Mitochondrial Electron Transport Complex I Is a Potential Source of Oxygen Free Radicals in the Failing Myocardium

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Abstract—Oxidative stress in the myocardium may play an important role in the pathogenesis of congestive heart failure (HF). However, the cellular sources and mechanisms for the enhanced generation of reactive oxygen species (ROS) in the failing myocardium remain unknown. The amount of thiobarbituric acid reactive substances increased in the canine HF hearts subjected to rapid ventricular pacing for 4 weeks, and immunohistochemical staining of 4-hydroxy-2-nonenal ROS-induced lipid peroxides was detected in cardiac myocytes but not in interstitial cells of HF animals. The generation of superoxide anion was directly assessed in the submitochondrial fractions by use of electron spin resonance spectroscopy with spin trapping agent, 5,5′-dimethyl-1-pyrroline-N-oxide, in the presence of NADH and succinate as a substrate for NADH–ubiquinone oxidoreductase (complex I) and succinate–ubiquinone oxidoreductase (complex II), respectively. Superoxide production was increased 2.8-fold (P<0.01) in HF, which was due to the functional block of electron transport at complex I. The enzymatic activity of complex I decreased in HF (274±13 versus 136±9 nmol · min⁻¹ · mg⁻¹ protein, P<0.01), which may thus have caused the functional uncoupling of the respiratory chain and the deleterious ROS production in HF mitochondria. The present study provided direct evidence for the involvement of ROS in the mitochondrial origin of HF myocytes, which might be responsible for both contractile dysfunction and structural damage to the myocardium. (Circ Res. 1999;85:357-363.)

Key Words: antioxidant ■ free radical ■ heart failure ■ myocardial contraction ■ reactive oxygen species

Congestive heart failure (HF) is an important cause of morbidity and mortality in patients with various heart diseases. Despite extensive studies, the fundamental mechanisms responsible for the development and progression of left ventricular (LV) failure have not yet been fully elucidated. Reactive oxygen species (ROS) such as superoxide anions (•O₂⁻) and hydroxyl radicals (•OH) cause the oxidation of membrane phospholipids, proteins, and DNAs, and they have been implicated in a wide range of pathological conditions including ischemia-reperfusion injury, neurodegenerative diseases, and aging. Under physiological conditions, their toxic effects are prevented by such scavenging enzymes as superoxide dismutase (SOD), glutathione peroxidase, and catalase as well as by other nonenzymatic antioxidants. However, when the production of ROS becomes excessive, oxidative stress might have a harmful effect on the functional integrity of biological tissue. Oxygen free radicals have been shown to cause contractile failure and structural damage in the myocardium.1,2 However, their significance has been demonstrated to limited subsets of cardiac diseases, which include ischemic heart disease3 and adriamycin-induced cardiac toxicity.4

Recent investigations have suggested the generation of ROS to increase in chronic HF. Lipid peroxides and 8-isoprostaglandin F₂α, which are the major biochemical consequences of ROS generation, have been shown to be elevated in plasma and pericardial fluid of patients with HF and also positively correlated to the severity of HF.5–7 In addition, a decrease of myocardial antioxidant reserve has been shown in animal models of HF.8–10 However, these experimental and clinical findings have provided only indirect evidence of ROS generation in failing myocardial tissue. Direct measurements of ROS using electron spin resonance (ESR) spectroscopy11 are therefore needed for direct quantitation within biological tissues.

The cellular sources and mechanisms for the increased ROS in HF also remain to be elucidated. Within the heart, possible cellular sources of ROS generation include cardiac myocytes, endothelial cells, and neutrophils. The contribution of neutrophils to ROS generation was found to be minor in an animal model of HF owing to rapid ventricular pacing, because the infiltration of inflammatory cells within the myocardium is not prominent. Within cardiac myocytes,
oxygen free radicals can be produced by several mechanisms including the mitochondrial electron transport and xanthine dehydrogenase/xanthine oxidase.3 Because of the very low xanthine oxidase activity in some species12 and an extreme abundance of mitochondria in cardiac myocytes, mitochondrial electron transport could thus be a major subcellular source of ROS in the failing myocardium.13,14 Therefore, the ROS production was directly monitored in the mitochondria isolated from canine failing hearts induced by rapid ventricular pacing by means of ESR spectroscopy in the presence of a spin trap.11 The magnitude and mechanisms of ROS generation in the failing hearts were also compared with the findings in similar preparations from normal control hearts.

**Materials and Methods**

**Animal Model of HF**

HF was produced in adult mongrel dogs (15 to 25 kg body weight) by rapid ventricular pacing at 240 bpm for 4 weeks, in accordance with methods described previously.15 All operative procedures were carried out under full surgical anesthesia with sodium pentobarbital (25 mg/kg iv). Two-dimensional and M-mode echocardiograms were recorded by ultrasonography. LV short-axis (cross-sectional) views were recorded at the papillary muscle level, and the internal LV dimensions were measured. The LV ejection fraction (%) was calculated using the formula:

\[
\frac{\text{LV End-Systolic Dimension}^3 - \text{LV End-Diastolic Dimension}^3}{\text{LV End-Diastolic Dimension}^3} \times 100
\]

End-Systolic Dimension / (LV End-Diastolic Dimension)^3

After making echocardiographic recordings, the animals were generally anesthetized, intubated, and ventilated with a respirator on a heating pad to maintain body temperature at 37°C. A catheter was inserted into the aortic arch via the left carotid artery to measure the systemic arterial pressure. After a thoracotomy was performed, an externally calibrated 7F catheter-tipped pressure transducer was inserted into the LV through the left atrium for the measurement of LV pressure. After a thoracotomy was performed, an externally calibrated 7F catheter-tipped pressure transducer was inserted into the LV through the left atrium for the measurement of LV pressure. At the time of the terminal study, the animals were killed with a lethal dose of nitrogen for the subsequent ESR measurements.

All procedures and animal care were approved by the Committee on Ethics of Animal Experiments, Faculty of Medicine, Kyushu University, and were conducted according to the Guidelines for Animal Experiments of Faculty of Medicine, Kyushu University.

**Measurement of Thiobarbituric Acid Reactive Substances (TBARS)**

Lipid peroxidation is a major biochemical consequence of ROS attack on biological tissue. We therefore determined the degree of lipid peroxidation in the myocardial tissue through biochemical assay of TBARS.16 LV myocardial tissue was homogenized (10% wt/vol) in 1.15% KCl solution (pH 7.4). The homogenate was mixed with 0.4% sodium dodecyl sulfate, 7.5% acetic acid adjusted to pH 3.5 with NaOH, and 0.3% thiobarbituric acid. Butylated hydroxytoluene (0.01%) was added to the assay mixture to prevent autoxidation of the sample. The mixture was kept at 5°C for 60 minutes and was heated at 100°C for 60 minutes. After cooling, the mixture was extracted with distilled water and n-butanol/pyridine (15:1, vol/vol) and centrifuged at 16000g for 10 minutes. The absorbance of the organic phase was measured at 532 nm. The amount of TBARS was determined by the absorbance with the molecular extinction coefficient of 156 000 and expressed as μmol/g wet weight.

**Immunohistochemistry of 4-Hydroxy-2-Nonenal (HNE)–Modified Protein**

To assess the cellular localization of lipid peroxidation by histochemical analysis, sections of LV myocardium obtained from the free wall at the papillary muscle level were immunolabeled with an antibody raised against HNE-modified protein, an aldehydic byproduct of lipid peroxidation.17,18 Paraffin-embedded tissue sections (3 μm thick) were deparaffinized with xylene and refixed with Bouin’s solution for 20 minutes and immersed in 70%, 90%, and 100% ethanol to remove picric acid. To inhibit endogenous peroxidase, the sections were incubated with 0.3% H₂O₂ in methanol for 30 minutes. After rinsing in 0.01 mol/L PBS, the sections were incubated with normal goat serum (diluted to 1:10) to inhibit nonspecific binding of antibodies. The sections were further incubated with polyclonal antiserum raised against an HNE-modified histidyl peptide (Gly3-His-Gly3) conjugated with keyhole-limpet hemocyanin, which had been confirmed to be specific in our previous studies.17,18 After rinsing with 0.01 mol/L PBS, the sections were incubated with biotin-labeled goat anti-rabbit IgG antiserum (diluted 1:100; DAKO A/S) for 60 minutes and then with avidin-biotin complex (Vectastain ABC kit; 1:100) for 60 minutes. After rinsing, the sections were finally incubated with 0.02% 3,3’-diaminobenzidine and 0.03% hydrogen peroxide in deionized water for 6 to 9 minutes. As a negative control, the sections were also incubated with normal rabbit serum.

A morphometric analysis of HNE-positive myocardial area was performed with tissue sections stained with HNE. Briefly, each section was photographed under a microscope and magnified (final magnification, ×200). Three to four fields were randomly selected from one or two coronal sections in each animal. As a result, the HNE-positive areas were measured at approximately five to seven fields for each animal. Within each field, myocardial segments that stained positively with anti-HNE antibody were identified and were manually traced by using a digitizing pad with a computer to calculate the traced area.

**Preparation of Cardiac Subcellular Fractions**

The frozen LV tissues were homogenized at 4°C for 1 minute in 6 volumes of buffer consisting of 10 mmol/L HEPES-NaOH (pH 7.4) and 250 mmol/L sucrose with a Polytron homogenizer. The homogenate was centrifuged at 4°C and 700g for 10 minutes to remove any nuclear and myofibrillar debris, and the resultant supernatant was centrifuged at 7000g for 10 minutes to separate any cardiac subcellular fractions. To isolate the mitochondria, the pellet was resuspended at 4°C in a buffer consisting of 10 mmol/L HEPES-NaOH (pH 7.4), 1 mmol/L EDTA, and 250 mmol/L sucrose (HES) and was washed three times with HES buffer. The frozen and thawed mitochondrial fraction was suspended in a buffer consisting of 10 mmol/L HEPES-NaOH (pH 7.4) and 1 mmol/L EDTA and kept at 4°C for 30 minutes. Submitochondrial particles were prepared by sonicating the mitochondria. The sonicated mitochondrial suspension was centrifuged at 21 000g for 10 minutes. The submitochondrial particle pellet was washed three times with HES buffer and stored at −80°C. The postmitochondrial supernatant was centrifuged at 170 000g for 4 hours and the final supernatant was used as the cytosolic fraction. The resultant pellet was resuspended at 4°C in HES buffer and was washed three times to obtain the microsomal fraction. These submitochondrial particle, cytosolic, and microsomal fractions were used for the ESR measurement of O₂⁻.

The cardiac SOD levels were determined in the submitochondrial particle, microsomal, and cytosolic fractions using the xanthine:xanthine oxidase:cytochrome c assay according to methods described previously.19 The protein concentration of each fraction was adjusted to obtain approximately a 50% inhibition of the rate of cytochrome c reduction produced by the xanthine:xanthine oxidase system. No SOD activity could be detected in the submitochondrial particle fractions from either control or HF hearts. There was no significant difference in the SOD activity of each fraction between the control and HF.

Special care was taken to minimize any artifactual generation of radical signals, in accordance with methods described by Zwie et al.20

**ESR Measurement of ROS**

To demonstrate ROS in the mitochondria obtained from control and HF hearts, the submitochondrial particle fractions were reacted with the substrate and a spin trapping agent, 5,5'-dimethyl-l-pyrroline-
N-oxide (DMPO; LABOTEC, Ltd), and processed for ESR spectroscopy. Immediately after starting the reaction (＜45 seconds), ESR spectra were recorded at an ambient temperature (25°C) with an ESR spectrometer (JES-RE-1X; JEOL) operating at X-band (4.95 GHz). The microwave power was 10 mW, the field modulation width was 0.063 mT, and the magnetic field range was swept at a scan rate of 5 mT/min. The quantitation of the DMPO signal intensity was performed by comparing the amplitude of the observed signal to the standard Mn⁻²/MgO marker.

Two segments of the respiratory chain are primarily responsible for ROS generation in the mitochondria, the NADH–ubiquinone reductase in complex I and the ubiquinol–cytochrome c reductase in complex III (Figure 1). 21–23 NADH (200 μmol/L) was used as a substrate for complex I and succinate (10 mmol/L) for complex II.

Assay of Mitochondrial Complex I Activity
To delineate the basis for the block of electron transfer at complex I, its enzymatic activity was measured. To measure the complex I activity, the submitochondrial particle fractions were assayed for a reduction of ubiquinone analog, decylubiquinone, using a spectrophotometer, in accordance with the method of Trounce et al. 24 with some modifications. Enzyme activity was expressed in nmol·min⁻¹·mg⁻¹ protein.

Statistical Analysis
All data are expressed as mean±SEM. To compare the data between control and HF, Student’s unpaired t test was used. For multiple comparisons, one-way ANOVA was used in conjunction with the post hoc test using Scheffe’s correction. All tests were considered to be statistically significant at P<0.05.

Results
Contractile Dysfunction in HF Model
Rapid pacing caused an approximately 125% increase in end-systolic LV dimension and a 60% decrease in LV ejection fraction (Table 1). For HF dogs, LV peak positive dP/dt was depressed, and LV end-diastolic pressure was elevated in comparison to the control values. As a result, HF dogs showed similar hemodynamic characteristics to those of humans with dilated cardiomyopathy. 15,25

Lipid Peroxidation of Failing Myocytes
TBARS significantly increased in HF animals (Figure 2A). Most importantly, an immunohistochemical analysis of HNE-modified protein revealed the lipid peroxides to be positively stained in myocytes from HF dogs, whereas no labeling was observed in the control myocardium (Figure 2B). The myocardial area stained positively with HNE was 18±6% in HF hearts, whereas it was 2±0.2% in control (P<0.01).

Mitochondria as the Source of ROS Production
At baseline conditions, only small ESR signals appeared for the submitochondrial particle fractions obtained from the normal heart in the presence of NADH or succinate as a substrate (Figure 3). When submitochondrial particle fractions were treated with rotenone (200 μmol/L), which selectively blocks the electron transport at the distal site of complex I, in the presence of NADH, prominent ESR signals produced in the microsomal and cytosolic fraction, each fraction obtained from control and HF hearts was reacted with NADH (200 μmol/L), and ESR spectra of DMPO were recorded in accordance with the same methods as those performed in the submitochondrial fractions.

\[
\text{TBARS} = \frac{\text{TBARS}_{\text{control}} - \text{TBARS}_{\text{HF}}}{\text{TBARS}_{\text{control}}} \times 100
\]

<table>
<thead>
<tr>
<th>TABLE 1. Characteristics of Rapid Pacing-Induced HF Model</th>
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<tbody>
<tr>
<td>Control (n=7)</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Body weight, kg</td>
</tr>
<tr>
<td>LV weight, g</td>
</tr>
<tr>
<td>LV end-diastolic dimension, mm</td>
</tr>
<tr>
<td>LV end-systolic dimension, mm</td>
</tr>
<tr>
<td>LV ejection fraction, %</td>
</tr>
<tr>
<td>LV peak +dP/dt, mm Hg/s</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mm Hg</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. Statistical comparisons are by unpaired t test. *P<0.05 and †P<0.01 indicate a statistically significant difference from control.

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Figure 1. Components of the respiratory chain in mitochondria. FAD indicates flavin adenine nucleotide; FMN, flavin mononucleotide; Fe-S, iron-sulfur protein; Q, ubiquinone, and Cyt, cytochrome.

Figure 2. A, Lipid peroxidation as indicated by TBARS in the myocardial tissue from control (open bar, n=7) and HF (filled bar, n=7) hearts. Statistical comparisons are by unpaired t test. Each assay was performed in triplicate. *P<0.01 for difference from control value. B, Immunohistochemical micrograph analysis of an HNE-modified histidine peptide in the myocardial tissue sections from control (left) and HF (right) dogs. Bar=50 μm.
of DMPO–superoxide adduct, DMPO-OOH, were observed with characteristic hyperfine splittings that yielded 12 resolved peaks (Figure 3A). A preliminary dose-response assay of rotenone concentration versus DMPO-OOH signal magnitude showed a maximal effect of rotenone to be achieved at a concentration of 200 μmol/L. We verified that the observed DMPO signals reflected the presence of \( \text{O}_2^- \) by a comparison of the signals with the identical hyperfine splittings elicited in the presence of pure \( \text{O}_2^- \) generated from the reaction of hypoxanthine (500 μmol/L) and xanthine oxidase (20 mU) in vitro. DMPO revealed \( \text{O}_2^- \) formation, as indicated by the DMPO-OOH spectra (•). Instrumental conditions were as follows: X-band (9.43 GHz) ESR; microwave power 10 mW; field modulation width 0.063 mT; and sweep time 5 mT/min.

The DMPO-OOH signals elicited by succinate and antimycin A were similarly enhanced by antimycin A. C, When hypoxanthine (500 μmol/L) and xanthine oxidase (20 mU) in vitro. DMPO revealed \( \text{O}_2^- \) formation, as indicated by the DMPO-OOH spectra (•). Instrumental conditions were as follows: X-band (9.43 GHz) ESR; microwave power 10 mW; field modulation width 0.063 mT; and sweep time 5 mT/min.

### Table 2. Magnitude of DMPO-OOH Signals in Normal and HF Mitochondria in the Presence of NADH or Succinate as a Substrate

<table>
<thead>
<tr>
<th>Reaction System (Substrate + Drugs)</th>
<th>n</th>
<th>Magnitude of DMPO-OOH Signals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mitochondria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH (200 μmol/L)</td>
<td>5</td>
<td>2.58±0.84</td>
</tr>
<tr>
<td>+ Rotenone (200 μmol/L)</td>
<td>5</td>
<td>8.95±0.76*</td>
</tr>
<tr>
<td>+ Rotenone+SOD (100 U/mL)</td>
<td>5</td>
<td>0.73±0.36†</td>
</tr>
<tr>
<td>+ Rotenone+SOD+catalase (500 U/mL)</td>
<td>5</td>
<td>0.08±0.09†</td>
</tr>
<tr>
<td>+ Antimycin A (200 μmol/L)</td>
<td>5</td>
<td>13.73±3.28*</td>
</tr>
<tr>
<td>+ Antimycin A+SOD (100 U/mL)</td>
<td>5</td>
<td>0.44±0.09‡</td>
</tr>
<tr>
<td>+ Antimycin A+SOD+catalase (500 U/mL)</td>
<td>5</td>
<td>0.12±0.09‡</td>
</tr>
<tr>
<td>Succinate (10 mmol/L)</td>
<td>5</td>
<td>0.71±0.39</td>
</tr>
<tr>
<td>+ Antimycin A (200 μmol/L)</td>
<td>5</td>
<td>11.64±1.46*</td>
</tr>
<tr>
<td>+ Antimycin A+SOD (100 U/mL)</td>
<td>5</td>
<td>0.42±0.26§</td>
</tr>
<tr>
<td>+ Antimycin A+SOD+catalase (500 U/mL)</td>
<td>5</td>
<td>0.52±0.35§</td>
</tr>
<tr>
<td>HF mitochondria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH (200 μmol/L)</td>
<td>5</td>
<td>6.66±1.08</td>
</tr>
<tr>
<td>+ Rotenone (200 μmol/L)</td>
<td>5</td>
<td>8.17±0.96</td>
</tr>
<tr>
<td>+ Antimycin A (200 μmol/L)</td>
<td>5</td>
<td>15.98±1.52*</td>
</tr>
<tr>
<td>Succinate (10 mmol/L)</td>
<td>5</td>
<td>0.33±0.26</td>
</tr>
<tr>
<td>+ Antimycin A (200 μmol/L)</td>
<td>5</td>
<td>14.66±1.11*</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. n indicates number of preparations. Effects of mitochondrial electron transport inhibitors (rotenone and antimycin A) and radical scavengers were examined in 5 preparations in each normal and HF group. Statistical comparisons are by one-way ANOVA with a post hoc test using Scheffé’s correction.

*P<0.05, statistically different from the substrate (NADH or succinate); †P<0.05, statistically different from NADH+rotenone; ‡P<0.05, statistically different from NADH+antimycin A; and §P<0.05, statistically different from succinate+antimycin A.

ROS Production in HF Mitochondria

To determine whether \( \text{O}_2^- \) production is enhanced in mitochondria isolated from HF and to identify its production site, submitochondrial particles obtained from HF hearts were reacted with NADH (200 μmol/L), and the ESR spectra of DMPO were recorded. Only small signals appeared for normal control heart, whereas HF exerted a prominent ESR signal of DMPO-OOH, thus indicating the generation of \( \text{O}_2^- \) (Figure 4A). The magnitude of \( \text{O}_2^- \) was 2.8-fold (P<0.01) higher in HF than in control mitochondria (Figure 5). For the HF mitochondria studied, each sample demonstrated DMPO-OOH spectra. Repeated experiments on the same heart exerted the same spectra (repeated measurements of DMPO-OOH magnitude: 100±5% of initial value, P=NS). These ESR signals were attenuated in the presence
of SOD (100 U/mL) alone or SOD (100 U/mL) plus catalase (500 U/mL), thus suggesting that most ESR signals derived from \( \text{O}_2^- \) (Figure 5).

To examine the production of \( \text{O}_2^- \) at complex III, the submitochondrial particles were incubated with succinate (10 mmol/L). In contrast to NADH as a substrate, ESR demonstrated very few DMPO signals in the HF heart (Figure 4B). The production of \( \text{O}_2^- \) in normal mitochondria treated with complex I inhibitor, rotenone, in the presence of NADH (Figure 3A) was comparable to the baseline \( \text{O}_2^- \) production in the HF mitochondria (Figure 4A). An important finding was that \( \text{O}_2^- \) production was initiated by the addition of NADH but not of succinate, thus indicating that the electron transfer function at complex I was primarily responsible for such production in HF.

\( \text{O}_2^- \) production by complex I in the submitochondrial particles from HF hearts was maximally enhanced and there was no significant “rotenone”-recruitable reserve (Figure 4 and Table 2). We next examined whether the inhibition of complex III in HF mitochondria could further increase the \( \text{O}_2^- \) production as shown in normal mitochondria. Antimycin A further increased the DMPO-OOH signals in the HF mitochondria in the presence of either NADH or succinate as a substrate (Figure 4 and Table 2), thus indicating that the additive nature of complex I and complex III in the \( \text{O}_2^- \) production is also present in HF. Most importantly, the combination of Figure 3 and Figure 4 suggests that \( \text{O}_2^- \) is produced via a functional block at complex I in HF.

The magnitude of DMPO-OOH signals obtained from the microsomal fractions was found to be comparable between control and HF (Table 3). However, the signals could not be detected in the cytosolic fractions.

**Complex I Enzymatic Activity**

Complex I activity significantly decreased in HF myocardium in comparison to control values (274\( \pm \)13 versus 136\( \pm \)9 nmol \( \cdot \) min\(^{-1} \) \cdot mg\(^{-1}\) protein, \( P<0.01 \)).

**Discussion**

The present study using ESR spectroscopy provided the first direct evidence for the increased generation of \( \text{O}_2^- \) in the mitochondria isolated from the failing heart. The decrease in complex I enzymatic activity and the resultant impairment of electron transfer lead to the deleterious production of ROS in the intracellular space, which could result in the myocyte injury in HF.

Mitochondria produce ROS through one electron carrier in the respiratory chain. Under physiological conditions, small quantities of ROS are formed during mitochondrial respiration, which, however, can be detoxified by the endogenous scavenging mechanisms of myocytes. In line with the findings of previous studies,\(^{26}\) the inhibition of electron transport at the sites of complex I and complex III in the normal submitochondrial particles resulted in a significant production of \( \text{O}_2^- \) (Figure 3). The most important findings of the present study were that HF mitochondria produce more \( \text{O}_2^- \) than normal mitochondria in the presence of NADH but

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**Table 3. Magnitude of DMPO-OOH Signals in the Subcellular Fractions Isolated From Control and HF in the Presence of NADH as a Substrate**

<table>
<thead>
<tr>
<th>Subcellular Fraction</th>
<th>Control (n=5)</th>
<th>HF (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsome</td>
<td>2.17( \pm )0.69</td>
<td>3.74( \pm )1.18</td>
</tr>
<tr>
<td>Cytosol</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are expressed as mean\( \pm \)SEM. Statistical comparisons are made by Student’s unpaired \( t \) test. ND indicates not detectable.
not succinate (Figure 4) and that complex I is the predominant source of such \( \cdot \text{O}_2^\cdot \) production (Figures 3 and 4, Table 2). Furthermore, HF mitochondria were also found to be associated with a decrease in the complex I activity. Although the present study could not prove the mechanisms for this decreased activity, it has provided compelling evidence that the defects in electron transfer function lead to mitochondrial ROS production. In aging and neurodegenerative diseases such as Parkinson disease, the mitochondria are the predominant source of ROS. In this respect, our results demonstrated a similar pathophysiological link between mitochondrial dysfunction and oxidative stress in failing hearts.

Cardiac myocytes are the likely targets of ROS attack in the failing heart (Figure 2B). It is conceivable that free radicals cause damage at or near the site of their formation, given that they are a highly reactive and short-lived species. Therefore, as major sources of ROS production, mitochondria also could be major targets of ROS attack and thus be particularly susceptible to its attack, which further impairs the function of the respiratory chain (T.I., unpublished data, 1998) and accelerates \( \cdot \text{O}_2^\cdot \) production within the mitochondria. Mitochondrial ROS production may underlie the mutations and/or deletions of mitochondrial DNA, which subsequently should lead to a further impairment of the mitochondrial function. The high susceptibility of mitochondrial DNA to mutation and oxidative damage is likely a mitochondrial function. The high susceptibility of mitochondrial DNA to mutation and oxidative damage is likely a reflection of this localized production of ROS by electron transport oxidation.

To determine whether the decreased antioxidant capacity may further aggravate the ROS accumulation in HF, SOD was quantified in the myocardial tissues. However, there was no significant difference in the SOD content between control and HF (H.T., unpublished data, 1998), which thus indicates that oxidative stress in HF is primarily due to the enhancement of mitochondrial prooxidant generation rather than the decline in antioxidant defenses. It should be acknowledged that other potential sources of ROS generation within the heart including vascular endothelial cells (via xanthine oxidase and/or NADPH oxidase) and activated leukocytes (via NADPH oxidase) could not be completely ruled out in the present study. Although mitochondrial electron transport plays an important role in the ROS production in HF, other mechanisms also might be involved. The activation of neurotransmitter factors commonly seen in HF, including catecholamines and cardiac sympathetic tone, renin-angiotensin system, and nitric oxide, can contribute to the generation of ROS.

The present study demonstrated that the production of ROS and ROS-induced lipid peroxidation are enhanced in HF heart. Oxygen radicals \( \cdot \text{OH} \) and \( \cdot \text{O}_2^- \) have been demonstrated to impair myocardial contractile function in vivo and in vitro. Further, lipid peroxides, byproducts of ROS generation, could also cause myocardial contractile defects and ultimately lead to structural damage. Although the pathophysiology of intense cellular damage resulting from the radical exposure commonly seen under ischemia and reperfusion could not be equated to the cardiac damage in HF, ROS production exceeding cellular antioxidant defense capabilities can have potentially lethal consequences for cardiac myocytes over longer periods. Recently, oxygen radicals have been suggested to be involved in apoptotic cell death. As a result, apoptosis might play an important role in the pathogenesis of HF.

Although the ESR technique is a direct method to detect ROS within biological tissue, several crucial steps must be performed carefully. First, given the highly transient nature of ROS, the ESR measurement procedures themselves may modify the status of ROS by destroying the antioxidant defenses and/or activating the ROS production system. Therefore, we took special care to minimize the artifactual generation of ROS and to preserve the in situ status of ROS by using freeze-clamped myocardial tissues, rigorously maintaining the sample under anaerobic conditions in the liquid nitrogen. More importantly, a differential effect of the experimental procedures on HF tissue seems unlikely. Second, although DMPO has been the most versatile and commonly used spin trap for measuring \( \cdot \text{O}_2^\cdot \), the half-life of DMPO–superoxide adduct, DMPO-OOH, is as short as 50 seconds in aqueous media, and it spontaneously decomposes to form the DMPO–OH adduct, which was identical to that formed from trapping the \( \cdot \text{OH} \) radical. To overcome these limitations, ESR spectra were recorded immediately after (\(<\ 45 \) seconds) the addition of DMPO to the sample. We confirmed that the DMPO signals detected in HF mitochondria are specific for \( \cdot \text{O}_2^\cdot \) by use of SOD (Figure 5), and identical DMPO-OOH signals were observed in the presence of pure \( \cdot \text{O}_2^\cdot \) generated by the interaction of hypoxanthine and xanthine oxidase in vitro (Figure 3). Furthermore, we obtained the same results by using 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide, a more sensitive and stable spin trap than DMPO (T.I., unpublished data, 1998).

Our findings do not prove a causal relationship. Indeed, given the importance of ROS in the pathophysiology of HF, the effects of radical scavengers on the reversibility or prevention of HF and the establishment of a cause-and-effect relationship will need to be determined in future studies.

HF is associated with alterations of various signal molecules in the myocardium, including the renin-angiotensin and sympathetic nervous systems, peptide growth factors, cytokines, nitric oxide, and ROS. All of these have the potential to exert profound effects on the phenotype of the myocardium. There are probably multiple levels of autocrine and paracrine interactions among these mediators resulting from both positive and negative feedback loops; therefore, studies in isolated tissues may not replicate the in vivo environment. It must be stated that a purely reductionistic approach might overlook the critical interactions among the various signal molecules in the intact heart. Although a more focused experimental approach is essential for determining the cellular and subcellular mechanisms stimulating these systems in HF, it will be important to search for a better understanding of the integrated regulation of various mediators in HF in future studies.

The present study provides, for the first time, direct evidence for the increased production of \( \cdot \text{O}_2^\cdot \) at the complex I site in the mitochondria isolated from failing cardiac myocytes. Although the focus of this study was the heart, these mechanisms of ROS production probably have a common foundation with other pathological conditions in other
organs such as the brain, liver, and kidney. These results provide a direct link among ROS generation, mitochondrial dysfunction, and contractile defects in HF and suggest that oxidative stress plays an important role in the pathogenesis of HF.

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References

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