Mitochondrial DNA Damage and Dysfunction Associated With Oxidative Stress in Failing Hearts After Myocardial Infarction

Tomomi Ide,* Hiroyuki Tsutsui,* Shunji Hayashidani, Dongchon Kang, Nobuhiro Suematsu, Kei-ichiro Nakamura, Hideo Utsumi, Naotaka Hamasaki, Akira Takeshita

Abstract—Mitochondria are one of the enzymatic sources of reactive oxygen species (ROS) and could also be a major target for ROS-mediated damage. We hypothesized that ROS may induce mitochondrial DNA (mtDNA) damage, which leads to defects of mtDNA-encoded gene expression and respiratory chain complex enzymes and thus may contribute to the progression of left ventricular (LV) remodeling and failure after myocardial infarction (MI). In a murine model of MI and remodeling created by the left anterior descending coronary artery ligation for 4 weeks, the LV was dilated and contractility was diminished. Hydroxyl radicals, which originated from the superoxide anion, and lipid peroxide formation in the mitochondria were both increased in the noninfarcted LV from MI mice. The mtDNA copy number relative to the nuclear gene (18S rRNA) preferentially decreased by 44% in MI by a Southern blot analysis, associated with a parallel decrease (30% to 50% of sham) in the mtDNA-encoded gene transcripts, including the subunits of complex I (ND1, 2, 3, 4, 4L, and 5), complex III (cytochrome b), complex IV (cytochrome c oxidase), and rRNA (12S and 16S). Consistent with these molecular changes, the enzymatic activity of complexes I, III, and IV decreased in MI, whereas, in contrast, complex II and citrate synthase, encoded only by nuclear DNA, both remained at normal levels. An intimate link among ROS, mtDNA damage, and defects in the electron transport function, which may lead to an additional generation of ROS, might play an important role in the development and progression of LV remodeling and failure. (Circ Res. 2001;88:529-535.)

Key Words: mitochondria ★ free radicals ★ heart failure ★ myocardial infarction ★ remodeling

Reactive oxygen species (ROS) induce the functional and structural damage of cardiac myocytes and may play an important role in the pathophysiology of heart failure (HF). Recent evidence has suggested an intimate link between an excessive generation of ROS and the development of myocardial remodeling and failure.1–3 We have demonstrated by electron spin resonance (ESR) spectroscopy that the amount of ROS increases in the noninfarcted left ventricle (LV) in a murine model of myocardial infarction (MI) with HF.4 Furthermore, we also demonstrated that mitochondrial electron transport is a possible site of superoxide anion (O$_2^{-}$) generation in failing hearts,1 and H$_2$O$_2$, generated via O$_2^{-}$ dismutation by superoxide dismutase (SOD), reacted in the presence of transition metals to yield more reactive hydroxyl radicals (OH$^•$).5

ROS can damage various cellular components, such as proteins, lipids, and DNAs. They can damage mitochondrial macromolecules either at or near the site of their formation. Therefore, in addition to the role of mitochondria as a source of ROS, the mitochondria themselves can be damaged by ROS.6,7 Mitochondrial injury is reflected by mitochondrial DNA (mtDNA) damage as well as by a decline in the mitochondrial RNA (mtRNA) transcripts, protein synthesis, and mitochondrial function. mtDNA is more susceptible to oxidative attack than nuclear DNA, possibly because of its proximity to the respiratory chain in the mitochondrial inner membrane, the lack of protective histone-like proteins, and its poor repair activity against damage.8 mtDNA mutations may prevent its replication or expression. Therefore, mitochondrial ROS may result in the progressive destruction of the mtDNA, and such mtDNA damage can lead to a decline of mtRNA transcription and a loss of function.9 Recent in vitro studies have clearly shown that ROS mediate mtDNA damage, alterations of gene expression, and mitochondrial dysfunction in cultured vascular endothelial and smooth muscle cells.10
Mitochondria contain closed circular, double-stranded DNA of ≈16.5 kb. Both strands of the mtDNA are transcribed. The mitochondrial genome encodes 13 polypeptides involved in oxidative phosphorylation, including 7 subunits (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6) of rotenone-sensitive NADH-ubiquinone oxidoreductase (complex I), 1 subunit (cytochrome b) of ubiquinol-cytochrome c oxidoreductase (complex III), 3 subunits (COI, COII, and COIII) of cytochrome c oxidase (complex IV), and 2 subunits (ATPases 6 and 8) of complex V along with 2 rRNA (12S and 16S) subunits. The polypeptides are translated by mitochondrial ribosomes and consist of components of the electron transport chain.\(^8\) As opposed to nuclear-encoded genes, mitochondrial-encoded gene expression is largely regulated by the copy number of mtDNA.\(^11\) A decrease in the mtDNA copy number results in a corresponding decrease in mtRNA and proteins, finally leading to mitochondrial dysfunction.

We thus hypothesized that mtDNA, mtRNA, and proteins all decrease in failing mitochondria, in which higher levels of ROS are produced. Therefore, the objective of the present study was to measure the amount of ROS using ESR techniques and to examine the changes in the mtDNA copy number and the steady-state content of mtRNA transcripts as well as the activity of the electron transport chain complex enzymes in failing hearts. In the present study, we used a murine model of myocardial infarction and consisted of components of the electron transport chain.\(^8\) As opposed to nuclear-encoded genes, mitochondrial-encoded gene expression is largely regulated by the copy number of mtDNA.\(^11\) A decrease in the mtDNA copy number results in a corresponding decrease in mtRNA and proteins, finally leading to mitochondrial dysfunction.

We thus hypothesized that mtDNA, mtRNA, and proteins all decrease in failing mitochondria, in which higher levels of ROS are produced. Therefore, the objective of the present study was to measure the amount of ROS using ESR techniques and to examine the changes in the mtDNA copy number and the steady-state content of mtRNA transcripts as well as the activity of the electron transport chain complex enzymes in failing hearts. In the present study, we used a murine model of myocardial infarction (MI) after a coronary artery ligation for 4 weeks, in which the LV cavity was dilated and contractility was reduced.\(^4\)

**Materials and Methods**

**Animal Model**
The study was approved by our institutional animal research committee and conformed to the animal care guidelines of the American Physiological Society. MI was created in male CD-1 mice by ligating the left anterior descending coronary artery.\(^4\)

**Echocardiography**
On the day the study was terminated, 4 weeks after surgery, one subset of investigators (S.H. and N.S.), who were not informed of the experimental groups, performed echocardiographic studies.\(^4\)

**Myocardial Tissue Preparation**
The myocardial tissue specimens with MI were carefully dissected into two parts, one consisting of the infarcted LV with the peri-infarct rim (a 0.5- to 1-mm rim of normal-appearing tissue), and the remaining consisting of noninfarcted (remote) LV. In all subsequent assays, the comparison was made between noninfarcted LV myocardium from MI animals and control LV myocardium from sham-operated animals.

**Myocardial ROS**
ROS were quantified in the LV by using ESR spectroscopy with 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl (hydroxy-TEMPO).\(^5\)

**Mitochondrial TBARS**
The formation of lipid peroxides was measured in the mitochondrial fraction isolated from LV myocardium through a biochemical assay of TBARS.\(^1\)

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**Figure 1.** Map of the mitochondrial genome. The 16.3-kb mitochondrial genome is diagrammed, showing the 13 mRNA, 2 rRNA (12S and 16S), and 21 tRNA coding genes. mRNA genes are shown as the areas labeled with the codes of the corresponding electron transport chain complexes I, III, IV, and V. mtDNA probes 1, 2, 3, and 4 used in the Northern blot analysis (Figure 4) are represented as large arcs. PH and PL refer to the promoters of heavy (H) and light (L) strand transcription, respectively.

**Myocardial Biochemical Measurements**
The myocardial content of DNA, RNA, and protein was measured and expressed as mg/g wet weight.\(^15\) To determine whether quantitative changes in specific contractile protein content occur with MI, an electrophoretic separation of myocardial protein homogenates was performed.

**DNA Isolation and Southern Blot Analysis of mtDNA**
DNA was extracted from LV, and a Southern blot analysis was performed to measure the mtDNA copy number.\(^12,13\) Primers for the mtDNA probe corresponded to nucleotides 2424–3605 of the mouse mitochondrial genome, and those for the nuclear-encoded mouse 18S rRNA probe corresponded to nucleotides 435–1951 of the human 18S rRNA genome. The mtDNA levels were normalized to the abundance of the 18S rRNA gene run on the same gel. The protein levels of transcription factor A (Tfam), a nucleus-coded protein with the capacity to recognize and bind to specific mtDNA sequences, were analyzed by Western blot analysis.\(^13\)

**RNA Isolation and Northern Blot Analysis**
Total RNA was isolated from frozen LV by the guanidinium method, and a Northern hybridization analysis was performed.\(^4\) Probes for mtDNA analysis were prepared by amplification of nucleotides 1209–2606 (probe 1), nucleotides 3351–7570 (probe 2), nucleotides 8861–14549 (probe 3), and nucleotides 14729–15837 (probe 4) of mtDNA from mouse genomic DNA (Figure 1). In addition, the mRNA levels for the contractile proteins, including cardiac α-actin and myosin heavy chain, as well as mitochondrial proteins, such as manganese superoxide dismutase (MnSOD), all of which are coded by nuclear DNA, were also measured in the LVs from both groups.

**Mitochondrial Enzyme Activity**
The specific activity of mitochondrial complex enzymes, including complexes I, II, III, and IV, was measured in the LV. The activity of citrate synthase, one of nuclear DNA-coded mitochondrial enzymes, was also measured.
**Myocardial Structure**

The coronal sections from mid-LV were fixed in 6% formaldehyde, and 5-μm-thick paraffin-embedded sections were stained with Mason’s trichrome. The mitochondrial ultrastructure was assessed by electron microscopy.16,17

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

**Results**

**Animal Characteristics**

Thirteen sham-operated and 10 MI mice were used for the present studies. Two-dimensional and M-mode echocardiography demonstrated LV dilatation and contractile impairment in the MI mouse. The summarized data of the echocardiographic measurements obtained from mice 4 weeks after the operation are presented in online Table 1 (available at http://www.circresaha.org.). In comparison with sham-operated animals, MI animals showed a significant increase in LV end-diastolic diameter (3.4 ± 0.1 versus 6.0 ± 0.3 mm; \( P<0.01 \)) and a decrease in percent fractional shortening (42.3 ± 1.2 versus 17.3 ± 2.6%; \( P<0.01 \)).

**Oxidative Stress in the Mitochondria**

ESR signals of hydroxy-TEMPO decreased more rapidly in the presence of myocardial homogenates from MI hearts compared with sham (Figure 2A). There was a linear relation in the semilogarithmic plot of peak signal intensity versus time, thus indicating the first order kinetics of the signal decay (Figure 2B). The rate constant of signal decay, calculated from the slope of this line, was significantly larger in MI than that in sham mice (Figure 2C). DMTU (50 mmol/L) added to the reaction mixture completely abolished an increase of signal decay in MI, indicating that OH contributed to this increase (Figure 2C). SOD (50 U/mL) plus catalase (50 U/mL) also attenuated an increased signal decay rate in MI, which implies the contribution of \( \text{O}_2^- \) to the production of OH. These results confirmed our recent results that \( \text{O}_2^- \) and OH increased in the LV from MI mice.4

MI mice had a 4-fold increase in TBARS formation in the mitochondrial fraction compared with sham-operated animals (2.7 ± 0.9 versus 11.4 ± 2.4 nmol/mg mitochondrial protein, \( P<0.01 \)), thus indicating enhanced lipid peroxidation.

**Myocardial Biochemical Composition**

The biochemical composition of LV from the sham and MI groups is shown in online Table 2. In the MI mice, no significant change was observed in the myocardial DNA, RNA, or protein content compared with sham mice. The quantification of actin revealed no significant change in the LV after MI (Figure 3 and online Table 2).

**mtDNA Copy Number and mtRNA Expression**

The copy number of mtDNA, expressed as the ratio of mtDNA to nuclear DNA (18S rRNA), as measured by a Southern blot analysis, decreased by 44% \( (P<0.05) \) in MI compared with sham mice (1.03 ± 0.02 versus 0.58 ± 0.13; Figure 3). Parallel to the mtDNA copy number, the Tfam protein level also decreased by 35% in MI animals (148 ± 9 versus 96 ± 6, \( P<0.05 \)).

To determine the effects of a decreased mtDNA copy number on mtRNA, mtRNA transcript levels were measured by Northern blot analysis. MI showed a significant decrease in ND1 + ND2, ND4, ND4L, ND5, cytochrome b, COI, COII, and COIII transcripts as well as 16S rRNA (Figure 4). In contrast, the steady-state mRNA levels for cardiac α-actin were virtually identical between the sham and MI mice (63.4 ± 5.9 versus 51.4 ± 5.6, \( P=NS \); Figure 4). Similarly, myosin heavy chain mRNA levels were comparable between the groups (125.2 ± 13.5 versus 102.0 ± 19.3 \( P=NS \)). Furthermore, the MnSOD mRNA level was also comparable (68.7 ± 3.3 versus 71.4 ± 3.4, \( P=NS \)). As a result, all mitochondrial mRNAs tested were downregulated by 30% to 50%
in MI (Figure 5), whereas the mRNA levels for the nucleus-coded mRNA levels remained unchanged.

**Mitochondrial Enzymes**

Having demonstrated that the mtRNA levels are downregulated in MI, the enzymatic activity of the mitochondria was quantified (Figure 6). The enzymatic activities of complexes I, III, and IV all decreased in MI. In contrast, there was no decrease in the enzymatic activity of complex II and citrate synthase in MI, both of which were exclusively encoded by nuclear DNA.

**Myocardial Structure**

A light microscopic analysis of myocardial tissue sections has indeed shown increased interstitial fibrosis in MI. However, \( \approx 90\% \) to 95\% of all cross sections were occupied by cardiac myocytes in either the sham or MI mice (Figure 7).

We detected no evidence of myocyte injury in MI at an ultrastructural level (Figure 7). Myofibrillar organization was maintained, and the membrane structure was preserved at the sarcolemma. In both the sham and MI myocytes, the mitochondria were present throughout the cytoplasm in a characteristic organized pattern around the Z line. The sarcomere (sm) length was comparable between sham and MI myocytes (1.59 \( \pm \) 0.03 versus 1.65 \( \pm \) 0.03 \( \mu \)m; \( P = \text{NS} \)). However, the overall number of interfibrillar mitochondria increased in MI compared with sham mice (117 \( \pm \) 14 versus 149 \( \pm \) 9/100 sm\(^2\); \( P \leq 0.05 \)), and the overall average size of the mitochondria decreased in MI (0.59 \( \pm \) 0.04 versus 0.45 \( \pm \) 0.03 \( \mu \)m\(^2\); \( P \leq 0.05 \)).

**Discussion**

The present study demonstrated that the increased generation of ROS was associated with mitochondrial damage and a dysfunction in the post-MI failing hearts, which were characterized by an increased lipid peroxidation in the mitochondria, decreased mtDNA copy number, decrease in the number of mtRNA transcripts, and reduced oxidative capacity attrib-

**Figure 4.** Northern blot analysis of mtDNA-derived transcripts as well as ribosomal RNA and cardiac \( \alpha \)-actin in the hearts from sham-operated and MI mice. mtDNA probes (probes 1, 2, 3, and 4) were created by the amplification of the corresponding mitochondrial DNA from mouse genomic DNA as shown in Figure 1. A probe hybridizing to 18S rRNA was used as a control for the RNA loading levels.

**Figure 5.** Summary data for Northern blot analysis of mtDNA-derived transcripts for sham-operated (n=5) and MI (n=5) mice. Data were obtained by a densitometric quantification of the Northern blots, such as those shown in Figure 4. Data are expressed as the percent of the sham values (mean \( \pm \) SEM).

**Figure 6.** Enzymatic activity of electron transport chain complexes I, II, III, and IV as well as citrate synthase in isolated mitochondria from MI animals (n=6). Data are expressed as the percent of sham values (mean \( \pm \) SEM). Statistical comparisons are performed by Student’s unpaired \( t \) test. Each assay was done in triplicate. \( **p < 0.01 \) indicates a significant difference from the sham values.

**Figure 7.** Representative light micrographs (A and B) and electron micrographs (C and D) of the left ventricle from sham-operated (A and C) and MI (B and D) mice. Bar=20 \( \mu \)m in A and B; bar=1 \( \mu \)m in C and D.
Membranes and, hence, ROS damage may be contained formed inside the mitochondria, cannot pass through the result from a ROS-induced mutation at the origins of repli-
number are still poorly understood, mtDNA depletion may 
induced myopathy. \textsuperscript{18,19} the pathogenesis of mitochondrial diseases and zidovudine-
decrease in the mtDNA copy number has been implicated in 
drial dysfunction, because the maintenance of mtDNA is 
decreased mtDNA copy number is responsible for mitochon-
distinct (online Table 2, available at http://www.circresaha.org) 
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DNA.
Complex enzymes, because they consist of iron-sulfur clusters that can be inactivated by $O_2^-$. 

**Clinical Implications**

Several pathogenic mtDNA base substitution mutations, such as missense mutations and mtDNA rearrangement mutations (deletions and insertions), have been identified in patients with mitochondrial diseases. An accumulation of the deleted forms of mtDNA in the myocardium frequently results in either cardiac hypertrophy, conduction block, or HF. Furthermore, there is now a consensus view that mutations in mtDNA and abnormalities in mitochondrial function are associated with common forms of cardiac diseases, such as ischemic heart disease and dilated cardiomyopathy. In these conditions, however, the strict causal relationships between abnormalities in mtDNA and cardiac dysfunction have yet to be fully elucidated. Even though the mechanisms by which mtDNA damage arises in these conditions have not been clarified, ROS have been proposed to be the primary contributing factor. In the present study, we provided direct evidence that mtDNA defects occur not only in a limited small subset of mitochondrial diseases but also in a more common HF phenotype occurring after MI in mice. In addition, these alterations in mtDNA and mitochondrial function may also result from oxidative stress. The present findings are also supported by the studies on mice lacking MnSOD, which show an accumulation of oxidative damage of mtDNAs and electron transport complexes in association with the development of dilated cardiomyopathy.

The remodeling of noninfarcted LV is characterized by myocyte hypertrophy and interstitial fibrosis, both of which are also recognized in pressure-overloaded cardiac hypertrophy. Therefore, we could not rule out the possibility that the similar alterations in mtDNA and electron transport complexes could be observed in hypertrophied hearts in general, especially because increased ROS generation has been demonstrated in an animal model of cardiac hypertrophy. 

**Conclusions**

The present study demonstrates that chronic increases in ROS production are associated with mitochondrial damage and dysfunction, which thus lead to a catastrophic cycle of mitochondrial functional decline, additional ROS generation, and cellular injury. Therefore, these cellular events might be involved in myocardial remodeling and failure.

**Acknowledgments**

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**References**


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Mitochondrial DNA damage and dysfunction associated with oxidative stress in failing hearts following myocardial infarction

Tomomi Ide, Hiroyuki Tsutsui, Shunji Hayashidani, Dongchon Kang, Nobuhiro Suematsu, Kei-ichiro Nakamura, Hideo Utsumi, Naotaka Hamasaki, Akira Takeshita

Methods

Animal Model

The study was approved by our Institutional Animal Research Committee and conformed to the animal care guidelines of the American Physiological Society. We used male CD-1 (Charles River) littermate mice, 5 - 8 wk old and 25 - 35 g body weight, which were randomly divided into sham-operated and MI groups. MI was created by ligating the coronary artery according to the methods described previously.¹ MI mice were both age-matched and gender-matched with the sham-operated animals. After general anesthesia using avertin (250 μg/g) via intraperitoneal injection, the mice were intubated, and ventilated with a small animal respirator (tidal volume = 0.5 mL, rate = 120 breaths/min). The left anterior descending coronary artery was ligated by a 8-0 silk suture under a dissecting microscope. The suture was positioned approximately midway between the apex and base (2.5 - 3.0 mm from the origin). The chest cavity was closed in layers with 5-0 silk, and the animal was gradually weaned from the respirator. Once spontaneous respiration resumed, the endotracheal tube was removed, and the animal was placed under a heating lamp. The animals remained in a supervised setting until fully conscious, when they were returned to their cages and given standard chow and water ad libitum. The sham-operated mice underwent the same procedures without ligating the coronary artery.
Echocardiography

On the day the study was terminated, 4 weeks after surgery, echocardiographic studies were performed under light anesthesia and spontaneous respiration as described previously. In brief, a commercially available echocardiography system (SSD-5500, Aloka) was utilized with a dynamically focused 10-MHz linear array transducer using a depth setting of 2.0 cm. Two-dimensional images and M-mode tracings were recorded from the short-axis view at the papillary muscle level. Care was taken not to apply too much pressure to the chest wall. The M-mode tracings were printed on glossy paper by using a digital printer. The LV end-diastolic diameter (EDD), end-systolic diameter (ESD), and wall thickness were measured, averaging three to five cardiac cycles. Fractional shortening (% FS) was calculated using the following equation; % FS = (LV EDD) - (LV ESD) / (LV EDD) X 100. One subset of investigators (S.H. and N.S.), who were not informed of the experimental groups, performed echocardiographic studies.

Myocardial Tissue Preparation

The myocardial tissue specimens with MI were carefully dissected into two parts; one consisting of the infarcted LV with the peri-infarct rim (a 0.5- to 1-mm rim of normal-appearing tissue) and the remaining noninfarcted (remote) LV. In all subsequent assays, the comparison was made between non-infarcted LV myocardium from MI animals and control LV myocardium from sham-operated animals.

Quantitation of Myocardial ROS by ESR Spectroscopy

ROS were quantified in the LV by using ESR spectroscopy with 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl (hydroxy-TEMPO) as a spin probe. In brief, ESR spectra of hydroxy-TEMPO were recorded in the presence of myocardial homogenates. The ESR measurements were performed at room temperature using an X-band (9.43 GHz) ESR spectrometer (JES-RE-1X; JEOL). The ESR settings were as follows: a microwave power of 10 milliwatt, a range of external magnetic field of 20 mT, and a scan rate of 10 mT/min. The amount of ROS was assessed by monitoring the time-
dependent decay of the amplitude of low field component of ESR spectra elicited by hydroxy-TEMPO. The rate of decay was determined by the kinetics of ESR signal attenuation.

Freeze-clamped LV myocardial samples (70 - 80 mg) were homogenized in 50 mmol/L sodium phosphate buffer (pH 7.4) containing protease inhibitors (leupeptin 10 μg/mL, phenylmethylsulfonyl fluoride 100 μg/mL, dithiothreitol 1 mmol/L, and trypsin inhibitor 10 g/mL). The homogenate was immediately reacted with hydroxy-TEMPO (0.1 mmol/L) in phosphate buffered saline (PBS) and its ESR spectra were recorded up to 10 min at the intervals of 10 to 15 sec. To determine the contribution of ROS in the attenuation of ESR signals, all measurements were performed in two parallel runs, in the presence and absence of DMTU (50 mmol/L) or catalase (50 U/mL) plus superoxide dismutase (SOD; 50 U/mL) as competitive reagents in the reaction mixture.

**Mitochondrial TBARS Assay**

The mitochondrial fraction from LV was isolated according to the methods reported previously with some modifications. Briefly, fresh myocardial tissues were minced and homogenized at 4 °C for 30 seconds in 12 volumes of HES buffer consisting of 10 mmol/L HEPES-NaOH (pH 7.4), 1 mmol/L EDTA, and 250 mmol/L sucrose with a Teflon pestle in a glass homogenizer. The homogenate was centrifuged at 4 °C and 700 x g for 10 min to remove any nuclear and myofibrillar debris, and the resultant supernatant was then subsequently centrifuged at 7,000 x g for 10 min. To isolate the mitochondrial fraction, the resultant pellet was resuspended and washed three times with HES buffer. Protein concentration was determined by a BCA assay.

The formation of lipid peroxides in the mitochondrial fraction was measured through a biochemical assay of TBARS as described previously with slight modifications. The mitochondrial fractions (30 μg), suspended in 10 mmol/L HEPES-NaOH (pH 7.4), were mixed with 0.4 % thiobarbituric acid and 1 mmol/L EDTA. Butylated hydroxytoluene (0.01 %) was added to the assay mixture in order to prevent autoxidation of the sample. The mixture was heated at 100 °C for 60 min. After cooling,
n-butanol:pyridine (15:1, vol/vol) was added to the assay mixture, which was centrifuged at 1,600 x g for 10 min to extract TBARS. TBARS were determined by a fluorometric assay with the excitation at 515 nm and the emission at 553 nm\textsuperscript{5} and expressed as nmol/mg mitochondrial protein. Commercially available malondialdehyde was used as a standard.

**Myocardial Biochemical Measurements**

LV myocardial content of DNA, RNA, and protein was measured as described previously and expressed as µg/mg wet weight.\textsuperscript{12} To determine whether quantitative changes in specific contractile protein content occur with MI, electrophoretic separation of myocardial protein homogenates from both groups was performed and actin content was determined. Samples were loaded in triplicate, and purified actin standards as well as molecular weight markers were routinely loaded on the gel. Quantification of the bands corresponding to actin (43 kDa) was performed using an image analysis system.

**DNA Isolation and Southern Blot Analysis of MtDNA**

To measure the mtDNA copy number, DNA was extracted from the myocardial tissue. Briefly, frozen minced LV (20 mg) was crushed into the powdered tissue and suspended in the digestion buffer consisted of 100 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 8.0), 25 mmol/L EDTA and 0.5% sodium dodecyl sulfate (SDS) with 100 µg/mL proteinase K (GIBCO BRL) at 50 °C for 16 hours. The digested sample was centrifuged at 1,800 x g for 10 min and the resultant supernatant was collected. DNA was extracted from the supernatant by adding phenol/chloroform/isoamyl alcohol (25:24:1) and the mixture was centrifuged at 1700 x g for 10 min to collect the aqueous layer. To purify DNA, an equal volume of 2.5 mol/L ammonium acetate and 2 volumes of 100 % ethanol were added to the aqueous layer. The precipitated DNA was collected by centrifugation at 9,000 x g for 20 min. DNA sample was rinsed with 70 % ethanol, air dried and
resuspended in the TE buffer consisting of 10 mmol/L Tris-HCl and 1 mmol/L EDTA (pH 7.4).

To measure the mtDNA copy number, a Southern blot analysis was performed as previously described. For each tissue specimen, 1 µg of total DNA was digested with Pst I restriction endonuclease (Takara), separated by electrophoresis through 0.7% agarose gel, and transferred onto the nylon membrane (Hybond-N+, Amersham) under alkaline conditions. All membranes were prehybridized at 57 °C for 60 min with hybridization buffer (AlkPhos Direct hybridization buffer, Amersham), hybridized to the labeled probe at 57 °C overnight, and then washed twice at 57 °C for 20 min each in wash buffer (2 mol/L urea, 0.1 % SDS, 50 mmol/L Na phosphate, 150 mmol/L NaCl, and 10 mol/L MgCl2) and twice at room temperature for 10 min each in the buffer consisted of 50 mmol/L Tris, 100 mmol/L NaCl, and 2 mmol/L MgCl2. The probe for mtDNA corresponded to nt2424-3605 of the mouse mitochondrial genome and that for the nuclear-encoded mouse 18S rRNA corresponded to nt435-1951 of the human 18S rRNA genome. Each probe was labeled with a labeling kit using alkaline phosphatase (AlkPhos Direct, Amersham). The signals were detected by chemiluminescent reagents (CDP-Star, Amersham) and quantified by an image analyzer (NIH Image). MtDNA levels were normalized to nuclear DNA content as expressed by the abundance of the 18S rRNA gene run on the same gel.

**RNA Isolation and Northern Blot Analysis**

Total RNA was isolated from frozen LV (30 - 40 mg) by the guanidinium method (ISOGEN, Nippon Gene). For each tissue specimen, 5 µg of total RNA was separated by electrophoresis (80 V, 2 hours) through 1 % (wt/vol) agarose gel, followed by blotting onto the nylon membrane (Hybond-N+, Amersham), and hybridized with probes. Probes for the RNA analysis of mtDNA was prepared by amplification of nt1209-2606 (Probe 1), nt3351-7570 (Probe 2), nt8861-14549 (Probe 3), and nt14729-15837 (Probe 4) of the mitochondrial DNA from mouse genomic DNA (Figure 1). The steady-state mRNA levels for the contractile proteins including cardiac α-actin and
myosin heavy chain (MHC) as well as mitochondrial proteins such as manganese superoxide dismutase (MnSOD), all of which are coded by nuclear DNA, were also measured. The cardiac α-actin cDNA was a 878-bp segment from mouse cardiac actin and the MHC cDNA was a 940-bp segment from mouse that binds equally to both α- and β-isoforms. Probes were radiolabeled with [α-32P]dCTP using the random primer DNA labeling kit (Takara) and the labeled probes were purified through columns (Takara SUPEREC-02). A Northern hybridization analysis was performed according to the methods described previously.8 The intensity of the bands was quantitated by an image analyzer (NIH Image). The steady-state abundance of mRNAs was expressed as relative to the intensity of the corresponding 18S rRNA and individual results were expressed as a percentage of the average value for the sham-operated animals.

**Mitochondrial Enzyme Activity**

The specific enzymatic activity of rotenone-sensitive NADH-ubiquinone oxidoreductase (complex I) was measured by a reduction of ubiquinone analog, decylubiquinone, using a spectrophotometer according to the methods described previously.3 For the activity of succinate-ubiquinone oxidoreductase (complex II), the reduction of 2,6-dichlorophenolindophenol, when coupled to complex II-catalyzed reduction of decylubiquinone, was measured.10 For the specific activity of ubiquinol-cytochrome c oxidoreductase (complex III), the reduction of cytochrome-c catalyzed by complex III in the presence of reduced decylubiquinone was monitored.10 The specific activity of cytochrome-c oxidase (complex IV) was measured by following the oxidation of reduced cytochrome-c, which had been prepared in the presence of dithionite.11 The activity of citrate synthase, one of nucleus-coded mitochondrial enzymes, was also measured. All enzymatic activities were expressed as nmol/min/mg protein.
Mitochondrial Transcription Factor A (Tfam)

The protein level of Tfam, a nucleus-coded protein with the capacity to recognize and bind to specific mtDNA sequences, was measured by a Western blot analysis. In brief, the mitochondrial fraction was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by western blotting using a rabbit polyclonal antiserum to Tfam. Each blot was blocked with 3% bovine serum albumin at 4°C overnight, and then was incubated with antiserum of Tfam (dilution: 1:2,500). This was followed by washing with Tris-buffered saline solution containing 0.05% Tween-20 and a reaction with HRP-labeled goat anti-rabbit IgG antiserum (dilution: 1:10,000). The reaction was visualized using a chemiluminescence reagent (Amersham) and the intensity was analyzed using an image analysis system.

Myocardial Structure

To assess the proportion of myocytes, the coronal sections from mid-LV were fixed in 6% formaldehyde and 5 μm-thick paraffin-embedded sections were stained with hematoxylin and eosin and Masson's trichrome.

For the assessment of mitochondrial ultrastructure by electron microscopy, LV tissues were fixed in a mixture of 3% glutaraldehyde and 3% paraformaldehyde in 0.1 mol/L cacodylate buffer at pH 7.4 for 3 hours at 4°C. After washing in 0.1 mol/L cacodylate buffer, they were postfixed with 1% osmium tetroxide for 1.5 hr at 4°C. After washing in distilled water, they were block-stained with 0.2% uranyl acetate in 50% methanol overnight, dehydrated in a graded series of ethanol, and then were embedded in Epon 812. Ultrathin sections were double stained with uranyl acetate and lead citrate, and then were observed under transmission electron microscope (JEM-1200EX; JEOL). For the quantitative morphometric analysis, the number and size of the mitochondria were examined according to the methods by Sabbah et al. The number of mitochondria and the cross-sectional area (size) of each mitochondrion was measured within a sampling region of 100 square sarcomeres (μm²). Eighteen regions were
selected at random for each specimen and for all regions the average of mitochondrial number and cross-sectional area were calculated.

**Statistical Analysis**

The values are expressed as the means±SEM. Statistical comparisons between the sham-operated and MI mice were performed by the unpaired t test. For multiple-group comparisons, one-way ANOVA followed by Scheffe's t test was performed. \( P<0.05 \) was considered to be statistically significant.
References


8. Igarashi-Saito K, Tsutsui H, Yamamoto S, Takahashi M, Kinugawa S, Tagawa H, Usui M, Yamamoto M, Egashira K, Takeshita A. Role of SR Ca^{2+}-ATPase in


Table 1. Echocardiographic data

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 13)</th>
<th>MI (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV EDD, mm</td>
<td>3.4 ± 0.1</td>
<td>6.0 ± 0.3**</td>
</tr>
<tr>
<td>LV ESD, mm</td>
<td>2.0 ± 0.1</td>
<td>4.9 ± 0.4**</td>
</tr>
<tr>
<td>% Fractional shortening, %</td>
<td>42.3 ± 1.2</td>
<td>17.3 ± 2.6**</td>
</tr>
</tbody>
</table>

Data are the means ± SE. n indicated the number of animals studied. Echocardiographic measurements were performed in mice 4 weeks after the operation.

L.V, left ventricular. EDD, end-diastolic diameter. ESD, end-systolic diameter.

**p < 0.01 vs. Sham.
### Table 2. Myocardial biochemical composition

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 5)</th>
<th>MI (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA content</td>
<td>1.18 ± 0.06</td>
<td>1.37 ± 0.10</td>
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<tr>
<td>RNA content</td>
<td>1.46 ± 0.12</td>
<td>1.27 ± 0.06</td>
</tr>
<tr>
<td>Protein content</td>
<td>84.9 ± 10.2</td>
<td>84.0 ± 4.1</td>
</tr>
<tr>
<td>Actin content</td>
<td>33.6 ± 3.3</td>
<td>31.8 ± 1.3</td>
</tr>
</tbody>
</table>

Data are the means ± SE in mg/g wet tissue weight. n indicated the number of animals studied.