET-1 levels were not different between groups; however, in doxorubicin-treated mice, ET-1 levels were more than twice that of sham-treated animals (Figure 3E and 3F). Furthermore, we found that, in doxorubicin-treated ECE-1−/− mice, tissue and serum ET-1 levels were significantly decreased compared with ECE-1+/+ mice despite the fact that expression of preproET-1 mRNA was not different between doxorubicin-treated groups. These findings suggest that the decrease of ET-1 levels in doxorubicin treated ECE-1−/− mice is induced by a rate-limiting effect of ECE-1, and this leads to a cardioprotective effect.

Attenuation of Mitochondrial Damage in Doxorubicin-Treated ECE-1+/− Mice
Disruption of bioenergetics and reactive oxygen species generation induced by the inhibition of mitochondrial biogenesis are thought to be critical for doxorubicin-induced cardiomyopathy.3–5 We found that cardiac ATP content was decreased in doxorubicin-treated ECE-1+/+ mice (Figure 4A); however, this decrease was attenuated in ECE-1−/− mice. Furthermore, mitochondrial superoxide dismutase 2 was decreased and cardiac lipid peroxidation was increased in ECE-1+/+ mice compared with ECE-1−/− mice (Figure 4B and 4C). These results indicate that the doxorubicin-induced mitochondrial damage is attenuated by the inhibition of ECE-1.

Protected Mitochondrial Biogenesis in Doxorubicin-Treated ECE-1+/− Mice
To assess the ability to overcome mitochondrial damage in ECE-1−/− mice, we evaluated mitochondrial biogenesis. After doxorubicin treatment, mitochondrial DNA copy number was decreased in ECE-1−/− mice (Figure 5A); however, this was preserved at a normal level in ECE-1+/+ mice. Mitochondrial biogenesis is known to be regulated by transcriptional events. Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) is a key transcriptional regulator for mitochondrial biogenesis24,25 and is downregulated in doxorubicin-induced cardiomyopathy.5 We found
that myocardial levels of PGC-1α mRNA were significantly decreased in doxorubicin-treated ECE-1+/+ mice compared with ECE-1+/− mice (Figure 5B). Between sham-treated animals, there were no significant differences in the levels of PGC-1α. The inhibitory effect of doxorubicin on the expression of PGC-1α was inhibited by decreased ECE-1 expression. It has been shown that transcriptional regulation of mitochondrial biogenesis is induced by nuclear respiratory factors, as well as PGC-1α.22,26 In the present study, the protein levels of PGC-1α and nuclear respiratory factor 1 were decreased in ECE-1+/+ mice; however, in ECE-1+/− mice, the protein levels were normal (Figure 5C and 5D). Doxorubicin has also been shown to decrease the level of mitochondrial transcription factors.5 We found that mitochondrial transcription factor A was also preserved in ECE-1 mice compared with wild-type mice despite better cardiac function (Figure 7A). cGMP generation was significantly higher in the ERA-treated group (Figure 6C). These results suggest that endothelin receptor blockade attenuates doxorubicin-induced cardiac injury and impairment of mitochondrial biogenesis; furthermore, decreased ET-1 induced by ECE-1 inhibition directly leads to cardioprotection and preserved mitochondrial biogenesis.

Effects of Endothelin Receptor Antagonists in Doxorubicin-Induced Cardiomyopathy

The effects of endothelin receptor antagonists (ERAs) in this model were examined to assess the direct beneficial effects of decreased ET-1. Echocardiography revealed that, in the ERA-treated group, cardiac injury was significantly attenuated compared with the control group (Figure 6A). Histological evaluation also showed that cardiac injury was less and cardiomyocyte size was larger in the ERA-treated group (Figure 6B). Furthermore, cardiac PGC-1α mRNA level was significantly higher in the ERA-treated group (Figure 6C). These results suggest that endothelin receptor blockade attenuates doxorubicin-induced cardiac injury and impairment of mitochondrial biogenesis; furthermore, decreased ET-1 induced by ECE-1 inhibition directly leads to cardioprotection and preserved mitochondrial biogenesis.

Tissue and Serum ANP Levels and cGMP Content of the Heart

Mitochondrial biogenesis is partly maintained by cGMP.27,28 In our model, cGMP levels were decreased in ECE-1+/+ mice compared with ECE-1+/− mice (Figure 7A). cGMP generation is activated by NO or ANP in the heart.29,30 The ANP-cGMP pathway is known to have a cardioprotective effect.31 We found that serum ANP levels were increased in ECE-1+/− mice compared with wild-type mice despite better cardiac function (Figure 7B and 7C). Tissue ANP contents were also higher in ECE-1+/− mice, but the difference was not statistically significant. These findings indicate that protection from doxorubicin-induced cardiotoxicity is partly caused by an enhanced ANP-cGMP pathway in ECE-1+/− mice.

Table. Cardiomyopathy Scores of Animals

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>Cardiomyopathy Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control ECE-1+/+</td>
<td>6</td>
<td>0 1.0 1.5 2.0 2.5 3.0</td>
</tr>
<tr>
<td>Control ECE-1+/−</td>
<td>6</td>
<td>0 0 0 0 0 0</td>
</tr>
<tr>
<td>ECE-1+/− + DOX</td>
<td>8</td>
<td>0 0 1 1 3 3</td>
</tr>
<tr>
<td>ECE-1+/− + DOX*</td>
<td>10</td>
<td>0 4 2 2 1 1</td>
</tr>
</tbody>
</table>

DOX indicates doxorubicin. Scores were significantly increased in doxorubicin-treated ECE-1+/+ mice compared with ECE-1+/− mice. Data show the Mann-Whitney test: control ECE-1+/+ (n=6), control ECE-1+/− (n=6), ECE-1+/− + DOX (n=9), and ECE-1+/− + DOX (n=10).

*P<0.05.
Discussion

Our data demonstrated that the genetic reduction of ECE-1 contributed to cardioprotection against doxorubicin-induced cardiomyopathy through prevention of mitochondrial biogenesis impairment. The cardiac dysfunction and cardiomyocyte injury induced by doxorubicin were attenuated by ECE-1 inhibition. We have shown that PGC-1α, nuclear respiratory factor 1, and mitochondrial transcription factor A, which are...
nuclear transcription regulators for mitochondrial biogenesis, were decreased by doxorubicin treatment, whereas those factors were preserved by ECE-1 inhibition. From the data presented here, we cannot rule out the involvement of other pathways including endothelin-3, brain natriuretic peptide, or NO-cGMP. Nevertheless, our current findings strongly support the idea that the cardioprotective effects were induced mainly by decreased levels of ET-1 and an enhanced ANP-cGMP pathway.

Previous studies have shown that apoptosis of cardiomyocytes contributes to doxorubicin-induced cardiac injury. In our model, apoptosis was not detected within 5 days after treatment with doxorubicin (data not shown). This indicates that the cardiac injury in our 5-day model did not depend on apoptosis of cardiomyocytes.

In the present study, we demonstrated the pathophysiological rate-limiting effect of ECE-1 in vivo for the first time. In our model, decreased ECE-1 activity did not affect ET-1 levels in nontreated ECE-1-/- mice. On the other hand, doxorubicin-induced upregulation of proET-1 expression made the difference of ET-1 levels between ECE-1-/- mice and ECE-1+/+ mice despite the same levels of propreET-1 expression. These results suggest that, under normal conditions, the production of ET-1 is not hindered by decreased ECE-1 activity, whereas in pathological conditions in which ET-1 is increased, decreased ECE-1 activity is not enough to convert big ET-1 into ET-1. Thus, the inhibition of ECE-1 could be a feasible target for the treatment of diseases in which upregulation of ET-1 is involved in pathogenesis.

Previous studies have reported that cardiac-specific overexpression of ET-1 leads to cardiomyopathy and that tissue ET-1 levels were increased in dilated cardiomyopathy. In doxorubicin-induced cardiomyopathy, increased ET-1 levels in the heart have been demonstrated. We found that tissue and serum ET-1 levels were increased more than twice by doxorubicin treatment. Moreover, in doxorubicin-treated ECE-1-/- mice, ET-1 levels were decreased compared with ECE-1+/+ mice. Our results suggest that decreased ET-1 levels by ECE-1 inhibition protect against cardiomyopathy.

Previous reports have suggested that ET-1-mediated cardiac injury is caused by inflammation and fibrosis. However, in the present study, cardiac inflammation and fibrosis were not observed (data not shown). Yuhki et al have shown that ET-1 induced mitochondrial dysfunction and cardiomyocyte injury in an in vitro study using primary cultured cardiomyocytes. Similarly, Marin-Garcia et al demonstrated that endothelin receptor A antagonist reversed the dysfunction of mitochondrial respiratory enzymes in a canine pacing–induced cardiomyopathy model. These reports suggest that ET-1 induces cardiac injury through myocardial mitochondrial dysfunction. In the present study, our results also demonstrated the protective effect of endothelin receptor

Figure 5. Cardiac mitochondrial DNA copy number and regulation of mitochondrial biogenesis. A, Mitochondrial DNA copy number in the heart. Values are mean±SEM of 5 to 7 mice per group. B, mRNA levels of PGC-1α. PGC-1α expression of doxorubicin-treated ECE-1-/- mice was higher than that of ECE-1+/+ mice. Each bar represents mean±SEM, control ECE-1+/+ (n=6), control ECE-1+/+ DOX (n=9), ECE-1+/+ DOX (n=9). C, Top, Expression of PGC-1α and nuclear respiratory factor (NRF) 1 by Western blot analysis compared with actin. Bottom, Mitochondrial transcription factor A (Tfam) is shown compared with HSP70. D, Graphs show densitometric quantification from 3 to 5 individual hearts per group. Each bar represents mean±SEM; *P<0.05. DOX indicates doxorubicin.
blockade against cardiac injury and mitochondrial biogenesis impairment. These results indicate that the reduction of ET-1 levels by ECE-1 inhibition directly leads to cardioprotection by preserving mitochondrial biogenesis.

In our model, it is important to recognize the role of the ANP-cGMP pathway in preventing doxorubicin-induced cardiomyopathy. ANP is a natriuretic peptide that regulates a variety of physiological and pathophysiological activities. ANP generates second messengers such as cGMP and is secreted by atrial myocytes under normal conditions. In a failing heart, ventricular myocytes also secrete ANP, and the circulating ANP level is a marker for cardiac overload. ANP is known to be a diuretic drug and is prescribed for heart failure; it is also known to have a cardioprotective effect. The cardioprotective effect of ANP is thought to be achieved through the generation of cGMP, which is recognized as regulating mitochondrial biogenesis.

In an in vitro study, ECE-1 was shown to have a broad substrate specificity. ECE-1 cleaves cardioprotective peptides, such as ANP and bradykinin, to inactivate them.

Previous reports have shown that the kallikrein-kinin system is involved in the development of cardiomyopathy. Because we did not detect differences in the serum bradykinin levels in both genotypes (data not shown), we feel that bradykinin does not contribute to the phenotypes in the present study. The inhibition of ECE-1 can increase ANP levels and lead to a cardioprotective effect through cGMP-dependent activation of mitochondrial biogenesis. Although a few studies have shown that ECE-1-neutral endopeptidase dual inhibitor increased serum ANP levels, these results had limitations for elucidating the specific effects of ECE-1. In the present study, we confirmed that serum ANP levels and cardiac cGMP levels were increased by ECE-1 selective inhibition in an in vivo cardiomyopathy model. ECE-1 inhibition could provide a cardioprotective effect by increasing the ANP level.

**Perspectives**

Recently, the beneficial effects of dual (ECE-1 and neutral endopeptidase) or triple peptidase inhibitors (ECE-1, neutral endopeptidase, and angiotensin-converting enzyme) were demonstrated in preclinical and clinical trials. These inhibitors are known to be effective in the treatment of congestive heart failure. For the blockade of the endothelin system, we can use the ERA bosentan, which is frequently used for the treatment of pulmonary arterial hypertension.
previous study reported that bosentan inhibited doxorubicin-induced cardiomyopathy. Although an ERA may be a beneficial drug for doxorubicin-induced cardiomyopathy, we expect that the inhibition of ECE-1 could be a more effective therapeutic strategy because of the beneficial effects of not only reducing ET-1 generation but also activating the cardioprotective ANP-cGMP pathway. ECE-1 inhibition can provide more effective therapy for some pathological conditions as compared with an ERA. In the future, these inhibitors might become beneficial therapeutic drugs for doxorubicin-induced cardiomyopathy.

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This study was supported in part by Grant-in-Aid for Scientific Research (C) to N.E. from the Japan Society for the Promotion of Science.

Disclosures
None.

References


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ONLINE SUPPLEMENT

Attenuation of Doxorubicin-Induced Cardiomyopathy by ECE-1 ablation through Prevention of Mitochondrial Biogenesis Impairment

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Methods

Physiological studies
Blood pressure and heart rate were measured using the tail-cuff method (Softron, Tokyo, Japan). After measurement of blood pressure, animals were anesthetized with pentobarbital (50 µg/g intraperitoneally). Transthoracic echocardiography was then performed with an echocardiographic system equipped with a 12-MHz transducer (Philips, Andover, MA). The images were taken in M mode. LV fractional shortening was calculated with the formula: (Left ventricular end–diastolic diameter [LVEDD] – Left ventricular end–systolic diameter) / LVEDD.

Histological analysis
After performing echocardiography, hearts were removed. The middle third of each heart was fixed in 4% phosphate-buffered paraformaldehyde and embedded in paraffin. Sections of 4-µm thickness were stained with hematoxylin-eosin. With light microscopic analysis of sections, cardiomyocyte size was measured, and the severity of doxorubicin-induced damage of cardiomyocyte was scored as described previously.

Electron microscopy
Myocardial tissue samples were fixed with phosphate-buffered 2.5% glutaraldehyde, 2% paraformaldehyde, and 0.1-M cacodylate buffer solution for 2 h. After fixation, standard blocks were prepared for transmission electron microscopy.

Gene expression analysis
Real-time reverse transcribed–polymerase chain reaction (RT-PCR) was performed on an ABI PRISM 7500 system (Applied Biosystems, Foster City, CA) with a One-Step SYBR PrimeScript RT-PCR Kit (Takara, Dalian, China) and Superscript III Platinum One-Step qRT-PCR System (Invitrogen, Carlsbad, CA). β-Actin was used as an internal control. Specific primers were used for ECE-1: forward primer, 5'-CGT TCT ACT ACT CGC CCA CCA AAG ACG-3', reverse primer, 5'-AAG TTC AAG GCG TTG GGT GAA G-3'; peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α): forward primer, 5'-CCG TAA ATC TGC GGG ATG ATG-3', reverse primer, 5'-CAG TTT CGT TCG ACC TGC GTA A-3'; and β-actin: forward primer, 5'-CAT CCG TAA AGA CCT CTA TGC CAA C-3', reverse primer, 5'-ATG GAG CCA CCG ATC CAC A-3'.

ECE-1 enzyme assay
Membrane fractions from heart were prepared as previously described. Heart samples
were minced in a 10× volume of homogenization buffer (20mM Tris-HCl (pH 7.4) and EDTA free protease inhibitor cocktail (nacalai tesque, Tokyo, Japan)) and then homogenized using Teflon homogenizer. Homogenates were centrifuged at 1,000g for 10 minutes and resulting supernatant was further centrifuged at 100,000g for 60 minutes. The pellet was resuspended in homogenization buffer and centrifuged again at 100,000g for 60 minutes. The pellet was resuspended in a 1× volume of homogenization buffer and protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA). Membrane fractions were incubated with 0.1μM human big ET-1 (PEPTIDE INSTITUTE, Osaka, Japan) in 0.1M sodium phosphate buffer (pH6.8), 0.5M NaCl at 37°C for 30 minutes. Enzyme reactions were terminated by adding 5mM EDTA and mature ET-1 productions were measured by QuantiGlo ET-1 immunoassay (R&D Systems, Minneapolis, MN).

**Tissue and serum levels of ET-1 and ANP**
Myocardial tissue samples were homogenized in 4% acetic acid containing 150 KIU/ml aprotinin, 2.5 μg/ml pepstatin, and 4 mM ethylenediaminetetraacetic acid and placed in boiling water for 10 min. The homogenates were centrifuged at 15000 × g for 30 min at 4°C. The supernatants were applied through Sep-Pak C-18 cartridges (Waters, Milford, MA) and eluted in 4% acetic acid, 86% ethanol solution. These samples were dried, lyophilized, and resuspended in assay buffer. The samples and serum were applied on QuantiGlo ET-1 immunoassay and Fluorescent ANP immunoassay kits (Phoenix Pharmaceuticals, Burlingame, CA) and analyzed.

**Tissue adenosine triphosphate content**
Tissue adenosine triphosphate (ATP) content was measured with a tissue ATP measurement kit (TOYO INK, Tokyo, Japan) according to manufacturer’s instructions. The luciferase-emitted light was measured by a luminescence reader (Aloca, Tokyo, Japan) as counts per minute and calculated against standards.

**Mitochondrial DNA copy number**
Cardiac mitochondrial deoxyribonucleic acid (DNA) copy number was quantified with real-time quantitative PCR (qPCR) as described previously. Mitochondria-encoded cytochrome b was collected for genomic DNA-encoded glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Specific primers were used for cytochrome b: forward primer, 5’-GCC ACC GCG GTC ATA CGA TT-3’, reverse primer, 5’-GGG TAT CTA ATC CCA GTT TGG GTC TTA GC-3’ and GAPDH: forward primer, 5’-TGT GTC CGT CGT GGA TCT GA-3’, reverse primer, 5’-TTG CTG TTG AAG TCG CAG GAG-3’. The qPCR reaction was
performed with SYBR GreenER qPCR SuperMix (Invitrogen).

**Western blot analysis**
Protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. Membranes were incubated with antibodies to PGC-1α, nuclear respiratory factor-1 (NRF-1), actin, mitochondrial transcription factor A (Tfam), superoxide dismutase 2 (SOD2), ECE-1 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), and heat-shock protein 70 (HSP70) (1:1000; Abcam, Cambridge, UK). After three washes with Tris-buffered saline-Tween 20, membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit antibody, anti-mouse antibody (1:2000; Sigma), and anti-goat antibody (1:1000; Santa Cruz Biotechnology). The membranes were developed with an ECL Plus Western Blot Detection System (GE Healthcare, Piscataway, NJ), and proteins were quantified on digitized images from the mid-dynamic range and normalized to actin or HSP70.
References


