Decrease in Mitochondrial Complex I Activity in Ischemic/Reperfused Rat Heart

Involvement of Reactive Oxygen Species and Cardiolipin

Giuseppe Paradies, Giuseppe Petrosillo, Marilva Pistolese, Nicola Di Venosa, Antonio Federici, Francesca Maria Ruggiero

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_2O_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

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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 reaction[7–10] 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 I[14] and complex III[15] 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-

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roid hormones status,[20,21] aging,[22–24] and ischemia/reperfusion,[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.[27,28] In fact, the presence of this phospholipid has been shown to be an absolute necessity for regenerating enzyme activity from phospholipid-depleted preparation of complex I.[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.[30]

Complex I is considered an important site of superoxide anion generation in mitochondria.[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_2O_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.[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.[17,18] We recently reported that mitochondrial-mediated ROS production affects the complex I activity via cardiolipin peroxidation in beef-heart submitochondrial particles.[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.[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_2O_2 Production

The rate of mitochondrial hydrogen peroxide production was estimated by measuring the linear fluorescence increase induced by H_2O_2 oxidation of dichlorofluorescein to the fluorescent dichlorofluorescein in the presence of horseradish peroxidase.[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_2O_2 produced was calculated by measuring the fluorescence changes on addition of known amounts of H_2O_2. Alternatively, mitochondrial H_2O_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.[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 sonicating, under nitrogen atmosphere, 1 mg of rat heart mitochondria dissolved in 1 mL of 50 mmol/L 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 $mmol/L^{-1} \times cm^{-1}$ 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.[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[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.

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Preparation and Analysis of Peroxidized Cardiolipin

Bovine heart cardiolipin was autoxidized overnight in a thin film at 37°C.[37] Peroxidized cardiolipin was identified by normal-phase HPLC, as described above, with UV detection at 235 nm, indicative of conjugated dienes.[37,38] 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 the microtip probe of a Branson sonifier (model 250) at 40 W for six cycles of 2.5 minutes in an ice bath under N_2 stream.

Preparation of Peroxidized Liposomes

Liposomes were peroxidized using the Fe^{2+}-ADP ascorbic acid method. Briefly, aliquots of liposomes (≈1.7 mg of phospholipids) were dispersed in 1 mL of oxygenated buffer containing 20 μmol/L Fe^{2+} and 120 μmol/L ADP. The peroxidation reaction was initiated by adding 200 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.[39]

Fusion of Liposomes With Mitochondria

The liposomes-mitochondrial membrane fusion was carried out essentially as described by Hackenbrock and Chazotte,[40] with some modifications.[41] 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 10 000*g* 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.[36] 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 asses that the decrease in the cardiolipin content observed in ischemic/reperfused rat heart 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.[37,38] 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

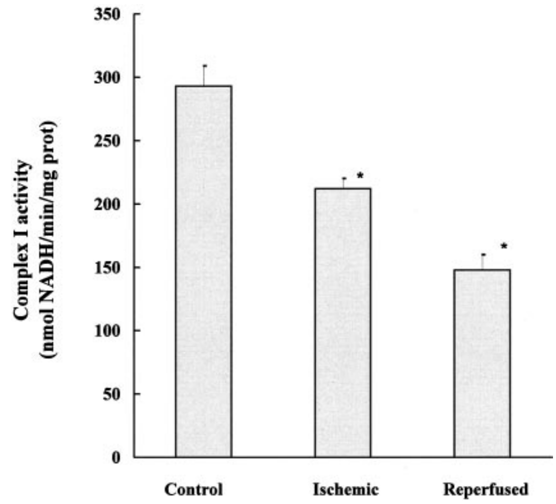

Figure 1. Activity of complex I in mitochondria isolated from control, ischemic, and ischemic/reperfused rat heart. The complex I activity was measured as described in Materials and Methods. Each value represents the mean±SE of 4 separate experiments. *$P<0.05$ vs control.

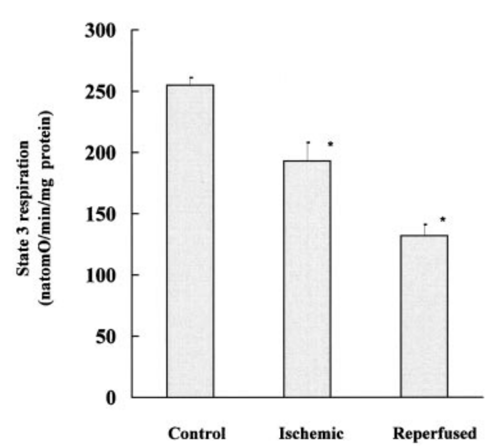

Figure 2. State 3 respiration in mitochondria isolated from control, ischemic, and reperfused rat heart. Mitochondrial respiratory activity was measured as described in Materials and Methods. Each value represents the mean±SE of 4 separate experiments. *$P<0.05$ vs control.

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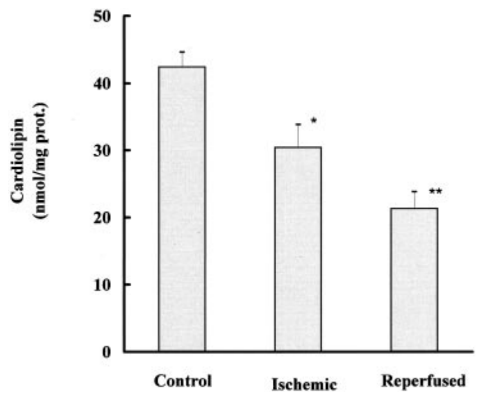

Figure 3. Cardiolipin content in mitochondria isolated from control, ischemic, and ischemic/reperfused rat heart. Mitochondrial cardiolipin content was determined by the HPLC technique as described in Materials and Methods. Each value represents the mean±SE obtained from 4 different experiments. *$P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

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

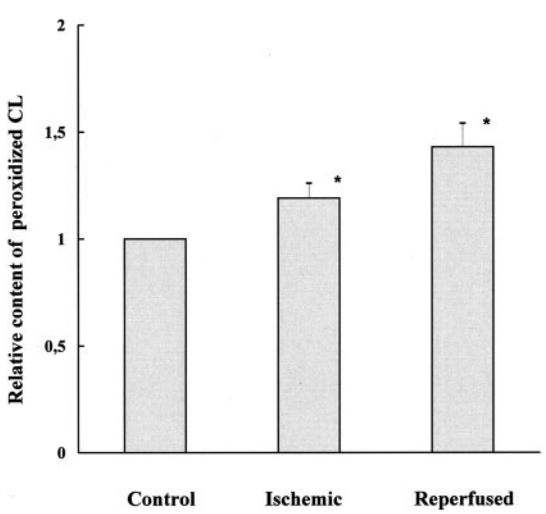

Figure 4. Relative content of peroxidized cardiolipin in mitochondria from ischemic and ischemic/reperfused rat heart. Mitochondrial content of peroxidized cardiolipin was determined by the HPLC technique described in Materials and Methods. The content of peroxidized cardiolipin is expressed as peak 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.

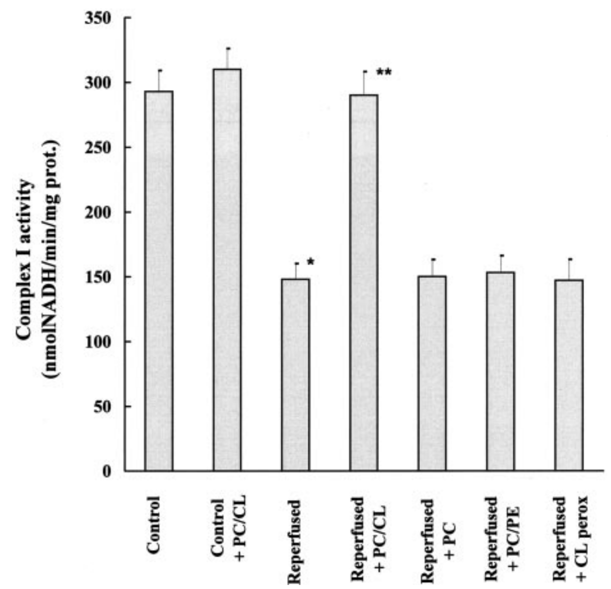

Figure 5. Decreased complex I activity in mitochondria isolated from ischemic/reperfused rat heart and specific reactivation by cardiolipin liposomes. The fusion of mitochondria with liposomes composed of various phospholipids was carried out as described in Materials and Methods. PC/CL liposomes (4:1 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.

of fusion of mitochondria from control and ischemic/reperfused heart with liposomes composed of various phospholipids PC, PC/CL, and PC/phosphatidylethanolamine (PE) on the activity of complex I. The results of these experiments are reported in Figure 5. As shown above, mitochondria from reperfused rat heart exhibited a 48% decline in complex I activity compared with control rats (see also Figure 1). This lower activity of complex I was almost completely restored to the level of control rats after fusion of mitochondria from reperfused rat heart with liposomes containing cardiolipin, whereas PC or PC/PE liposomes could not replace cardiolipin in this effect. Interestingly, no restoration was obtained with liposomes containing peroxidized CL, thus suggesting that integral molecules of cardiolipin are required for the reactivation of complex I. Fusion of mitochondria from control rats with PC/CL liposomes had practically no effect on the complex I activity, indicating that the liposome-mitochondrial membrane procedure used in our experiments did not affect the activity of this enzyme complex in control mitochondria (Figure 5).

It has been shown that addition of pyruvate plus malate to aerobic mitochondria causes detectable H_2O_2 production, which arises from superoxide anion formed at the level of complex I.[14,42] Mitochondria from control, ischemic, and reperfused rat heart were investigated for their capacity to generate oxygen radicals in the presence of pyruvate plus malate. As shown in Figure 6, the basal rate of H_2O_2 production was enhanced by 14% and 32% in mitochondria isolated from ischemic and ischemic/reperfused heart, respec-

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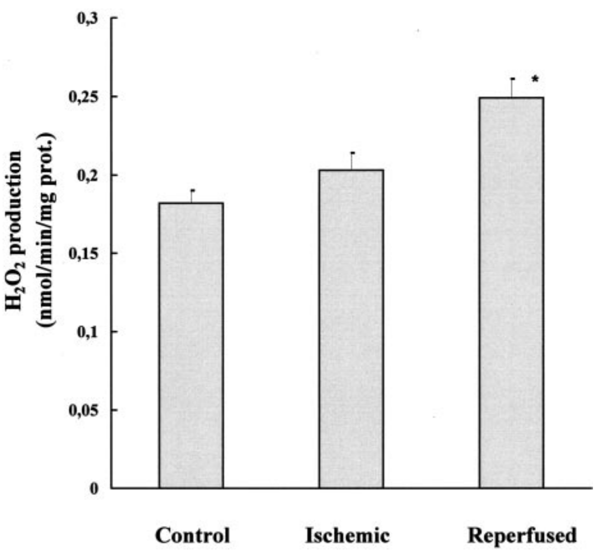

Figure 6. H_2O_2 production in mitochondria isolated from control, ischemic, and ischemic/reperfused rat heart. The H_2O_2 formation was induced by the addition of 2 mmol/L pyruvate plus 5 mmol/L malate and measured as described in Materials and Methods. Each value represents the mean±SE of 4 different experiments. **P*<0.05 vs control.

tively, compared with control heart. Similar results were obtained using glutamate plus malate as substrates (results not shown).

Discussion

ROS have been implicated in the cardiac tissue injury that follows ischemia and reperfusion. The mechanisms for the enhanced ROS generation and the cellular and subcellular targets of ROS attack are not well established. Mitochondria consume >90% of the oxygen used by the cell, and the mitochondrial respiratory chain generates a continuous flux of oxygen radicals. It has been estimated that ≈2% of the oxygen reacting with the respiratory chain leads to formation of superoxide radical. Subsequent dismutation of superoxide anion generates H_2O_2, which in turn can lead to production of $OH^{\cdot}$. Ischemia may cause mitochondrial alterations that would favor oxygen radical production when the oxygen concentration is reestablished by reperfusion and oxidative phosphorylation resumes. In this respect, in vitro studies have demonstrated that mitochondrial production of oxygen radicals mostly occurs during state 4 respiration, because oxygen concentration increases and the level of reduced one-electron donors in the respiratory chain is concomitantly increased due to a decreased availability of ADP.[12] A similar situation is likely to be encountered during ischemia. When ischemic tissue is reoxygenated, electron transport through the respiratory chain is impaired because of depletion of ADP during ischemia, and this leads to a burst of ROS generation during the first minutes of reoxygenation.[4–6,13]

It is conceivable that mitochondrial-mediated ROS generation leads to primary reaction and damage in the immediate area surrounding where these ROS are produced, 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. 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.

Complex I is considered an important source of oxygen radicals in mitochondria,[45–48] although the mechanism responsible for this production is not well established. The lower activity of complex I can account for the enhanced production of H_2O_2 observed in mitochondria isolated from ischemic and ischemic/reperfused rat heart, supplemented with pyruvate plus malate (see Figure 6). This finding is consistent with the results reported by others showing that mitochondrial electron transport complex I is a potential source of oxygen free radical in intact heart subjected to ischemia and reflow[13] during ischemia[49] and in the failing myocardium.[50]

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 nonylacridine orange, a compound that interacts specifically with cardiolipin,[51] 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-

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thetic process[52,53] or as a consequence of oxidative damage by ROS. In fact, cardiolipin, because of its high content of unsaturated fatty acids (90% represented by linoleic acid) and because of its location in the mitochondrial membrane near the site of oxygen radical production (mainly at the level of complexes I and III), is particularly susceptible to peroxidative attack by oxyradicals. This situation is likely to occur on reperfusion of ischemic heart, characterized by an increased production of ROS. Accordingly, our results demonstrate a pronounced loss in the cardiolipin content in mitochondria isolated from ischemic/reperfused rat heart, associated with an increase in peroxidized cardiolipin content (Figure 4) compared with control hearts.

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.

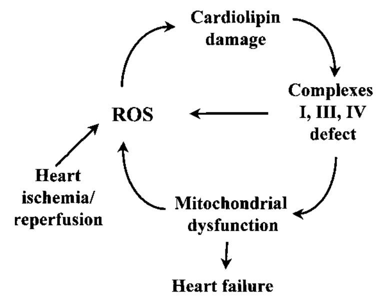

Figure 7. Schematic diagram of the role of ROS and cardiolipin in mitochondrial dysfunction associated with heart ischemia/reperfusion.

Acknowledgments

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References

1. Hess ML, Manson NH. Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion injury. *J Mol Cell Cardiol*. 1984;16:969–985.
2. McCord JM. Free radicals and myocardial ischemia: overview and outlook. *Free Radic Biol Med*. 1988;4:9–14.
3. Das DK. Cellular biochemical and molecular aspects of reperfusion injury. *Ann N Y Acad Sci*. 1994;723:116–127.
4. Zweier JL, Flaherty JT, Weisfeldt ML. Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc Natl Acad Sci U S A*. 1987;84:1404–1407.
5. Ambrosio G, Zweier JL, Flaherty JT. The relationship between oxygen radical generation and impairment of myocardial energy metabolism following post-ischemic reperfusion. *J Mol Cell Cardiol*. 1991;23: 1359–1374.
6. Vanden Hoek TL, Shao Z, Li C, Zak R, Schumaker PT, Becker LB. Reperfusion injury in cardiac myocytes after stimulated ischemia. *Am J Physiol*. 1996;270:H1334–H1341.
7. Chambers DE, Parks DA, Patterson G, Roy R, McCord JM, Yoshida S, Parmley LF, Downey JM. Xanthine oxidase as a source of free radical damage in myocardial ischemia. *J Mol Cell Cardiol*. 1985;17:145–152.
8. Thompson-Gorman SL, Zweier JL. Evaluation of the role of xanthine oxidase in myocardial reperfusion injury. *J Biol Chem*. 1990;265: 6656–6663.
9. Xia Y, Zweier JL. Substrate control of free radical generation from xanthine oxidase in the postischemic heart. *J Biol Chem*. 1995;270: 18797–18803.
10. Xia Y, Khatchikian G, Zweier JL. Adenosine deaminase inhibition prevents free radical-mediated injury in the postischemic heart. *J Biol Chem*. 1996;271:10096–10102.
11. Shandelya SM, Kuppusamy P, Weisfeldt ML, Zweier JL. Evaluation of the role of polymorphonuclear leukocytes on contractile function in myocardial reperfusion injury: evidence for plasma-mediated leukocyte activation. *Circulation*. 1993;87:536–546.
12. Chance B, Seis H, Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev*. 1979;59:527–605.
13. Ambrosio G, Zweier JL, Duilio C, Kuppusamy P, Santoro G, Elia PP, Tritto I, Cirillo P, Condorelli M, Chiariello M, Flaherty JT. Evidence that

mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. *J Biol Chem*. 1993;268:18532–18541.
14. Hansford RG, Hogue BA, Mildaziene VJ. Dependence of H_2O_2 formation by rat heart mitochondria on substrate availability and donor age. *J Bioenerg Biomembr*. 1997;29:89–95.
15. Boveris A, Cadenas E, Stoppani AOM. Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. *Biochem J*. 1976;156:435–444.
16. Hoch FL. Cardiolipins and biomembrane function. *Biochim Biophys Acta*. 1992;1113:71–133.
17. Robinson NC. Functional binding of cardiolipin to cytochrome *c* oxidase. *J Bioenerg Biomembr*. 1993;25:153–163.
18. Schlame M, Rua D, Greenberg ML. The biosynthesis and functional role of cardiolipin. *Prog Lipid Res*. 2000;39:257–288.
19. Paradies G, Petrosillo G, Pistolese M, Ruggiero FM. The effect of reactive oxygen species generated from the mitochondrial electron transport chain on the cytochrome c oxidase activity and on the cardiolipin content in bovine heart submitochondrial particles. *FEBS Lett*. 2000;466:323–326.
20. Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E. Enhanced cytochrome oxidase activity and modification of lipids in heart mitochondria from hyperthyroid rats. *Biochim Biophys Acta*. 1994;1225:165–170.
21. Paradies G, Petrosillo G, Ruggiero FM. Cardiolipin-dependent decrease of cytochrome c oxidase activity in heart mitochondria from hypothyroid rats. *Biochim Biophys Acta*. 1997;1319:5–8.
22. Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E. Age-dependent decrease in the cytochrome *c* oxidase activity and changes in phospholipid in rat-heart mitochondria. *Arch Gerontol Geriatr*. 1993;16:263–272.
23. Paradies G, Ruggiero FM, Petrosillo G, Gadaleta MN, Quagliariello E. Effect of aging and acetyl-L-carnitine on the activity of cytochrome oxidase and adenine nucleotide. *FEBS Lett*. 1994;350:213–215.
24. Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E. Age-dependent decline in the cytochrome *c* oxidase activity in rat heart mitochondria: role of cardiolipin. *FEBS Lett*. 1997;406:136–138.
25. Paradies G, Petrosillo G, Pistolese M, Di Venosa N, Serena D, Ruggiero FM. Lipid peroxidation and alterations to oxidative metabolism in mitochondria isolated from rat heart subjected to ischemia and reperfusion. *Free Radic Biol Med*. 1999;27:42–50.
26. Lesnefsky EJ, Slabe TJ, Stoll MS, Minkler PE, Hoppel CL. Myocardial ischemia selectively depletes cardiolipin in rabbit heart subsarcolemmal mitochondria. *Am J Physiol Heart Circ Physiol*. 2001;280:H2770–H2778.
27. Ragan CI. The role of phospholipids in the reduction of ubiquinone analogues by the mitochondrial reduced nicotinamide-adenine dinucleotide-ubiquinone oxidoreductase complex. *Biochem J*. 1978;172:539–547.
28. Fry M, Green DE. Cardiolipin requirement for electron transfer in complex I and III of the mitochondrial respiratory chain. *J Biol Chem*. 1981;256:1874–1880.
29. Drose S, Zwicker K, Brandt U. Full recovery of the NADH. ubiquinone activity of complex I (NADH: ubiquinone oxidoreductase) from *Yarrowia lipolytica* by the addition of phospholipids. *Biochim Biophys Acta*. 2002;1556:65–72.
30. Paradies G, Petrosillo G, Pistolese M, Ruggiero FM. Reactive oxygen species affect mitochondrial electron transport complex I activity through oxidative cardiolipin damage. *Gene*. 2002;282:135–141.
31. Petrosillo G, Ruggiero FM, Di Venosa N, Paradies G. Decreased complex III activity in mitochondria isolated from rat heart subjected to ischemia and reperfusion: role of reactive oxygen species and cardiolipin. *FASEB J*. 2003;17:714–716.
32. Paradies G. Interaction of α-cyano[^{14}C]cinnamate with the mitochondrial pyruvate translocator. *Biochim Biophys Acta*. 1984;766:446–450.
33. Black MJ, Brandt RB. Spectroflurometric analysis of hydrogen peroxide. *Anal Biochem*. 1974;58:246–254.
34. Loschen G, Flohè L, Chance B. Respiratory chain linked H_2O_2 production in pigeon heart mitochondria. *FEBS Lett*. 1971;18:261–264.
35. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*. 1959;37:911–917.
36. Ruggiero FM, Landriscina C, Gnoni GV, Quagliariello E. Lipid composition of liver mitochondria and microsomes in hyperthyroid rats. *Lipids*. 1984;19:171–178.
37. Parinardi NL, Weis BK, Schimid HHO. Assay of cardiolipin peroxidation by high-performance liquid chromatography. *Chem Phys Lipids*. 1988;49:215–220.
38. Shidoji Y, Hayashi K, Komura S, Ohishi N, Yagi K. Loss of molecular interaction between cytochrome *c* and cardiolipin due to lipid peroxidation. *Biochem Biophys Res Commun*. 1999;264:343–347.
39. Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol*. 1978;52:302–310.
40. Hackenbrock CR, Chazotte B. Lipid enrichment and fusion of mitochondrial inner membranes. *Methods Enzymol*. 1986;125:35–45.
41. Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E. Peroxidative damage to cardiac mitochondria: cytochrome oxidase and cardiolipin alterations. *FEBS Lett*. 1998;424:155–158.
42. Lopez-Torres M, Gredilla R, Sanz A, Barja G. Influence of aging and long-term caloric restriction on oxygen radical generation and oxidative DNA damage in rat liver mitochondria. *Free Radic Biol Med*. 2002;32:882–889.
43. Hardy L, Clark JB, Darley-Usmar VM, Smith DR, Stone D. Reoxygenation-dependent decrease in mitochondrial NADH:CoQ reductase (complex I) activity in the hypoxic/reoxygenated rat heart. *Biochem J*. 1995;274:133–137.
44. Kovaltowski AJ, Castilho RF. Vercesi A. Ca^{2+}-induced mitochondrial membrane permeabilization: role of coenzyme Q redox state. *Am J Physiol Cell Physiol*. 1995;38:C141–C147.
45. Turrens JF. Superoxide production by the mitochondrial respiratory chain. *Biosci Rep*. 1997;17:3–8.
46. Lenaz G. Role of mitochondria in oxidative stress and ageing. *Biochim Biophys Acta*. 1998;1366:53–67.
47. Cadenas E, Boveris A, Ragan CI, Stoppani AOM. Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinl-cytochrome *c* reductase from beef-heart mitochondria. *Arch Biochem Biophys*. 1977;180:248–257.
48. Turrens JF, Boveris A. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem J*. 1980;191:421–427.
49. Becker LB, Vanden Hoek TL, Shao Z, Li C, Shumacker PT. Generation of superoxide in cardiomyocytes during ischemia before reperfusion. *Am J Physiol*. 1999;277:H2240–H2246.
50. Ide T, Shintaro T, Utsumi U, Kang D, Hattori N, Uccida K, Arimura K, Egashira K, Takeshita A. Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium. *Circ Res*. 1999;85:357–363.
51. Petit JM, Maftah A, Ratinaud MH, Julien R. 10-*N*-nonylacridine orange interact with cardiolipin and allows the quantitation of this phospholipid in isolated mitochondria. *Eur J Biochem*. 1992;209:267–273.
52. Schlame M, Hostetler KY. Cardiolipin synthase from mammalian mitochondria. *Biochim Biophys Acta*. 1997;1348:207–213.
53. Hatch GM. Regulation of cardiolipin biosynthesis in the heart. *Mol Cell Biochem*. 1996;159:139–148.
54. Zhang M, Mileykovskaya E, Dowhan W. Gluing the respiratory chain together: cardiolipin is required for supercomplex formation in the inner mitochondrial membrane. *J Biol Chem*. 2002;277:43553–43556.

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