Oxidative Stress Causes Heart Failure with Impaired Mitochondrial Respiration

Received for publication, March 6, 2006, and in revised form, July 21, 2006. Published, JBC Papers in Press, September 6, 2006, DOI 10.1074/jbc.M602118200

Hidetoshi Nojiri*, Takahiko Shimizu*,†, Masabumi Funakoshi*,**, Osamu Yamaguchi†,‡, Heying Zhou†,‡, Satoru Kawakami*,†, Yutaka Ohta**, Manabu Sami**, Toshiaki Tachibana*, Hiroshi Ishikawa*, Hisashi Kurosawa*, Ronald C. Kahn†, Kinya Otsu*, and Takuji Shirasawa*†,§

From the *Research Team for Molecular Biomarkers, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173-0015, Japan, the †Department of Orthopedics, Juntendo University School of Medicine, Bunkyo-ku, Tokyo 113-8421, Japan, ‡Biological Science, Graduate School of Science, Tokyo Metropolitan University, Hachioji-shi, Tokyo 192-0397, Japan, §Osaka University Graduated School of Medicine, Suita, Osaka, 565-0871, Japan, ¶Fundamental Research Laboratory, Asahi Breweries, Ltd., Moriga-shi, Ibaraki 302-0106, Japan, and the ¶¶Department of Anatomy II, Jikei University School of Medicine, Minato-ku, Tokyo 105-8461, Japan. Research Division, Joslin Diabetes Center and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02215, §Applied Biological Chemistry, United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Fuchu-shi, Tokyo 183-8509, Japan, and Anti-Aging Science, Inc., Chiyoda-ku, Tokyo 100-0001, Japan.

Elderly people insidiously manifest the symptoms of heart failure, such as dyspnea and/or physical disabilities in an age-dependent manner. Although previous studies suggested that oxidative stress plays a pathological role in the development of heart failure, no direct evidence has been documented so far. In order to investigate the pathological significance of oxidative stress in the heart, we generated heart/muscle-specific manganese superoxide dismutase-deficient mice. The mutant mice developed progressive congestive heart failure with specific molecular defects in mitochondrial respiration. In this paper, we showed for the first time that the oxidative stress caused specific morphological changes of mitochondria, excess formation of superoxide (O$_2^-$), reduction of ATP, and transcriptional alterations of genes associated with heart failure in response to cardiac contractility. Accordingly, administration of a superoxide dismutase mimetic significantly ameliorated the symptoms. These results implied that O$_2^-$ generated in mitochondria played a pivotal role in the development and progression of heart failure. We here present a bona fide model for human cardiac failure with oxidative stress valuable for therapeutic interventions.

Heart failure is a leading cause of mortality worldwide, affecting about 1–2% of the population in Japan, Europe, and the United States (1). Heart failure results from an imbalance between left ventricular (LV)$^2$ performance and myocardial energy consumption, a phenomenon that is best described as mechanoenergetic uncoupling. Despite the markedly impaired work of the left ventricle in this process, the oxygen used by muscle contraction remains relatively unchanged, which results in a decline in the contractile function of the myocardium.

The mechanisms responsible for the progression of heart failure are emerging, whereas there is increasing evidence to suggest that reactive oxygen species (ROS) play a major pathological role (2, 3). In fact, several studies on human cases have revealed increased ROS production in the hearts of individuals with congestive heart failure. Biomarkers for ROS have been detected in the pericardial fluid (4) as well as in the peripheral blood (5, 6) of heart failure patients. Although it is still controversial whether antioxidative enzymes are inactivated in failing hearts (7, 8), polymorphisms in the candidate genes that are responsive to oxidative stress affect the susceptibility to and contribute to the risk of heart failure (2). It is therefore hypothesized that heart failure is attributable to an imbalance between the generation of ROS and cellular antioxidative defensive mechanisms.

Superoxide (O$_2^-$), one of the ROS generated in mitochondrial respiration, is involved in a variety of biological processes in the myocardium, including alterations in mitochondrial DNA (9), contractile proteins (10), ion channels (11), or mitochondrial respiratory enzymes (12, 13). Manganese superoxide dismutase (Mn-SOD) is a principal scavenger enzyme located in mitochondrial matrix, which detoxifies O$_2^-$ by dismutation, protecting cells from oxidative stress. Interestingly, polymorphisms in the targeting signal of the Mn-SOD gene affect the efficiency of its transport to mitochondria. The allele frequency is then associated with idiopathic dilated cardiomyopathy (14, 15). These genetic data suggested that Mn-SOD plays an
important role in the susceptibility to and/or the progression of dilated cardiomyopathy.

In previous studies, two groups independently reported on Mn-SOD-deficient mice (16, 17). The Mn-SOD deficiency on a CD1 background resulted in neonatal death by day 10 from severe dilated cardiomyopathy, liver dysfunction, and metabolic acidosis (16). Other Mn-SOD-deficient mice in a mixed genetic background of C57BL/6 and 129/Sv, on the other hand, died by day 18 with neuronal degeneration, particularly in the basal ganglia and brainstem (17). In this context, it was hard to investigate the pathological consequences of oxidative damages in adult tissues by using these Mn-SOD-deficient mice. These authors also argued that the phenotypes are too complex to sequestrate the specific aging processes in each tissue of mutant mice in vivo. In the present study, we successfully generated heart/muscle-specific Mn-SOD-deficient mice (H/M-Sod2−/−) using the Cre-loxP system under the control of the muscle creatine kinase (MCK) promoter and established a murine model for congestive heart failure as well as age-related physical disability induced by oxidative stress.

Since heart failure still remains a significant health problem, with mortality rates as high as 45% within 1 year (1), more effective treatments should be established. Since the biological effects of oxidative stress in heart failure have been precisely analyzed in the last decade, we expect an emergence of new cardiovascular medicine or new nutritional interventions that will improve the quality of life in patients with heart failure. Many of the antioxidants and oxidative stress-related medicines currently under investigation are controversial (3, 18). With this model, we provide a means to evaluate the efficacy of medicine or interventions over a physiological as well as pathological range of heart functions and failures.

**EXPERIMENTAL PROCEDURES**

**Animals and Genotyping**—The generation of Mn-SOD flox mice was described previously (19). The Mn-SOD flox mice were back-crossed to C57BL/6/C5CrSlc mice for five or six generations. The cross-breeding of homozygous Mn-SOD flox mice with MCK-Cre transgenic mice of FVB background (20) gave rise to H/M-Sod2−/− mice. All genotyping of the Cre transgene and the Mn-SOD flox was performed by PCR using genomic DNA isolated from the tail tip. The primers for identifying carriers of the MCK-Cre transgene (5′-TTC CAG GTA GAG AGA CTA CCA ACC C-3′ and 5′-TCG ACC AGT TTA GTT ACC C-3′) and Mn-SOD flox allele (5′-TTA GGC CTC AGG TTT GTC CAG AA-3′, 5′-CGA GGG GCA TCT AGT GGA GAA-3′, and 5′-AGC TTG GCT GGA CGT AA-3′) were used. The deleted alleles were confirmed as described previously (19). DNA isolated from cytomegalovirus enhancer/chicken β-actin (CAG) promoter Cre Mn-SOD-deficient neonates was used as a positive control (19). Mice were maintained and studied according to protocols approved by the Animal Care Committee of the Tokyo Metropolitan Institute of Gerontology.

**Western Blot Analysis**—Western blot analysis was performed with heart, skeletal muscle (tibialis anterior), brain, liver, and kidney homogenates (5 μg each) as previously described (19). Antibodies against Mn-SOD (1:10,000; catalog number SOD-111; StressGen, Victoria, Canada), Complex I α9 (1:2000; Molecular Probes, Inc., Eugene, OR), succinate dehydrogenase A (SDHA) flavoprotein (1:10,000; Molecular Probes), SDHB iron-sulfur protein (1:2000; Molecular Probes), Complex III Rieske iron-sulfur protein (1:2000; Molecular Probes), Complex III Core I (1:10,000; Molecular Probes), cytochrome c oxidase (COX) subunit I (1:500; Molecular Probes), Complex V α subunit (1:2000; Molecular Probes), Complex V β subunit (1:5,000; Molecular Probes), cleaved caspase-3 (1:1000; Cell Signaling, Danvers, MA), glyceraldehyde-3-phosphate dehydrogenase (1:2000; Biogenesis Ltd., Poole, UK), and actin (1:100; Sigma) were used.

**Generation of an Antibody against Mn-SOD for Immunohistochemistry**—An immunogen peptide corresponding to residues Lys32-Ala37 (KHSLDPYLDPYGA) of mouse Mn-SOD with an additional cysteine residue plus amide at the C terminus was synthesized. The synthetic peptide corresponding to the N-terminal region of the mature form of mouse Mn-SOD (21) was immunized to rabbits as described previously (22). The specific antibody was affinity-purified using the immunogen peptide immobilized on FMP-activated Cellulofine (Seikagaku Kogyo, Tokyo, Japan).

**Histological and Histochemical Studies**—Organs were dissected, fixed overnight in a 20% formalin neutral buffer solution (Wako, Osaka, Japan), embedded in paraffin, and sectioned on a microtome using standard techniques. To detect the fibrosis or glycogen accumulation in tissues, sections were stained by Azan staining or periodic acid-Schiff (PAS) staining methods, respectively. For immunohistochemistry, 4-μm deparaffinized transverse sections prepared from heart or tibialis anterior were immunostained with an anti-Mn-SOD antibody (1:1000) using a Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s protocol. For enzymatic histochemical staining, tissues were frozen in isopentane in liquid nitrogen and embedded in Tissue Tek O.C.T. compound on dry ice. Sections were cut 8 μm thick and mounted on silane-coated slide glasses. Frozen sections were dried and incubated in 50 mM sodium phosphate (pH 7.4), 84 mM succinate acid, 0.2 mM phenazine methosulfate, 2 mg/ml nitro blue tetrazolium, and 4.5 mM EDTA for SDH (Complex II) activity or in 50 mM sodium phosphate (pH 7.4), 1.0 mg/ml 3′,3′-diaminobenzidine, 24 units/ml catalase (Wako), 1 mg/ml cytochrome c (Wako), and 75 mg/ml sucrose for COX (Complex IV) activity. These reactions were performed in the dark at room temperature for 20 min. The nuclei with DNA fragmentation on 4-μm deparaffinized sections were stained with an ApopTag Plus fluorescein in situ apoptosis detection kit (Sero-logicals Corp., Norcross, GA).

**Echocardiology**—The mice were anesthetized with 2.5% avertin (8 μl/g) and echocardiography was performed using ultrasonography (SONOS-5500, equipped with 15-MHz linear transducer; Philips Medical Systems) (23). The heart was imaged in the two-dimensional parasternal short axis view, and an M-mode echocardiogram of the midventricle was recorded at the level of the papillary muscles. Heart rate, anterior and posterior wall thickness, and end diastolic and end systolic internal dimensions of the left ventricle were obtained from the M-mode image.
Transmission Electron Micrographs of Myocardium and Skeletal Muscle—Hearts from 16-week-old mice were fixed by immersion in 2.5% glutaraldehyde in 0.1 M sodium phosphate (pH 7.3) for 48 h at 4 °C and postfixed in 1% osmium tetroxide in the same buffer for 2 h at 4 °C. Dehydrated in ethanol, immersed in absolute propylene oxide, and embedded in Epon 812 (Structure Probe, West Chester, PA). Regions of interest were localized and characterized with the light microscope on 1-μm sections stained with toluidine blue. Ultrathin sections from selected areas were cut with a diamond knife and stained with uranyl acetate and lead citrate and observed with a JEOL JEM 1200 EXII electron microscope (JEOL, Tokyo, Japan) at 60 kV.

Measurement of \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \)—

\( \text{O}_2 \) formation was measured using the chemiluminescent probe 2-methyl-6-p-methoxyphenylethynylimidazopyrazinone (ATTO, Tokyo, Japan) (24). \( \text{H}_2\text{O}_2 \) generation was measured using horseradish peroxidase (1 unit/ml), homovanillic acid (0.1 mM), and substrate (5 mM malate and 0.5 mM glutamate) (25) or horseradish peroxidase (0.2 unit/ml), Amplex red (Molecular Probes) (1 μM), and substrate (5 mM succinate) (26). Fluorescence was determined at 312-nm excitation and 420-nm emission in cases using homovanillic acid or at 544-nm excitation and 590-nm emission in cases using Amplex red with a SPECTRAMax Gemini XS (Molecular Devices, Inc., Sunnyvale, CA). Fluorescence units were converted by the standard curve of known concentration of \( \text{H}_2\text{O}_2 \). Results were expressed as pmol of \( \text{H}_2\text{O}_2/\text{min/mg of protein} \).

Lipid Peroxidation—Lipid peroxidation in cytoplasmic and mitochondrial fraction prepared from heart was measured with a colorimetric assay kit for lipid peroxidation (BIOXYTECK MDA-586 Oxis Research, Portland, OR) according to the manufacturer’s protocol.

Activities of Mitochondrial Respiratory Chain Enzymes and ATP Synthesis—Mitochondria were isolated from heart, tibialis anterior muscle, and liver by homogenization, followed by differential centrifugation (24). Oxidative phosphorylation (OXPHOS) enzyme activity was normalized for the protein concentration. Succinate-cytochrome c reductase (Complex II + III) activity and NADH-cytochrome c reductase (Complex I + III) activity were measured as described previously (24). ATP synthesis was measured in intact heart and liver mitochondria. Incubation buffer (250 mM sucrose, 5 mM KCl, 5
Analysis of Heart/Muscle-specific Mn-SOD-deficient Mice

mm KH₂PO₄, 5 mm MgCl₂, 1 mm EDTA, and 10 mm Tris-HCl, pH 7.4) was added to test tubes followed by the addition of mitochondria (0.01 mg of protein/ml). The tubes were incubated at 25 °C for 10 min. After that, 0.5 mm ADP, 5 mm malate, and 0.5 mm glutamate as substrates were added to test tubes and incubated at 25 °C for 5 min. Then the test tubes were placed on ice and centrifuged at 10,000 × g, 4 °C, for 10 min. A 190-μl aliquot of the supernatant was added to 10 μl of luciferase and luciferin (Wako), and bioluminescence was assessed on a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). Results were expressed as nmol of ATP/mg of protein. ATP content was measured with a cell Titer-Glo® luminescent cell viability assay (Promega Corp., Madison, WI) according to the manufacturer’s protocol.

**RNA Preparation, Affymetrix Microarray, and RT-PCR—**Total RNA was extracted from hearts of three 16-week-old mice with an RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). For the microarray analysis, the synthesis of cDNA and array hybridization was conducted with Affymetrix MOE430 2.0 array (Affymetrix, Santa Clara, CA) according to the manufacturer’s protocol. The raw microarray data files are available on the World Wide Web at www.ncbi.nlm.nih.gov/geo/ (Gene Expression Omnibus accession number GSE2236).

For the RT-PCR analysis, cDNAs were synthesized using an avian myeloblastosis virus reverse transcriptase first-strand cDNA synthesis kit (Takara Bio, Shiga, Japan). The primers used in PCR were 5’-`AGG TTC ATG GAG AGA TAC GC-3’ and 5’-`GCA ATA CAC TTC CCA CAC G-3’ for Sdhα, 5’-`ACC TCG AAT GAC GTA C-3’ and 5’-`TTC TGC AAT CGC GCC TTT CCC-3’ for Sdhβ, 5’-`TCT GCA GAT CCA AGG GGA GAA CC-3’ and 5’-`GTT GAG ACT CCA GCA ACT TCT TTT CC-3’ for Ucp3, 5’-`TGG CTG GCC ATG TAC AGA GCT-3’ and 5’-`TCC TGC TCC ATC TGA GCC GCC-3’ for Abcc9 (ATP-binding cassette, subfamily C, member 9), 5’-`GCA AGG AGG CAG GCC ACC AG-3’ and 5’-`GTC TTG GCC CGG GTG TCA TA-3’ for Aeg (angiotensin-converting enzyme), 5’-`TGC AAA AAG AAG TCT CCA CAG T-3’ and 5’-`AGG TGT GTC TCC CTG AAG CAG T-3’ for peristin, 5’-`GCC CCA GGA GCA AAT AGC AG-3’ and 5’-`TGG TCT GCC ATC TTC CCG CT for Ibg1bp3 (integrin β1-binding protein 3), and 5’-`GTC GCC CGC CGT TCT AGG CAC AA-3’ and 5’-`CTC TTG ATG TCA CGC ACG ATT TC-3’ for β-actin under the following conditions: 1 cycle of 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min; followed by 1 cycle of 72 °C for 10 min.

**Rescue Experiments—**Manganese 5,10,15,20-tetrakis (4-benzoic acid) porphyrin (MnTBAP) (Calbiochem) dissolved in PBS at 1 mg/ml was injected intraperitoneally into mutant mice once a day as previously described (27). The injections, the dose of which was 10 μg/g of body weight/day, began from 8 weeks of age for 4–8 weeks. PBS was injected into the littermate mutant mice as control.

**Analysis of Physical and Cardiac Contractile Activities—**Mice were voluntarily exercised using a running wheel apparatus (KI-101; Aptec, Kyoto, Japan) as described previously (28). The rotarod task was analyzed using a rotarod apparatus (MK-630; Muromachi Kikai, Tokyo, Japan). The period for which a mouse could remain on a rotating axis (30-mm diameter; rotational speed 20 rpm) without falling was measured. The test was stopped after an arbitrary limit of 300 s, and three sessions of training were required before mice attained a stable performance. Isolated hearts from 12-week-old mice were excised quickly to establish Langendorff perfusion and perfused with modified Krebs-Henseleit solution (containing 116.0 mm NaCl, 25.0 mm NaHCO₃, 2.5 mm CaCl₂, 1.2 mm MgSO₄, 4.7 mm KCl, 1.2 mm KH₂PO₄, and 5.5 mm glucose, pH 7.4) in a retrograde direction at a constant flow rate of 2.0 ml/min without recirculation. The perfusate was warmed to 38 °C and oxygenated with a 95% O₂, 5% CO₂ gas mixture to elevate the PO₂ to over 400 mm Hg. A hook through the apex of the heart was connected to an isotometric force transducer for monitoring tension and heart rate. The initial loaded tension was adjusted to the same force in each experiment. The cardiac contractile activity was evaluated with the tension rate product (tension times heart rate). The data were normalized with heart weight.

**Statistical Analysis—**We analyzed data using Student’s unpaired t test and considered p values of <0.05 to be statistically significant. Data are expressed as the mean ± S.D.
Table 1

Physiological parameters and analysis of in vivo cardiac size and function by echocardiography

Data are expressed as mean ± S.E. IVSd, diastolic intraventricular septum thickness; LVIDd, end diastolic interval dimensions of the LV; LVPWd, diastolic LV posterior wall thickness; IVSs, systolic intraventricular septum thickness; LVIDs, LV end systolic interval dimensions; LVPWs, systolic LV posterior wall thickness; HR, heart rate; FS, fractional shortening; EF, ejection fraction.

|                | 2 months               | 4 months               |
|----------------|------------------------|------------------------|
|                | H/M-Sod2−/− (n = 3)    | Control (n = 4)        |
| IVSd (mm)      | 0.70 ± 0.06            | 0.72 ± 0.04            |
| LVIDD (mm)     | 5.28 ± 0.13a           | 3.17 ± 0.05            |
| LVPWD (mm)     | 0.65 ± 0.03            | 0.65 ± 0.02            |
| IVSs (mm)      | 0.82 ± 0.09b           | 1.30 ± 0.08            |
| LVIDs (mm)     | 4.69 ± 0.10a           | 1.69 ± 0.03            |
| LVPWs (mm)     | 0.91 ± 0.11            | 1.00 ± 0.12            |
| HR (min)       | 599.00 ± 31.15         | 583.24 ± 17.24         |
| FS (%)         | 11.23 ± 0.79         a  | 46.40 ± 1.53           |
| EF (%)         | 21.06 ± 1.51a          | 71.37 ± 1.64           |
| Body weight (g)| 16.10 ± 0.76           | 14.40 ± 0.45           |
| Heart weight (mg)| 179.00 ± 8.5b       | 76.50 ± 7.46           |
| Lung weight (mg)| 109.30 ± 3.18        | 95.50 ± 3.93           |
| Heart weight/body weight (%) | 1.10 ± 0.03a | 0.69 ± 0.02 |

|                | H/M-Sod2−/− (n = 3)    | Control (n = 6)        |
| IVSd (mm)      | 0.73 ± 0.1             | 0.74 ± 0.02            |
| LVIDD (mm)     | 7.49 ± 0.12a           | 3.55 ± 0.08            |
| LVPWD (mm)     | 0.71 ± 0.08            | 0.76 ± 0.05            |
| IVSs (mm)      | 0.85 ± 0.05b           | 1.27 ± 0.06            |
| LVIDs (mm)     | 6.86 ± 0.10a           | 2.02 ± 0.09            |
| LVPWs (mm)     | 0.91 ± 0.06b           | 1.35 ± 0.12            |
| HR (min)       | 528.14 ± 8.58          | 533.87 ± 16.52         |
| FS (%)         | 8.30 ± 0.25b           | 43.17 ± 1.39           |
| EF (%)         | 16.03 ± 0.43b          | 67.65 ± 1.60           |
| Body weight (g)| 19.00 ± 0.4            | 22.63 ± 0.50           |
| Heart weight (mg)| 285.70 ± 17.3b      | 117.33 ± 7.07          |
| Lung weight (mg)| 140.00 ± 8.5          | 135.67 ± 5.57          |
| Heart weight/body weight (%) | 1.42 ± 0.05a | 0.52 ± 0.03 |

Results

Generation of Heart/Muscle-specific Mn-SOD-deficient Mice—In order to investigate the physiological as well as pathological role of Mn-SOD in the heart and muscle, we generated conditional Mn-SOD knock-out mice using the Cre-loxP system. We used MCK-Cre transgenic mice for the selective expression of Cre protein in muscle tissues (20). As shown in Fig. 1, cross-breeding of homozygous Mn-SOD flox mice (control) with MCK-Cre transgenic mice gave rise to H/M-Sod2−/−. Genomic DNAs extracted from various tissues were analyzed by PCR for the detection of the deleted fragment from the genomic Mn-SOD gene. A 401-bp DNA fragment corresponding to the deletion allele was specifically amplified by PCR from the heart and skeletal muscle of H/M-Sod2−/− mice, whereas no fragment was amplified in other tissues of H/M-Sod2−/− mice or in any tissues of control mice (Fig. 1B). Western blot analyses further showed a specific loss of Mn-SOD expression in the heart and skeletal muscle of H/M-Sod2−/− mice (Fig. 1C), but no loss was observed in control mice. In an immunohistochemical analysis, specific immunoreactivity for Mn-SOD was detected in the endothelial cells of the heart or muscle but not in the cardiac myocytes or the striated muscle cells of the tibialis anterior (TA) from H/M-Sod2−/− mice. On the other hand, specific immunoreactivity for Mn-SOD was detected in the myocardium and in the striated muscle from the TA of control mice (Fig. 1D).

Heart/Muscle-specific Mn-SOD-deficient Mice Developed Dilated Cardiomyopathy Associated with Progressive Physical Disabilities—In the neonatal stage, we were unable to find any differences in the outer appearance or body size between H/M-Sod2−/− and control mice. However, at 8 weeks of age, the H/M-Sod2−/− mice began to exhibit growth retardation. At 16 weeks of age, the H/M-Sod2−/− mice showed a 25% reduction in body weight compared with control mice without distinct muscle atrophy (Fig. 2, A and C). Phenotypically, H/M-Sod2−/− mice developed signs of fatigue at as early as 8 weeks of age, when some of these animals began to die. By 22 weeks of age, all H/M-Sod2−/− mutant mice died, with a median survival rate of 15.4 ± 4.0 weeks (Fig. 2B). When examined macroscopically, all of the hearts of H/M-Sod2−/− mice showed cardiac enlargement at 16 weeks of age without an exception (Fig. 2A). Under normal diet, the H/M-Sod2−/− mice exhibited 51% reduction in food intake compared with control mice (Fig. 2D). In order to evaluate daily physical activities of the H/M-Sod2−/− mutant mice, we placed a running wheel apparatus in their cages. The H/M-Sod2−/− mutant mice did not exhibit any signs of ataxia but hardly ran on the apparatus, whereas control mice ran more than 10 km every day after 4 days of the exercise session (Fig. 2E). When heart weight was standardized to body weight, the hearts of H/M-Sod2−/− mice were found to be 2.1-fold heavier at 2 months of age and 2.7-fold heavier at 4 months of age than those of control mice (Fig. 2F, Table 1).

To determine whether deficiency of Mn-SOD in heart and muscle would affect the cardiac function, cardiac performance was evaluated by means of echocardiography in 2-month-old mice (Fig. 3, Table 1). Compared with control mice, cardiac contractility was significantly depressed in H/M-Sod2−/− mice as assessed by fractional shortening (FS) and ejection fraction (EF) (Fig. 3B, Table 1). The LV end-diastolic and end-systolic diameters were significantly increased in H/M-Sod2−/− mice compared with control mice (Fig. 3B, Table 1). The decreased contractility and LV dilatation were also observed at 4 months compared with control mice. However, diastolic intraventricular septum thickness (IVSd), diastolic LV posterior wall thickness (LVPWd), and systolic LV posterior wall thickness (LVPWs) were not significantly increased in H/M-Sod2−/− mice compared with control mice (Fig. 3C, Table 1).

The Myocardium Showed Specific Pathological Findings Compatable with Typical Idiopathic Dilated Cardiomyopathy—Transverse sections of hearts from the H/M-Sod2−/− mice showed a marked dilatation of both left and right ventricles, which is compatible with the end stage of dilated cardiomyopathy (Fig. 4, A and C).
FIGURE 3. Echocardiographic analysis on H/M-Sod2−/− mice. A, transthoracic M-mode echocardiographic tracings from a H/M-Sod2−/− mouse (left) and a control mouse (right). Echocardiography was performed on 2- and 4-month-old mice. B, echocardiographic parameters, such as end diastolic (LVIDd) and end systolic (LVIDs) LV diameters, diastolic LV posterior wall thickness (LVPWd), systolic LV posterior wall thickness (LVPWs), ejection fraction (EF), and fractional shortening (FS). Closed and open bars represent H/M-Sod2−/− (n = 3) and control mice (n = 6), respectively. *, p < 0.05 versus control.
Heart/Muscle-Sod2\(^{-/-}\) Control

**FIGURE 4. Histopathology of myocardium.** A–D, transverse sections of hearts from a 15-week-old H/M-Sod2\(^{-/-}\) mouse (A and C) and a littermate control mouse (B and D) through the midportion with hematoxylin and eosin staining. Lower power views revealed distinct dilated cardiomyopathy (A and B, scale bar = 1 mm). Higher power views showed myocardial degeneration and malformation with cytoplasmic vacuolization as well as irregular myofilaments and pleomorphic nuclei (C and D, scale bar = 10 \(\mu\)m). E and F, Azan staining revealed diffuse myocardial fibrosis (blue) in H/M-Sod2\(^{-/-}\) (E) but not in control hearts at 15 weeks of age (F, scale bar = 10 \(\mu\)m). Nuclei, acidophilic cytoplasm, and so on were stained red. G and H, PAS staining showed interstitial storage of excess glycogen (purple) in the hearts of H/M-Sod2\(^{-/-}\) mice (G) but not in control hearts at 15 weeks of age (H, scale bar = 10 \(\mu\)m).

B). High power photomicrographs of the LV wall showed myocardial degeneration, myocyte disarray, vacuolization, and bizarre myocardial cells with irregular myofilaments and pleomorphic nuclei (Fig. 4C). In the histological sections with Azan staining, diffuse fibrotic scars surrounded myocardial cells. Some of the thickened fibrotic foci were due to necrotic changes of the myocardium, whereas the majority of the thin layer of interstitial thickened fibrotic foci were due to necrotic changes of the myocytes. Some of the affected areas were stained pink in the hearts of H/M-Sod2\(^{-/-}\) mice but not in control hearts (Fig. 4G and H).

Electron micrographs of the LV wall of H/M-Sod2\(^{-/-}\) mutant mice showed small mitochondria associated with scattered abnormal vacuoles (Fig. 5A). However, we were unable to find any ultrastructural changes in sarcomeric structures between H/M-Sod2\(^{-/-}\) mice and control mice. The cristae of mutant mitochondria were rough, irregular, abnormally

**FIGURE 5. Transmission electron micrographs of myocardium from H/M-Sod2\(^{-/-}\) mice.** A and B, small mitochondria and abnormal vacuoles were observed in 15-week-old H/M-Sod2\(^{-/-}\) hearts (A) but not in control hearts (B, scale bar = 2 \(\mu\)m). C and D, the cristae of mutant mitochondria (C) were rough, irregular, and concentrated in the center, whereas control cristae (D) formed regularly (scale bar = 200 nm).

**FIGURE 6. Apoptosis was not induced in H/M-Sod2\(^{-/-}\) mice.** A and B, nuclear morphology of H/M-Sod2\(^{-/-}\) and control myocardium by transmission electron microscopy (scale bar = 2 \(\mu\)m). C and D, TUNEL staining in myocardium of 15-week-old H/M-Sod2\(^{-/-}\) (C) and control (D) mice. Views of fluorescence staining using anti-digoxigenin conjugate (green) were merged with those of 4,6-diamidino-2-phenylindole staining (blue). E, Western blot analysis of protein extracted from heart (H), skeletal muscle (Sm), and liver (L) of 15-week-old H/M-Sod2\(^{-/-}\) mice using antibodies against activated caspase-3, glyceraldehyde-3-phosphate dehydrogenase, and Mn-SOD. We used NIH-3T3 cells treated with staurosporine as apoptotic positive control (P). F, ATP content in heart (n = 9), TA muscle (n = 6), and liver (n = 6) of 15-week-old H/M-Sod2\(^{-/-}\) mice (*, p < 0.001).
Analysis of Heart/Muscle-specific Mn-SOD-deficient Mice

**Figure 7.** Impaired mitochondrial respiratory activities. A, enzymatic histochemical staining for SDH (top) and COX activities (bottom) in hearts from 15-week-old mice of the indicated genotypes (scale bar = 30 μm). B and C, OXPHOS enzyme activities of heart, TA muscle, and liver mitochondria in H/M-Sod2<sup>−/−</sup> (n = 3) or control mice (n = 3) at 15 weeks of age. Activities of OXPHOS Complex I + III (B) and Complex II + III (C) are shown (*, p < 0.05; **, p < 0.005; ***, p < 0.0005). D, production of ATP in heart and liver mitochondria. ATP production in heart mitochondria from H/M-Sod2<sup>−/−</sup> (n = 5) was lower than that from control mice (n = 4; *, p < 0.05). In liver mitochondria, ATP production was not significantly different between H/M-Sod2<sup>−/−</sup> (n = 3) and control mice (n = 3). Results are represented by mean ± S.E. E, Western blot analysis of proteins extracted from heart of 15-week-old H/M-Sod2<sup>−/−</sup> using antibodies against OXPHOS subunits and actin. F, transcriptional alterations of Sdha, Sdhb, Ucp3, Abcc9, Ace, periostin, Itgb1bp3, and actin in the hearts (n = 3).

wound, and concentrated in the central zone of the matrix (Fig. 5, C and D). Similar but modest ultrastructural findings were observed in the skeletal muscle of the tibialis anterior (data not shown). However, we found no abnormal crystals or droplets in mitochondria as shown in the case of human dilated cardiomyopathy (29).

The Heart/Muscle-specific Mn-SOD-deficient Mice Failed to Show Apoptotic Cell Death in Myocardium and Skeletal Muscle—To determine the manner of cell death in myocardium and skeletal muscle, we investigated the nuclear morphology by electron microscopy, TUNEL staining, expression of cleaved caspase-3, and ATP content in 15-week-old H/M-Sod2<sup>−/−</sup> mice and control mice. In the study of electron microscopy as far as we investigated, we hardly detected the apoptotic nuclei with DNA fragmentation in myocardium of H/M-Sod2<sup>−/−</sup> mice as well as control mice (Fig. 6, A and B). Furthermore, no difference was observed in the number of TUNEL-positive apoptotic cells between H/M-Sod2<sup>−/−</sup> mice and control mice (Fig. 6, C and D). We also failed to detect the activated form of caspase-3 in myocardium and skeletal muscle of H/M-Sod2<sup>−/−</sup> mice and control mice (Fig. 6E). Moreover, we examined ATP content in heart, skeletal muscle, and liver, because ATP is required to induce the activation of caspase-3. ATP contents in heart and skeletal muscle of H/M-Sod2<sup>−/−</sup> mice both decreased to about 30% of those of control mice (Fig. 6F), whereas we failed to detect any difference in livers between H/M-Sod2<sup>−/−</sup> mice and control mice (Fig. 6F).

The Heart/Muscle-specific Mn-SOD-deficient Mice Showed Suppressed OXPHOS in Myocardium—To understand the biochemical alterations in pathogenesis of cardiomyopathy, we examined the mitochondrial respiratory functions in the heart of H/M-Sod2<sup>−/−</sup> mice. Using an enzymatic histochemical analysis for SDH, we assessed biochemical activity of Complex II in cardiac muscle and skeletal muscle of 15-week-old H/M-Sod2<sup>−/−</sup> mice and compared them with those of control mice. In the cardiac muscle of H/M-Sod2<sup>−/−</sup> mice, the activity of Complex II was hardly detected except in endothelial cells of intramuscular vessel walls (Fig. 7A). Likewise, we detected no enzymatic activity of Complex II in the skeletal muscle of H/M-Sod2<sup>−/−</sup> mice (data not shown), whereas a strong enzymatic staining of SDH (Complex II) activity was detected in the cardiac and skeletal muscles of control mice. We also assessed the activity of Complex IV, COX, in the cardiac muscle (Fig. 7A) and skeletal muscle (data not shown) of H/M-Sod2<sup>−/−</sup> mice. The enzymatic activity of Complex IV in H/M-Sod2<sup>−/−</sup> mice showed a strong staining that was comparable with control mice. The data clearly demonstrated a selective loss of enzymatic activity of Complex II but not of Complex IV in the cardiac and skeletal muscles of H/M-Sod2<sup>−/−</sup> mice.

In order to biochemically confirm the suppression of OXPHOS, we isolated mitochondria from heart, muscle, and liver of H/M-Sod2<sup>−/−</sup> mice as well as control mice. We then assessed NADH-cytochrome c reductase (Complex I + III) activity and succinate-cytochrome c reductase (Complex II + III) activity of H/M-Sod2<sup>−/−</sup> mice. NADH-cytochrome c reductase activity in the heart and skeletal muscle was signifi-
Analysis of Heart/Muscle-specific Mn-SOD-deficient Mice

The up- and down-regulated genes of 16-week-old H/M-Sod2−/− mice are listed. The listed genes showed at least 4-fold changes in the transcriptional level compared with control mice. The genes are categorized by biological functions.

### Up-regulated genes

| Fold change | Description |
|-------------|-------------|
| 52.51       | Igfbp3, integrin β1-binding protein 3 |
| 14.84       | Wnt2, WNT1-inducible signaling pathway protein 2 |
| 6.18        | Emp1, epithelial membrane protein 1 |
| 4.99        | Rp91, retinol-binding protein 1, cellular |
| 4.84        | Col5a2, procollagen, type V, α2 |
| 4.46        | Apod, apolipoprotein D |
| 4.31        | Frzb, frizzled-related protein |
| 4.22        | Krt1-18, keratin complex 1, acidic, gene 18 |

### Cell adhesion

| Description |
|-------------|
| 20.60       | Comp, cartilage oligomeric matrix protein |
| 15.16       | Tlshd3, thrombospondin 4 |
| 13.05       | Lgals3, lectin, galactoside binding, soluble 3 |
| 11.45       | Integrin, β-like 1 |
| 9.44        | Col1a1, procollagen, type VIII, α1 |
| 7.33        | Peristin |
| 5.62        | Col1a2, procollagen, type I, α2 |

### Organogenesis

| Description |
|-------------|
| 6.14        | Ctgf, connective tissue growth factor |
| 4.56        | Rltn, reelin |
| 4.31        | Ttfrs12a, tumor necrosis factor receptor superfamily, member 12a |
| 4.22        | Dbn1, drebrin 1 |

### Biosynthesis

| Description |
|-------------|
| 5.70        | Moxo1, mesenchyme homeobox 1 |
| 4.45        | Adcy7, adenylate cyclase 7 |
| 4.36        | Dlap1, dimethylarginine dimethylaminohydrolase 1 |
| 4.20        | Ich1l1, ubiquitin carboxyl-terminal hydrolase L1 |
| 4.11        | Asna, asparagine synthetase |

### Down-regulated genes

| Fold change | Description |
|-------------|-------------|
| 0.08        | Ucp3, uncoupling protein 3, mitochondrion |
| 0.20        | Efas3, ephrin B3 |
| 0.24        | Pis, Fbl osteosarcoma oncogene |
| 0.09        | Pah, phenylalanine hydroxylase |
| 0.18        | Adr, adipin |
| 0.16        | Fbp2, fructose bisphosphatase 2 |

Results indicated that H/M-Sod2−/− mice showed an enhanced posttranscriptional alteration of the subunits, we investigated the gene expression by RT-PCR. As shown in Fig. 7F, we detected comparable amounts of transcripts for sdha and sdhb in both H/M-Sod2−/− and control mice. Thus, these results suggested that OXPHOS suppression was caused by selective posttranscriptional modifications of specific enzymes in the mitochondrial respiratory chain.

Identification of Up- and Down-regulated Genes in Mn-SOD-deficient Heart—In order to investigate the transcriptional alterations in the hearts with dilatation of developed H/M-Sod2−/− mice (15 weeks of age), we carried out an Affymetrix microarray analysis. We found 419 genes up-regulated and 118 genes down-regulated with transcriptional alterations of more than 2-fold compared with the control. The genes with more than 4-fold alternations are presented in biological categories (Table 2). The transcriptional up-regulation of genes for cell growth, cell maintenance, cell adhesion, and organogenesis suggested active remodeling processes in fibrotic myocardial degeneration, whereas the transcriptional down-regulation of Ucp3 (uncoupling protein 3) and Fbp2 (fructose bisphosphatase 2) was not observed. However, transcripts for other members of Igfbp1 and Igfbp2 (melusin), were not changed. Melusin was associated with mechanical stress signaling in the heart (31). This suggests that Igfbp1 is a specific biomarker for cardiac oxidative stress (Table 2). We failed to detect the transcriptional down-regulation of antioxidant enzymes, such as Cu/Zn-SOD, extracellular SOD, and catalase, suggesting that the Mn-SOD system was not compensated by another antioxidant system (data not shown). Next, we investigated the transcriptional alterations of candidate genes that had been reported in heart failure and/or dilated cardiomyopathy. Of more than 30 candidates, six genes were up-regulated, whereas only one gene, Abcc9, was significantly down-regulated 0.48-fold. The up-regulated transcripts included Ace, Acta1 (skeletal muscle actin α1), Gbe1 (glucan (1,4-α)-branching enzyme 1), Lmna (lamin A), Nppa (natriuretic peptide precursor type A), and Nppb (natriuretic peptide precursor type B) in Mn-SOD-deficient heart (supplemental Table 1). Transcriptional alterations were also confirmed by RT-PCR (Fig. 7F).

Heart/Muscle Sod2−/− Mice Showed Enhanced ROS Generation with Increased Lipid Peroxidation in Mitochondria—Because H/M-Sod2−/− mice showed suppressed OXPHOS in the myocardium, we measured the formation of ROS, such as O₂⁻ and H₂O₂. As shown in Fig. 8A, when we used 5 mM succinate as a substrate, O₂⁻ formation in heart mitochondria from H/M-Sod2−/− mice increased to 161% of that from control mice (Fig. 7D). In the liver, we failed to detect any difference in ATP production between H/M-Sod2−/− mice and control mice (Fig. 7D). As shown in Fig. 7E, immunoblot analyses on the expression of the SDHA and SDHB subunit from Complex II revealed significant reductions in the heart of H/M-Sod2−/− mice, whereas we detected moderate suppression of Complex I α9, Rieske iron-sulfur protein (FeS) and Core I subunit of Complex III, and α and β subunits of Complex V and detected no suppression of COX I, a component of Complex IV (Fig. 7E). In order to clarify whether the suppression of the subunits is due to the transcriptional down-regulation or the...
Analysis of Heart/Muscle-specific Mn-SOD-deficient Mice

**O$_2^*$ generation in mitochondria of both heart and skeletal muscles.** Interestingly, H$_2$O$_2$ formation was down-regulated in skeletal muscles but not down-regulated in heart muscles of H/M-Sod2$^{-/-}$ mice.

In order to evaluate oxidative damage of H/M-Sod2$^{-/-}$ mice, we measured the amount of malondialdehyde (MDA). We detected higher levels of MDA in heart mitochondria from H/M-Sod2$^{-/-}$ mice than in those from control mice (Fig. 8D). In the cytoplasmic fraction of heart, however, we failed to detect any difference in the amount of MDA between H/M-Sod2$^{-/-}$ mice and control mice. These results indicated that oxidative damages were specifically localized in mitochondria of H/M-Sod2$^{-/-}$ mice.

Antioxidants Partially Ameliorated Symptoms of Heart/Muscle-specific Mn-SOD-deficient Mice—In order to ameliorate the symptoms of H/M-Sod2$^{-/-}$ mice, we intraperitoneally administered MnTBAP to mutant mice. We then assessed the heart weights and the cardiac function of 12-week-old H/M-Sod2$^{-/-}$ mice. Although we failed to detect a significant pharmacological effect on the standardized heart weights of MnTBAP-treated H/M-Sod2$^{-/-}$ mice (Fig. 9A), we found a significant improvement in the cardiac function (Fig. 9B). The result suggested that MnTBAP reversed the weakened function of the failing heart, which was caused by the loss of Mn-SOD activity.

In the assessment of physical activities, we injected MnTBAP into 8-week-old H/M-Sod2$^{-/-}$ mice for 5 weeks. We then compared the daily running distances of MnTBAP-treated mice with those of PBS-treated control mice. MnTBAP significantly improved the physical activity of H/M-Sod2$^{-/-}$ mice, whereas PBS failed to improve the physical activity (Fig. 9C). Likewise, rotarod tasks with 14-week-old mutant mice showed a significant recovery on the administration of MnTBAP (Fig. 9D). These results suggested that the administration of MnTBAP significantly rescued the impaired cardiac contractility as well as the physical disabilities of the H/M-Sod2$^{-/-}$ mice.

**DISCUSSION**

Total Mn-SOD KO mice have been independently reported by two laboratories (16, 17). These authors showed that Mn-SOD-deficient mice die with complex pathologies, including growth immaturity, dilated cardiomyopathy, metabolic abnormalities, such as ketosis and lactic acidosis, steatosis or fatty change of the liver, and central nervous system damage within 3 weeks after birth (16, 17). In the present study, we established mice carrying a specific deletion of the Mn-SOD gene in heart and muscle tissues to define the phenotypes of heart/muscle-specific Mn-SOD-deficient mice. These results indicated that MnTBAP reversed the impaired cardiac contractility as well as the physical disabilities of the H/M-Sod2$^{-/-}$ mice.
Sod1<sup>−/−</sup> mice should provide important information to clarify the differences of H<sub>2</sub>O<sub>2</sub> production from mitochondria between heart and skeletal muscles.

Experimental evidence suggests that ROS can mediate apoptosis by a variety of mechanisms (35). However, an increased level of ATP is a requisite to the process of apoptotic cell death (36). Leist et al. (37) have reported that depletion of cellular ATP could switch the type of cell death from apoptosis to necrotic cell death. Apoptosis is characterized by activation of caspase-3 and internucleosomal DNA fragmentations. Thus, the level of ATP is critical to the activation of caspase-3 (36). In the present study, dysfunction of mitochondrial respiratory chain led to reduction of ATP production (Fig. 7D) as well as ATP content (Fig. 6F), which could account for the absence of apoptosis in H/M-Sod2<sup>−/−</sup> mice. Spontaneous apoptosis was reported to be observed in heterozygous Mn-SOD-deficient mice (38). We speculate that the apoptosis occurred because there was sufficient ATP available to induce it.

In the heart mitochondria, H/M-Sod2<sup>−/−</sup> mice showed significantly reduced ATP production (Fig. 7D). Two possibilities for the reduction of ATP production were suggested. Brand et al. (39) reported that excess O<sub>2</sub><sup>−</sup> generation in mitochondria uncoupled the respiration by activation of UCPs, which resulted in decreased ATP production (39). In microarray analysis, however, we failed to show up-regulation of Ucp1 and Ucp2 genes in the hearts of H/M-Sod2<sup>−/−</sup> mice (data not shown). Inversely, the Ucp3 gene was markedly down-regulated 0.08-fold in hearts of H/M-Sod2<sup>−/−</sup> mice compared with control mice (Table 2). Another possibility is global reduction in mitochondrial respiration. In the present study, we showed marked reduction of Complex II (SDH) subunits and moderate suppression of subunits in Complexes I, III, and V (Fig. 7E). Therefore, it is suggested that reduced ATP production in mutant mitochondria is due to a decrease in mitochondrial respiration associated with suppression of OXPHOS proteins in H/M-Sod2<sup>−/−</sup> mice.

In the hearts of H/M-Sod2<sup>−/−</sup> mice, Complex II (SDH) was selectively inactivated among mitochondrial respiratory enzymes. As presented under “Results,” protein expression of SDHA and SDHB decreased significantly without down-regulation of mRNA (Fig. 7E and F). The result suggested the pathological role of oxidative stress in the modification of the enzymes, which might induce the degradation of SDH protein. It implies that Complex II is specifically vulnerable to O<sub>2</sub><sup>−</sup> among mitochondrial respiratory chain complexes.
Analysis of Heart/Muscle-specific Mn-SOD-deficient Mice

It has been reported that oxidative stress activates redox-sensitive transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP1). NF-κB is also known to be a key molecule of myocardial ischemia-reperfusion injury that generated elevated levels of free radicals (41). In this context, we investigated the expression of NF-κB and its signaling molecules, such as inducible NO synthase, heme oxygenase-1, and interleukin-1β. In microarray analysis, we could not detect any up- or down-regulation of these transcripts between H/M-Sod2−/− and control mice (data not shown). Based on the transcriptional analysis, we speculated that NF-κB is hardly attributable to the development and progression of dilated cardiomyopathy in H/M-Sod2−/− mice.

Should free radicals play a pivotal role in the pathogenesis of dilated cardiomyopathy, dietary supplementation of anti-oxidants would confer a nutritional benefit. Broqvist et al. (42), however, reported that such clinical trials failed to show a significant benefit for dilated cardiomyopathy. In the present study, we investigated whether a diet high in glucose improved the physical activities of the mutant mice. The result showed that a high glucose diet did not improve physical activity as assessed using the running wheel apparatus (data not shown). Since some Mn-SOD mimetics, such as MnTBAP (43), EUK-8, and EUK-134 (44, 45), showed antioxidant effects in model organisms, such as C. elegans and mice, we administered MnTBAP to the H/M-Sod2−/− mice. The results revealed that MnTBAP ameliorates the phenotypic symptoms, physical disabilities, and cardiac contractility, suggesting that free radicals play an important role in the progression of dilated cardiomyopathy. Furthermore, antioxidant regimens can prevent the further progression of heart failure. Since MnTBAP only partially rescued the phenotypes, oxidative stress would have caused irreversible damage that could not be rescued using antioxidants. This suggests that patients with genetic susceptibility to heart failure might benefit from treatment with antioxidants in the early presymptomatic stage. In conclusion, we presented a murine model of heart/muscle-specific oxidative stress and devised a strategy for preventive medicine with a therapeutic treatment for age-related or oxidative stress-dependent heart diseases. Furthermore, we demonstrated the efficacy and limitations of antioxidant SOD mimetics. Future studies with our model mice should provide better ways to develop novel medicine or new dietary supplements for the improvement of physical disabilities as well as the quality of senescent life.

Acknowledgments—We thank Drs. E. Morizumi, M. Takahashi, M. Ogawara, S. Uchiyama, D. Nakai, T. Ikegami, T. Baba, F. Huang, H. Kuwahara, and H. Sakuramoto (Tokyo Metropolitan Institute of Gerontology) for technical support. We also thank W. Zhou (Tokyo Metropolitan Institute of Gerontology) for manuscript preparation. We are also grateful to Drs. T. Okada, M. Watanabe, and C. Z. Li (Dept. of Physiology, Juntendo University School of Medicine) for support and valuable suggestions regarding measurement of cardiac contractile activity.

REFERENCES

1. Liew, C. C., and Dzau, V. J. (2004) Nat. Rev. Genet. 5, 811–825
2. Alameddine, F. M., and Zafari, A. M. (2002) Congest. Heart Fail. 8, 157–164, 172
3. Givertz, M. M., and Colucci, W. S. (1998) Lancet 352, Suppl. 1, SI34–SI38
4. Mallat, Z., Philip, I., Lebret, M., Chatel, D., Maclouf, J., and Tedgui, A. (1998) Circulation 97, 1536–1539
5. Diaz-Velez, C. R., Garcia-Castineiras, S., Mendoza-Ramos, E., and Hernandez-Lopez, E. (1996) Am. Heart J. 131, 146–152
6. Keith, M., Gerammaryan, A., Sole, M. J., Kurian, R., Robinson, A., Omran, A. S., and Jeejeebhoy, K. N. (1998) Am. Coll. Cardiol. 31, 1352–1356
7. Dietrich, S., Bieniek, U., Beulich, K., Hasenfuss, G., and Prestle, J. (2000) Circulation 101, 33–39
8. Khaper, N., Kaur, K., Li, T., Farahmand, F., and Singh, P. K. (2003) Mol. Cell. Biochem. 251, 9–15
9. Ide, T., Tsutsui, H., Hayashidani, S., Kang, D., Suematsu, N., Naka- muru, K., Uchiyama, S., Harada, N., Hamasaki, N., and Takeshita, A. (2001) Circ. Res. 88, 529–535
10. Suzuki, S., Kaneko, M., Chapman, D. C., and Dhalla, N. S. (1991) Biochim. Biophys. Acta 1074, 95–100
11. Jahr, R. I., and Cole, W. C. (1995) Circ. Res. 76, 812–824
12. Wallace, D. C. (2002) Methods Mol. Biol. 197, 3–54
13. Melov, S., Coskun, P., Patel, M., Tuinastra, R., Cottrell, B., Jun, A. S., Zastawny, T. H., Dizdaroglu, M., Goodman, S. L., Huang, T. T., Mziorko, H., Epstein, C. J., and Wallace, D. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 846–851
14. Hiroi, S., Harada, N., Nishi, H., Satoh, M., Nagai, R., and Kimura, A. (1999) Biochim. Biophys. Res. Commun. 261, 332–339
15. Valenti, L., Conte, D., Piperno, A., Dongiovanni, P., Cracanzani, A. L., Fraqielli, M., Vergani, A., Gianni, C., Carmagnola, L., and Fargion, S. (2004) J. Med. Genet. 41, 946–950
16. Li, Y., Huang, T. T., Carlson, E. J., Melov, S., Ursell, P. C., Olson, J. L., Noble, L. J., Yoshimura, M. P., Berger, C., and Chan, P. H. (1995) Nat. Genet. 11, 376–381
17. Lebovitz, R. M., Zhang, H., Vogel, H., Cartwright, J. J., Dionne, L., Lu, N., Huang, S., and Matzuk, M. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9782–9787
18. Tang, W. H., and Francis, G. S. (2003) Expert Opin. Investig. Drugs 12, 1791–1801
19. Ikegami, T., Suzuki, Y., Shimizu, T., Isokao, K., Koike, H., and Shirasawa, T. (2002) Biochem. Biophys. Res. Commun. 296, 729–736
20. Bruning, J. C., Michael, M. D., Winny, I. N., Hayashi, T., Horsch, D., Accili, D., Gooyear, L. J., and Kahn, C. R. (1998) Mol Cell 2, 559–569
21. Kong, B. W., Kim, H., and Foster, D. N. (2003) Biochim. Biophys. Acta 1625, 98–108
22. Shimizu, T., Ikegami, T., Ogawara, M., Suzuki, Y., Takahashi, M., Morio, H., and Shirasawa, T. (2002) J. Neurosci. Res. 69, 341–352
23. Yanaguchi, O., Higuchi, Y., Hirotani, S., Kashiwase, K., Nakayama, H., Hikoso, S., Takeda, T., Watanabe, T., Asahi, M., Taniki, M., Matsumura, Y., Tsujiom, I., Hongo, K., Kusakari, Y., Kurihara, S., Nishida, K., Ichijo, H., Hori, M., and Otsu, K. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 15883–15888
24. Nakai, D., Shimizu, T., Nosiri, H., Uchiyama, S., Koike, H., Takahashi, M., Hirokawa, K., and Shirasawa, T. (2004) Aging Cell 3, 273–281
25. Barja, G. (2002) J. Bioenerg. Biomembr. 34, 227–233
26. Morten, K. J., Ackrell, B. A., and Melov, S. (2006) J. Biol. Chem. 281, 3534–3539
27. Scortegagna, M., Ding, K., Oktay, Y., Gaur, A., Thurmond, F., Yan, L. J., Mark, B. T., Matsumoto, A. M., Shetton, J. M., Richardson, J. A., Bennett, M. J., and Garcia, J. A. (2003) Nat. Genet. 35, 331–340
28. Shirasawa, T., Izumizaki, M., Suzuki, Y., Ishihara, A., Shimizu, T., Tamaki, M., Huang, F., Koizumi, K., Iwase, M., Sakai, H., Tsuchida, E., Ueshima, K., Inoue, H., Koike, H., Senda, T., Kuriyama, T., and Homma, I. (2003) J. Biol. Chem. 278, 5035–5043
29. Arbustini, E., Diegoli, M., Farin, R., Grasso, M., Morbini, P., Banchieri, N., Bellini, O., Dal Bello, B., Pitloot, A., Magrini, G., Campagna, C., Fortina, P., Gavazzi, A., Narula, J., and Viganò, M. (1998) Am. J. Pathol. 153, 1501–1510
30. Li, J., Mayne, R., and Wu, C. (1999) J. Cell Biol. 147, 1391–1398
31. Brancaccio, M., Fratta, L., Notte, A., Hirsch, E., Poulet, R., Guazzzone, S., De Acetis, M., Vecchione, C., Marino, G., Altruda, F., Silengo, L., Tarone, G., and Lembo, G. (2003) Nat. Med. 9, 68–75
32. Brown, J. M., Terada, L. S., Grosso, M. A., Whitmann, G. J., Velasco, S. E., Patt, A., Harken, A. H., and Repine, J. E. (1988) J. Clin. Invest. 81, 1297–1301
33. Ide, T., Tsutsui, H., Kinugawa, S., Suematsu, N., Hayashidani, S., Ichikawa, K., Utsumi, H., Machida, Y., Egashira, K., and Takeshita, A. (2000) Circ. Res. 86, 152–157
34. Okado-Matsumoto, A., and Fridovich, I. (2001) J. Biol. Chem. 276, 38388–38393
35. Giordano, F. J. (2005) J. Clin. Invest. 115, 500–508
36. Zamarraeva, M. V., Sabirov, R. Z., Maeno, E., Ando-Akatsuka, Y., Bessonova, S. V., and Okada, Y. (2005) Cell Death Differ. 12, 1390–1397
37. Leist, M., Single, B., Castoldi, A. F., Kuhnle, S., and Nicotera, P. (1997) J. Exp. Med. 185, 1481–1486
38. Strassburger, M., Bloch, W., Sulyok, S., Schuller, J., Keist, A. F., Schmidt, A., Wenk, J., Peters, T., Wlaschek, M., Lenart, J., Krieg, T., Hafner, M., Kumin, A., Werner, S., Muller, W., and Scharffetter-Kochanek, K. (2005) Free Radic. Biol. Med. 38, 1458–1470
39. Brand, M. D., Affourtit, C., Esteves, T. C., Green, K., Lambert, A. J., Miwa, S., Pakay, J. L., and Parker, N. (2004) Free Radic. Biol. Med. 37, 755–767
40. Bowie, A., and O’Neill, L. A. (2000) Biochem. Pharmacol. 59, 13–23
41. Bolli, R., and Marban, E. (1999) Physiol. Rev. 79, 609–634
42. Broqvist, M., Arnbjörnsson, H., Dahlstrom, U., Larsson, J., Nylander, E., and Permentier, J. (1994) Eur. Heart J. 15, 1641–1650
43. Melov, S., Schneider, J. A., Day, B. J., Hinerfeld, D., Coskun, P., Mirra, S. S., Crapo, J. D., and Wallace, D. C. (1998) Nat. Genet. 18, 159–163
44. Melov, S., Ravenscroft, J., Malik, S., Gill, M. S., Walker, D. W., Clayton, P. E., Wallace, D. C., Malfroy, B., Doctrow, S. R., and Lithgow, G. J. (2000) Science 289, 1567–1569
45. Melov, S., Doctrow, S. R., Schneider, J. A., Haberson, J., Patel, M., Coskun, P. E., Huffman, K., Wallace, D. C., and Malfroy, B. (2001) J. Neurosci. 21, 8348–8353