Decrease in Mitochondrial Complex I Activity in Ischemic/Reperfused Rat Heart
Involvement of Reactive Oxygen Species and Cardiolipin

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Abstract—Reactive oxygen species (ROS) are considered an important factor in ischemia/reperfusion injury to cardiac myocytes. Mitochondrial respiration is an important source of ROS production and hence a potential contributor to cardiac reperfusion injury. In this study, we have examined the effect of ischemia and ischemia followed by reperfusion of rat hearts on various parameters related to mitochondrial function, such as complex I activity, oxygen consumption, ROS production, and cardiolipin content. The activity of complex I was reduced by 25% and 48% in mitochondria isolated from ischemic and reperfused rat heart, respectively, compared with the controls. These changes in complex I activity were associated with parallel changes in state 3 respiration. The capacity of mitochondria to produce H$_2$O$_2$ increased on reperfusion. The mitochondrial content of cardiolipin, which is required for optimal activity of complex I, decreased by 28% and 50% as function of ischemia and reperfusion, respectively. The lower complex I activity in mitochondria from reperfused rat heart could be completely restored to the level of normal heart by exogenous added cardiolipin. This effect of cardiolipin could not be replaced by other phospholipids nor by peroxidized cardiolipin. It is proposed that the defect in complex I activity in ischemic/reperfused rat heart could be ascribed to a ROS-induced cardiolipin damage. These findings may provide an explanation for some of the factors responsible for myocardial reperfusion injury. (Circ Res. 2004;94:53-59.)

Key Words: complex I ■ cardiolipin ■ reactive oxygen species ■ mitochondria ■ ischemia/reperfusion

It is generally acknowledged that reactive oxygen species (ROS) play an important role in producing lethal cell injury associated with cardiac ischemia and reperfusion.1–3 Experimental evidence has shown that during the first minutes after cardiac postischemic reperfusion, a burst of ROS generation occurs.4–6 Oxygen radicals can be generated by several mechanisms, including the xanthine/xanthine oxidase reaction7–10 and the activity of NADPH oxidase.11 Another potential source of oxygen radicals is thought to be the mitochondrial respiratory chain.12 Because of the abundance of mitochondria in cardiac myocytes, mitochondrial electron transport might be an important subcellular source of ROS and hence a potential contributor to heart reperfusion injury.13

Peroxidation of membrane lipid components has been hypothesized to be a major mechanism of oxygen free radical toxicity, resulting in generalized impairment of membrane function. Cardiolipin, a phospholipid of unusual structure localized almost exclusively within the mitochondrial inner membrane, is particularly rich in unsaturated fatty acids (90% represented by linoleic acid). Thus, mitochondrial cardiolipin molecules are a possible and early target of oxygen-free radical attack, either because of their high content of unsaturated fatty acids or because of their location in the inner mitochondrial membrane, near the site of ROS production, mainly at the level of complex I14 and complex III15 of the mitochondrial respiratory chain. This phospholipid plays an important role in mitochondrial bioenergetics, optimizing the activity of key mitochondrial inner membrane proteins, including several anion carriers and some electron transport complexes.16–18 It seems likely that mitochondrial oxidative stress may lead to cardiolipin modification and hence to loss of mitochondrial enzyme function. This condition is likely to be encountered on reperfusion of ischemic cardiac tissue. In this regard, recent results from this laboratory have demonstrated that mitochondrial-mediated ROS generation affects the activity of cytochrome c oxidase via oxidative damage of cardiolipin.19 These results have been useful to explain the molecular basis of the decline in the cytochrome c oxidase activity, observed in mitochondria isolated from animals in certain physiopathological conditions, such as different thy-
roid hormones status,\textsuperscript{20,21} aging,\textsuperscript{22–24} and ischemia/reperfusion,\textsuperscript{25,26} which are characterized by an increase in the basal rate of ROS production.

Complex I, also known as NADH-ubiquinone oxidoreductase, is a multisubunit integral membrane complex of the mitochondrial electron transport chain that catalyzes electron transfer from NADH to ubiquinone. Results from different laboratories have shown that cardiolipin is specifically required for electron transfer in complex I of the mitochondrial respiratory chain.\textsuperscript{27,28} In fact, the presence of this phospholipid has been shown to be an absolute necessity for reactivating enzyme activity from phospholipid-depleted preparations of the mitochondrial fraction.\textsuperscript{29} The involvement of cardiolipin in the function of complex I is also supported by the results of our recent study showing inactivation of this enzyme complex by micromolar concentrations of nonylacridine, a compound that interacts specifically with cardiolipin.\textsuperscript{30}

Complex I is considered an important site of superoxide anion generation in mitochondria.\textsuperscript{14} The formation of superoxide occurs via the transfer of a free electron to molecular oxygen. This reaction occurs at specific sites of the electron transport chain, which resides in the inner mitochondrial membrane. Complex I produces most of the superoxide, which is then scavenged by the mitochondrial enzyme Mn-superoxide dismutase to produce H$_2$O$_2$. Thus, a defect of complex I activity can be considered a potential source of ROS in heart ischemia/reperfusion.

Previous results from this and other laboratories have shown a decline in complexes III and IV activity in mitochondria isolated from ischemic and ischemic/reperfused rat heart.\textsuperscript{25,26,31} This decline was attributed to a loss of mitochondrial cardiolipin, a phospholipid that is needed for full functioning of these enzyme complexes.\textsuperscript{17,18} We recently reported that mitochondrial-mediated ROS production affects the complex I activity via cardiolipin peroxidation in beef-heart submitochondrial particles.\textsuperscript{30} Thus, it is likely that oxidative damage to cardiolipin may affect the activity of mitochondrial complex I in ischemic and ischemic/reperfused rat heart as well. This possibility was explored in the present investigation. We have also evaluated other changes induced by heart ischemia/reperfusion related to mitochondrial function, such as oxygen consumption, mitochondrial ROS production rates, and cardiolipin content.

**Materials and Methods**

**Preparation and Perfusion of Isolated Rat Heart**

Male Wistar rats (250 to 300 g) were used throughout the studies. After intraperitoneal injection of heparin (1000 U/kg) and administration of thiopental (50 mg), hearts were removed and then placed in ice-cold Krebs-Henseleit buffer. The aorta was cannulated, and the heart was perfused in retrograde fashion according to Langendorff with Krebs-Henseleit buffer at 37°C, saturated with 95% O$_2$ and 5% CO$_2$. Hearts were placed in a water-jacketed chamber at 37°C, and perfusion pressure was maintained at 60 mm Hg. One group of hearts was subjected to 30 minutes of global ischemia at 37°C, and another group of hearts was subjected to 30 minutes of global ischemia followed by 15 minutes of reperfusion. In control perfusions, the ischemic period was replaced by an equal period of flow-through perfusion.

**Isolation of Mitochondria**

Rat heart mitochondria were isolated in a medium of 250 mmol/L sucrose, 10 mmol/L Tris-HCl, and 1 mmol/L EGTA, pH 7.4, by differential centrifugation of heart homogenates, essentially as described previously.\textsuperscript{32} Mitochondria were resuspended in 250 mmol/L sucrose and 10 mmol/L Tris-HCl (pH 7.4) and stored in ice. The yield of mitochondrial proteins (mg/g heart wet weight) within each group of animals was consistent, suggesting minimal variation in the preparations of the mitochondrial fraction. Mitochondrial protein concentration was measured by the biuret method using serum albumin as standard.

**Determination of Mitochondrial H$_2$O$_2$ Production**

The rate of mitochondrial hydrogen peroxide production was estimated by measuring the linear fluorescence increase induced by H$_2$O$_2$ oxidation of dichlorofluorescein to the fluorescent dichlorofluorescein in the presence of horseradish peroxidase.\textsuperscript{33} Rat heart mitochondria (0.3 mg protein) were suspended in 2.5 mL of a medium of 100 mmol/L sucrose, 100 mmol/L KCl, and 5 mmol/L Tris, pH 7.4, supplemented with 7.5 μg horseradish peroxidase and 1 μmol/L dichlorofluorescein. The production of hydrogen peroxide was induced by addition of 5 mmol/L malate plus 2 mmol/L pyruvate as substrates (state 4). The amount of H$_2$O$_2$ produced was calculated by measuring the fluorescence changes on addition of known amounts of H$_2$O$_2$. Alternatively, mitochondrial H$_2$O$_2$ production was measured using 1 μmol/L scopoletin plus 1 μmol/L horseradish peroxidase at 365 nm for excitation and 450 nm for emission.\textsuperscript{34}

**Mitochondrial Oxygen Consumption**

Mitochondrial ADP-dependent state 3 respiration was measured polarographically with an oxygen electrode at 25°C. Respiration was initiated by the addition of 2 mmol/L pyruvate plus 5 mmol/L malate. After 2 minutes, state 3 respiration was induced by the addition of 0.5 mmol/L ADP.

**Complex I Activity**

The complex I (NADH-CoQ reductase) activity was measured in mitochondrial particles prepared by sonication, under nitrogen atmosphere, 1 mg of rat heart mitochondria dissolved in 1 mL of 50 mmol/mL phosphate buffer, pH 7.2. The assay mixture contained 3 mmol/L sodium azide, 1.2 μmol/L antimycin A, 50 μmol/L decylubiquinone, and 50 mmol/L phosphate buffer, pH 7.2. The mitochondrial sample (50 μg) was added to 3 mL of the assay mixture, and the reaction was started by the addition of 60 μmol/L NADH. The reaction was measured by following the decrease in absorbance of NADH at 340 nm with a diode-array spectrophotometer. The activity was calculated using an extinction coefficient of 6.22 mmol/L cm$^{-1}$ for NADH. The specific activity of the enzyme is expressed as nanomole of NADH oxidized per minute per milligram of SMP.

**Analysis of Cardiolipin in Mitochondrial Membranes**

Cardiolipin was analyzed by high-pressure liquid chromatography (HPLC) using a Hewlett Packard series 1100 gradient liquid chromatograph. Lipids from heart mitochondria were extracted with chloroform/methanol by the procedure of Bligh and Dyer.\textsuperscript{35} Lipid extraction was carried out on ice immediately after the preparation of mitochondria in the presence of BHT and under nitrogen atmosphere. Phospholipids were separated by the HPLC method previously described\textsuperscript{36} with an Lichrosorb Si60 column (4.6×250 mm). The chromatographic system was programmed for gradient elution using two mobile phases: solvent A, hexane/2-propanol (6:8, vol/vol), and solvent B, hexane/2-propanol/water (6:8:1.4, vol/vol/vol). The percentage of solvent B in solvent A was increased in 15 minutes from 0% to 100%. Flow rate was 1 mL/min, and detection was at 206 nm. The peak of cardiolipin was identified by comparison with the retention time of standard cardiolipin and rechromatographed by thin-layer chromatography.
Preparation and Analysis of Peroxidized Cardiolipin

Bovine heart cardiolipin was autoxidized overnight in a thin film at 37°C. Peroxidized cardiolipin was identified by normal-phase HPLC as described above, with UV detection at 235 nm, indicative of conjugated dienes. The resulting peak was rechromatographed by thin-layer chromatography and used as standard.

Preparation of Liposomes

Liposomes (small unilamellar vesicles) were prepared by sonicating 1.7 mg of phospholipids in 1 mL of incubation medium of 25 mmol/L phosphate buffer (pH 6.7) with constant stirring. After 40 minutes of incubation, phospholipid-phosphate buffer pH 6.7 was added to 1 mg of mitochondria at 30°C. The mitochondrial pellet was then washed and resuspended in 250 mmol/L sucrose and 10 mmol/L Tris, pH 7.4.

Preparation of Peroxidized Liposomes

Liposomes were peroxidized using the Fe2+/ADP ascorbic acid method. Briefly, aliquots of liposomes (~1.7 mg of phospholipids) were dispersed in 1 mL of freshly sonicated liposomes were dispersed in 1 mL of oxygenated buffer containing 20 μmol/L FeNO3 and 120 μmol/L ADP. The peroxidation reaction was initiated by adding 20 μmol/L ascorbic acid. Incubation was carried out at 37°C in a shaking water bath for 6 minutes. The extent of lipid peroxidation was monitored by conjugated dienes formation, as described by Buege and Aust.

Fusion of Liposomes With Mitochondria

The liposomes-mitochondrial membrane fusion was carried out essentially as described by Hackenbrock and Chazotte, with some modifications. Briefly, 1 mL of freshly sonicated liposomes in phosphate buffer pH 6.7 was added to 1 mg of mitochondria at 30°C with constant stirring. After 40 minutes of incubation, phospholipid-enriched mitochondria were centrifuged at 10 000g for 20 minutes to remove the phospholipid excess. The mitochondrial pellet was then washed and resuspended in 250 mmol/L sucrose and 10 mmol/L Tris, pH 7.4.

Statistical Analysis

Results are expressed as mean±SE. Statistical significances were determined by the Student’s t test.

Results

The activity of complex I was measured in mitochondria isolated from control, ischemic, and ischemic/reperfused rat heart (Figure 1). In ischemic heart, mitochondrial complex I activity was decreased by 25% compared with the normal heart. In ischemic/reperfused heart, the decrease of complex I activity was much more pronounced, reaching 48% compared with control heart.

Respiratory activities of mitochondria isolated from control, ischemic, and reperfused rat heart, measured in presence of pyruvate and malate as substrates and ADP to stimulate respiration (state 3), are reported in Figure 2. Mitochondria from ischemic heart exhibited a 24% and those from reperfused heart a 48% reduction in the rate of state 3 respiration compared with the control value.

The site of the ischemia and ischemia/reperfusion damage to complex I was approached in subsequent experiments. As mentioned above, cardiolipin is specifically required for full activity of complex I. Thus, it is possible that ROS-induced oxidative damage to mitochondrial cardiolipin may be responsible for the observed defect in complex I activity in ischemia and ischemia/reperfusion rat heart. The content of cardiolipin was determined in mitochondria isolated from normal, ischemic, and ischemic/reperfused rat hearts by a very sensitive HPLC technique set up in our laboratory. As shown in Figure 3, the content of cardiolipin decreased by 28% and 50% in mitochondria from ischemic and ischemic/reperfused rat hearts, respectively, compared with the values obtained with mitochondria isolated from control hearts.

To assess that the decrease in the cardiolipin content observed in ischemic/reperfused rat hearts could be attributable to cardiolipin peroxidation because of the ROS attack to its double bonds, the content of peroxidized cardiolipin was measured in mitochondria by an HPLC method based on the absorbance at 235 nm, indicative of the formation of conjugated dienes. As shown in Figure 4, an increase in the content of peroxidized cardiolipin was observed in mitochondria from ischemic rat heart, which was more pronounced on reperfusion compared with control heart.

The changes in the cardiolipin content observed in mitochondria from ischemic and reperfused rat heart paralleled
the changes in the complex I activity, thus suggesting a possible involvement of cardiolipin in this effect. To demonstrate this more directly, we investigated whether addition of exogenous cardiolipin to mitochondria from reperfused heart was able to reverse the observed defect of complex I activity. Because cardiolipin is poorly permeable to mitochondrial membrane, a previously reported method of fusion of vesicular lipids with mitochondrial membrane was used to enrich the inner mitochondrial membrane with cardiolipin.40,41 We have recently shown that fusion of mitochondria from reperfused rat heart, which contains a lower level of cardiolipin, with phosphatidylcholine/cardiolipin (PC/CL) liposomes resulted in a significant enrichment in the mitochondrial cardiolipin content.31 Using this procedure, we studied the effect of fusion of mitochondria from control and ischemic/reperfused heart with liposomes composed of various phospholipids. The fusion of mitochondria with liposomes composed of various phospholipids was carried out as described in Materials and Methods. PC/CL liposomes (4:1 molar ratio) and PC/PE liposomes (1:1 molar ratio). Control and ischemic/reperfused mitochondria were treated in the same manner as the liposome-treated mitochondria but in the absence of liposomes. Each value represents the mean±SE of 5 separate experiments. *P<0.05 vs control; **P<0.05 vs ischemic/reperfused heart.

![Figure 3](image3.png)

Figure 3. Cardiolipin content in mitochondria isolated from control, ischemic, and ischemic/reperfused rat heart. Mitochondrial cardiolipin content was determined by the HPLC technique as described in Materials and Methods. Each value represents the mean±SE obtained from 4 different experiments. *P<0.05, **P<0.01 vs control.

![Figure 4](image4.png)

Figure 4. Relative content of peroxidized cardiolipin in mitochondria from ischemic and ischemic/reperfused rat heart. Mitochondrial content of peroxidized cardiolipin was determined by the HPLC technique described in Materials and Methods. The content of peroxidized cardiolipin is expressed as peak area (at 235 nm) per milligram of phospholipids, and the peak area of the control is assumed as a unit. Each value represents the mean±SE obtained from 4 different experiments. *P<0.05 vs control.

![Figure 5](image5.png)

Figure 5. Decreased complex I activity in mitochondria isolated from ischemic/reperfused rat heart and specific reactivation by cardiolipin liposomes. The fusion of mitochondria with liposomes composed of various phospholipids was carried out as described in Materials and Methods. PC/CL liposomes (4:1 molar ratio) and PC/PE liposomes (1:1 molar ratio). Control and ischemic/reperfused mitochondria were treated in the same manner as the liposome-treated mitochondria but in the absence of liposomes. Each value represents the mean±SE of 5 separate experiments. *P<0.05 vs control; **P<0.05 vs ischemic/reperfused heart.
be major targets of ROS attack. The effects of ROS should be greatest at the level of mitochondrial membrane constituents, including the complexes of the respiratory chain and phospholipid constituents particularly rich in unsaturated fatty acids, such as cardiolipin. Previous studies from this and other laboratories have shown that the activity of complexes III and IV is reduced in mitochondria from ischemic and ischemic/reperfused rat hearts.25,26,31 In the present study, we demonstrate that the activity of complex I is reduced by 25% in mitochondria from ischemic heart and by 48% in those from ischemic/reperfused heart compared with control heart (see Figure 1). A defect in complex I activity in mitochondria isolated from hypoxic/reoxygenated rat heart has been already reported.43 This defect has been explained on the basis of an increase in the Ca$^{2+}$ uptake during reoxygenation by unknown mechanism. Interestingly, Ca$^{2+}$ overload has been shown to stimulate ROS production.44

Complex I is considered an important factor in the regulation of mitochondrial respiration. A decrease in the mitochondrial complex I activity, as observed in mitochondria from ischemic and ischemic/reperfused rat heart, should be associated with a decline in mitochondrial respiration. The results reported in Figure 2 clearly demonstrate that mitochondria from ischemic and reperfused rat heart exhibit lower rate of state 3 respiration compared with control heart. These changes in state 3 respiration are quantitatively related to changes in complex I activity, thus suggesting that the lowered complex I activity is probably the most important and rate-determining step responsible for the alteration to the mitochondrial oxidative metabolism in ischemic and reperfused rat heart.

Cardiolipin has a particularly important function in mitochondrial bioenergetics in that it interacts with several major inner membrane proteins, including anion carriers and complexes of the respiratory chain,16–18 even if its precise mechanism of action is still not well understood. It has been reported that cardiolipin is specifically required for electron transfer in complex I of the mitochondrial respiratory chain.27–29 Additional evidence for the cardiolipin involvement in the complex I functioning comes from our recent finding showing that nonylacriline orange, a compound that interacts specifically with cardiolipin,45 inactivates the complex I activity in SMP and that added cardiolipin fully prevented this inactivation.30

The content of cardiolipin in the inner mitochondrial membrane may change either as a consequence of an alteration of one of the enzymatic steps involved in its biosyn-

![Figure 6. H$_2$O$_2$ production in mitochondria isolated from control, ischemic, and ischemic/reperfused rat heart. The H$_2$O$_2$ formation was induced by the addition of 2 mmol/L pyruvate plus 5 mmol/L malate and measured as described in Materials and Methods. Each value represents the mean±SE of 4 different experiments. *P<0.05 vs control.](https://example.com/image)
The changes in complex I activity, observed in mitochondria from ischemic/reperfused rat heart, are quantitatively related to changes in the mitochondrial cardiolipin content. On this basis, it is reasonable to assume that the molecular basis for the ischemia/reperfusion defect in complex I activity can be mainly ascribed to a loss in mitochondrial content of cardiolipin, which is required for the optimal function of this enzyme complex. This assumption is also supported by the results of our recent study showing a close correlation between loss of cardiolipin content in submitochondrial particles and loss in complex I activity.30

More firm evidence for the involvement of cardiolipin in the loss of complex I activity, observed in mitochondria isolated from ischemic/reperfused hearts, comes from the results of the experiments reported in Figure 5. These results demonstrate that exogenously added cardiolipin to mitochondria from reperfused rat heart resulted in almost complete restoration of the normal activity of this enzyme complex. This effect of cardiolipin could not be replaced by other phospholipids, such as PC and PE, nor by peroxidized cardiolipin. These results clearly indicate that the defect in the complex I activity in mitochondria from reperfused heart could be mainly ascribed to a loss of cardiolipin content, attributable to ROS attack to double bonds of its fatty acid constituents.

In addition to the defect in mitochondrial complex I activity (present study), we have recently reported a defect in mitochondrial complex III and IV in ischemic/reperfused rat heart, similarly attributable to ROS-induced cardiolipin damage.25,31 Thus, a common mechanism seems to be involved in the defect of these three mitochondrial respiratory complex activities in ischemia/reperfusion. These data appear particularly interesting in light of recent findings in the literature indicating the association of complexes I, III, and IV in a supercomplex in the mitochondrial inner membrane. Cardiolipin seems to play a central role in higher-order organization of these components of the respiratory chain.54

Complex I exhibits a lower activity compared with the other respiratory complexes; therefore, it is considered an important factor in the regulation of oxidative phosphorylation. This enzyme complex is also considered the main site of oxygen radical production in mitochondria. Thus, the impairment of mitochondrial complex I activity, in addition to that of complexes III and IV previously reported,25,31 attributable to ROS-induced cardiolipin damage may increase the electron leak from the electron transport chain, generating more superoxide radical and perpetuating a cycle of oxygen-radical–induced damage, which ultimately leads to a decrease in oxidative phosphorylation and to heart failure on reperfusion (see Figure 7). The pattern of results presented here may prove useful in elucidating the molecular mechanism of ROS-induced alterations to mitochondrial structure and function, which could be responsible for contractile defect of ischemic heart on reperfusion and in the development of appropriate treatment strategies.

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