Dissociation of Cytochrome c from the Inner Mitochondrial Membrane during Cardiac Ischemia*

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Mitochondria isolated from ischemic cardiac tissue exhibit diminished rates of respiration and ATP synthesis. The present study was undertaken to determine whether cytochrome c release was responsible for ischemia-induced loss in mitochondrial function. Rat hearts were perfused in Langendorff fashion for 60 min (control) or for 30 min followed by 30 min of no flow ischemia. Mitochondria isolated from ischemic hearts in a buffer containing KCl exhibited depressed rates of maximum respiration and a lower cytochrome c content relative to control mitochondria. The addition of cytochrome c restored maximum rates of respiration, indicating that the release of cytochrome c is responsible for observed declines in function. However, mitochondria isolated in a mannitol/sucrose buffer exhibited no ischemia-induced loss in cytochrome c content, indicating that ischemia does not on its own cause the release of cytochrome c. Nevertheless, state 3 respiratory rates remained depressed, and cytochrome c release was enhanced when mitochondria from ischemic relative to perfused tissue were subsequently placed in a high ionic strength buffer, hypotonic solution, or detergent. Thus, events that occur during ischemia favor detachment of cytochrome c from the inner membrane increasing the pool of cytochrome c available for release. These results provide insight into the sequence of events that leads to release of cytochrome c and loss of mitochondrial respiratory activity during cardiac ischemia/reperfusion.

Cardiovascular disease characterized by diminished coronary blood flow is a leading cause of debilitation and mortality worldwide. Immediate consequences of oxygen deprivation are a reduction in the rate of oxidative phosphorylation, incomplete oxidation of glycolytic metabolites, depletion of creatine phosphate and ATP, and metabolic acidosis (1–11). Declines in both pH and ATP concentration adversely affect the maintenance of ionic gradients and are in part responsible for intracellular Ca²⁺ overload (4, 5). If the episode persists, mitochondria isolated from ischemic myocardial tissue exhibit intrinsic declines in the ability to carry out maximum rates of respiration (1–3, 7, 10, 11). This deficit in activity would limit restoration of normal cardiac function upon reintroduction of blood flow. It is therefore critical to define molecular mechanism(s) responsible for ischemia-induced inactivation of mitochondrial function.

We have previously reported that, in an isolated rat heart model, ischemia results in inhibition of complex I with no alterations in other respiratory chain complex activities (11). The results of in vitro studies revealed that respiratory complex activities must be reduced by greater than 50% to exert effects on maximum rates of mitochondrial respiration and ATP synthesis (11). Therefore complex I inactivation was of insufficient magnitude to account for ischemia-induced declines in the respiratory activity of isolated mitochondria (11). It is important to note, however, that complex activities are measured upon the addition of specific electron carriers. Thus, release of respiratory chain components during ischemia may be overlooked as contributing factors in the loss of mitochondrial respiration when standard assays for complex activities are utilized. One such electron transport chain component is cytochrome c, a protein required for the transfer of electrons from complexes III to IV.

In the current study, we utilized an isolated buffer-perfused rat heart model to investigate mechanism(s) by which mitochondrial respiratory function is impaired during cardiac ischemia. It has previously been reported that mitochondria isolated from ischemic myocardial tissue exhibit a decrease in the characteristic absorbance of cytochrome c (3, 7, 12). We sought immunochromatographic evidence that ischemia induces alterations in the association of cytochrome c with inner membrane components and that these events are responsible for diminished rates of NADH-linked ADP-dependent mitochondrial respiration. In addition, we utilized different mitochondrial isolation buffers and performed in vitro studies with intact cardiac mitochondria to determine when in the sequence of events cytochrome c dissociated from the inner mitochondrial membrane and was subsequently released into cytosolic extracts.

MATERIALS AND METHODS

Preparation and Perfusion of Isolated Rat Heart—The hearts were isolated from adult (6–8 months of age) male Fisher-344 rats (National Institute of Aging colony) and perfused in retrograde fashion as described previously (10, 11). Briefly, the rats were administered heparin (500 units) and sodium pentobarbital. The heart was rapidly removed through a midline incision and rinsed in ice-cold modified Krebs-Henseleit buffer (120 mM NaCl, 4.8 mM KCl, 2.0 mM CaCl₂, 1.25 mM MgCl₂, 1.25 mM KH₂PO₄, 22 mM NaHCO₃, and 10 mM glucose). After the extraneous tissue was removed, the aorta was cannulated, and the heart was perfused in retrograde fashion according to the Langendorff procedure (10, 11) with modified Krebs-Henseleit buffer saturated with 95% O₂, 5% CO₂ (60 mm Hg, 37 °C). Throughout each protocol, the hearts were immersed in a water-jacketed chamber (37 °C) and paced at 300 beats/min. Left ventricular pressure was monitored utilizing a pressure transducer within a latex balloon placed into the left ventricle. The balloon was inflated to an initial left ventricular end diastolic pressure of 5.0 mm Hg. Experiments consisted of: (i) 60-min normoxic...
perfusion or (ii) 30-min normoxic perfusion followed by 30 min no flow ischemia.

Isolation of Subsarcolemmal Rat Heart Mitochondria and Cytosolic Fractions—Subsarcolemmal mitochondria were isolated from the ventricles as follows. The ventricles were rapidly dissected and rinsed in ice-cold homogenization buffer (180 mM KCl, 5.0 mM MOPS, 2.0 mM EGTA, pH 7.4, or 210 mM mannitol, 70 mM sucrose, 5.0 mM MOPS, 1.0 mM EDTA, pH 7.4, as indicated). The ventricles were blotted dry, weighed, minced with scissors, and then homogenized in 20 ml/g of tissue in cold homogenization buffer (Polytron homogenizer, low setting, 3 s). The homogenate was then centrifuged at 500 × g for 5.0 min. The supernatant was filtered through cheese cloth and centrifuged at 5,000 × g for 10.0 min. The mitochondrial pellet was washed twice and resuspended into homogenization buffer at a final protein concentration of ∼3.0 mg/ml. The 5,000 × g supernatant represented the cytosolic fraction (protein concentration, ∼1.5 mg/ml). All manipulations were carried out at 4 °C. For in vitro studies, cardiac mitochondria and cytosolic fractions were prepared from rat hearts immediately following euthanasia with a lethal dose of sodium pentobarbital. Protein concentration was determined using the bicinchoninic assay (Pierce), with bovine serum albumin as standard.

Evaluation of Mitochondrial O2 Consumption—Rates of NADH-linked ADP-dependent (state 3) and ADP-independent (state 4) respiration were measured using a Clark-type oxygen electrode (Instech, Plymouth Meeting, PA) as described (10, 11). Briefly, mitochondria were diluted to a concentration of 0.5 mg/ml in assay buffer (120 mM KCl, 5.0 mM KH2PO4, 1.0 mM EDTA, pH 7.25, or 210 mM mannitol, 70 mM sucrose, 5.0 mM KH2PO4, 1.0 mM EDTA, pH 7.4, as indicated) and placed in a sealed oxygen chamber. Respiration was initiated by addition of glutamate (15 mM). After 2.0 min, state 3 respiration was induced by addition of ADP (0.5 mM). Upon depletion of ADP, the rate of state 4 respiration was evaluated. Where indicated, respiration was monitored upon addition of cytochrome c (1.0 μM) at the time of initiation of respiration.

Detection of Cytochrome c by Western Blot Analysis—Mitochondria prepared from isolated rat hearts exposed to perfusion (60 min) or ischemia (30 min perfusion followed by 30 min ischemia) were diluted to 1.0 mg protein/ml in 5.0 mM KH2PO4, 0.5 mM EDTA, pH 7.25. An equal volume of 2 × sample buffer (126 mM Tris-HCl, 20% glycerol, 4.0% SDS, 1.0% 2-mercaptoethanol, 0.005% bromphenol blue, pH 6.8) was then added. The samples were loaded onto 14% Tris-buffered polyacrylamide gels, and component proteins were resolved by SDS-PAGE (Novex Mini-Cell II). The proteins were electroblotted onto a nitrocellulose membrane (0.45 μm), and component proteins were resolved by SDS-PAGE (Novex Mini-Cell II). The proteins were electroblotted onto a nitrocellulose membrane (0.45 μm), and cytochrome c was detected utilizing monoclonal anti-cytochrome c (PharMingen) as primary antibody and anti-mouse IgG conjugated to alkaline phosphatase as secondary antibody. Primary antibody binding was visualized with an alkaline phosphatase-based chemiluminescence system (Tropix). As indicated, densitometric analyses were performed utilizing NIH Image software.

Quantitation of Mitochondrial Cytochrome c Content by Reverse Phase HPLC—Cardiac mitochondria (60.0 μl at 5.0 mg/ml) prepared from hearts following perfusion (60 min) or ischemia (30 min perfusion followed by 30 min no flow ischemia) were diluted into an equal volume (60.0 μl) of 5.0 mM KH2PO4, 0.5 mM EDTA, pH 7.25. An equal volume of 2× sample buffer (126 mM Tris-HCl, 20% glycerol, 4.0% SDS, 1.0% 2-mercaptoethanol, 0.005% bromphenol blue, pH 6.8) was then added. The samples were loaded onto 14% Tris-buffered polyacrylamide gels, and component proteins were resolved by SDS-PAGE (Novex Mini-Cell II). The proteins were electroblotted onto a nitrocellulose membrane (0.45 μm), and cytochrome c was detected utilizing monoclonal anti-cytochrome c (PharMingen) as primary antibody and anti-mouse IgG conjugated to alkaline phosphatase as secondary antibody. Primary antibody binding was visualized with an alkaline phosphatase-based chemiluminescence system (Tropix). As indicated, densitometric analyses were performed utilizing NIH Image software.

Electron Microscopy—Mitochondria were isolated from ischemic and reperfused myocardial tissue in mannitol/sucrose buffer by density centrifugation as detailed above. The mitochondrial fraction was resuspended and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (12 h). Mitochondria were post-fixed utilizing 1% OsO4 and reperfused myocardial tissue in mannitol/sucrose buffer by density centrifugation as detailed above. The mitochondrial fraction was resuspended and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (12 h). Mitochondria were post-fixed utilizing 1% OsO4. En bloc staining with uranyl acetate was followed by dehydration with increasing concentrations of ethanol. Resin PolyBed 812 was used for sample infiltration and embedding. Embedded samples were sectioned and affixed to grids according to standard protocols. Mitochondrial ultrastructure was then evaluated by transmission electron microscopy (JEOL 1200 CX electron microscope).

Statistical Analysis—The data were evaluated utilizing a two-tailed t test. Statistical significance was assigned for p ≤ 0.05, as indicated.

RESULTS

Effect of Cardiac Ischemia on Cytochrome c Content of Mitochondria—Cardiac ischemia was found to induce a loss in the cytochrome c content of isolated mitochondria. Hearts excised from adult rats were perfused for 60 min or for 30 min followed by 30 min no flow ischemia as described under “Materials and Methods.” Myocardial tissue was homogenized in a buffer composed of 180 mM KCl, 5.0 mM MOPS, 1.0 mM EDTA, pH 7.25. Mitochondria and cytosolic extracts were then isolated, and proteins were resolved by SDS gel electrophoresis using 14% gels (12.5 μg of total protein/lane) and transferred to a nitrocellulose membrane for Western blot analysis using anti-cytochrome c antibody. The gels were detected utilizing monoclonal antibody binding to cytochrome c. The blots depicted represent three separate rats for each experimental condition.

Effect of Cytochrome c Addition on Respiratory Activity of Mitochondria Isolated from Perfused and Ischemic Myocardial Tissue—Respiratory rates were assessed in a buffer composed of 125 mM KCl and 5.0 mM KH2PO4, pH 7.25. Ischemia (30 min) was found to induce a 41.8% loss in NADH-linked ADP-dependent (state 3) respiration relative to control mitochondria (p ≤ 0.0001). This decrement in respiratory activity was abolished upon addition of cytochrome c (14.0 nmol/mg mitochondrial protein) to isolated respiring mitochondria (Fig. 2). Thus, ischemia-induced release of cytochrome c from cardiac mitochondria was found to induce a loss in the cytochrome c content of isolated mitochondria. Hearts excised from adult rats were perfused for 60 min or for 30 min followed by 30 min no flow ischemia as described under “Materials and Methods.” Myocardial tissue was homogenized in a buffer composed of 180 mM KCl, 5.0 mM MOPS, 1.0 mM EDTA, pH 7.25. Mitochondria and cytosolic extracts were then isolated, and proteins were resolved by SDS gel electrophoresis using 14% gels (12.5 μg of total protein/lane) and transferred to a nitrocellulose membrane for Western blot analysis using anti-cytochrome c antibody. The gels were detected utilizing monoclonal antibody binding to cytochrome c. The blots depicted represent three separate rats for each experimental condition.
mitochondria (Fig. 1) is largely responsible for the observed declines in maximal rates of respiration (Fig. 2). Mitochondrial state 4 respiratory rates, ADP/O, and yield were not altered by ischemia, indicating that ischemia did not cause global disruption of mitochondria. Importantly, the respiratory chain appears accessible to exogenously added cytochrome c. This suggests that ischemia and/or isolation conditions induce alterations in the outer mitochondrial membrane, which provide exit and entry for cytochrome c.

**Ischemia-induced Isolation-dependent Release of Cytochrome c**—The results presented in Fig. 1 do not indicate when in the sequence of events cytochrome c is released from mitochondria. This may occur during ischemia and/or during the isolation process. There are two commonly used homogenization buffers for mitochondrial isolation. To reflect physiologic K⁺ ion in the cytosol, KCl is employed in the buffer to establish an appropriate osmotic strength. It is well known, however, that exposure of mitochondria to KCl induces extrusion of cytochrome c (13-15). Alternatively, inclusion of mannitol and sucrose instead of KCl in the homogenization buffer is generally considered less disruptive to mitochondrial integrity. Isolated rat hearts were therefore subjected to 60 min of perfusion or to 30 min of perfusion followed by 30 min of ischemia. The hearts were homogenized in 210 mM mannitol, 70 mM sucrose, 5.0 mM MOPS, 1.0 mM EDTA, pH 7.25. Mitochondria and cytosolic extracts were then prepared and evaluated for cytochrome c content. In striking contrast to mitochondria isolated in KCl containing buffer (Fig. 1), mitochondria isolated in mannitol/sucrose exhibited no detectable decrease in cytochrome c content as a result of ischemia (Fig. 3). In keeping with this result, relatively little cytochrome c was detected in cytosolic extracts of either perfused or ischemic tissue (Fig. 3). The potential exists that K⁺-dependent release of cytochrome c would occur in situ upon extended periods of ischemia. Nevertheless, mitochondrial and cytosolic extracts prepared in mannitol/sucrose homogenization buffer from rat hearts (n = 3) that had undergone 60 min of ischemia did not exhibit any decrease or increase in cytochrome c content, respectively, relative to perfused values (not shown). These findings indicate that, even with prolonged periods of ischemia, cytochrome c is not released from the mitochondria. Nevertheless, events that occur during ischemia enhance KCl-induced release of cytochrome c in vitro (Fig. 1).

**Effect of Ischemia on Respiratory Activity of Mitochondria**

**Fig. 2. Effect of ischemia on ADP-dependent NADH-linked mitochondrial respiration: reversal by addition of cytochrome c.** Hearts obtained from adult rats were exposed to perfusion (P, 60 min) (n = 8) or no flow ischemia (I, 30 min) (n = 9). Mitochondria were then isolated, and the respiratory activities were assessed using a Clark-type oxygen electrode. Mitochondria at a concentration of 0.5 mg/ml were incubated with 15 mM glutamate for 2.0 min at 25 °C in the absence or presence of 7.0 μM cytochrome c. State 3 respiration was then initiated by addition of 0.5 mM ADP.

**Fig. 3. Effect of ischemia on mitochondrial and cytosolic cytochrome c content.** Hearts obtained from adult rats were exposed to perfusion (P, 60 min) or no flow ischemia (I, 30 min). Myocardial tissue was homogenized in a buffer containing 210 mM mannitol, 70 mM sucrose, 5.0 mM MOPS, 1.0 mM EDTA, pH 7.25. Mitochondria (M) and cytosolic extracts (C) were then isolated, and proteins were resolved by SDS gel electrophoresis using 14% gels (12.5 μg of total protein/lane) and transferred to a nitrocellulose membrane for Western blot analysis using anti-cytochrome c antibody. The blots depicted represent three separate rats for each experimental condition.

*Isolated in Mannitol/Sucrose Homogenization Buffer—The occurrence of ischemia-induced alterations in state 3 respiratory rates were investigated in mitochondria isolated in mannitol/sucrose-based buffer. Despite the fact that ischemia did not result in a release of cytochrome c (Fig. 3), state 3 respiratory rates were markedly depressed in mitochondria isolated from ischemic relative to perfused tissue (Fig. 4). The relative decline in state 3 respiration was ~30% irrespective of whether mitochondria were assayed in mannitol/sucrose-based (p ≤ 0.0007) or KCl-based (p ≤ 0.009) respiratory buffer. The addition of exogenous cytochrome c to mitochondria from perfused and ischemic myocardial tissue isolated and assayed in mannitol/sucrose buffers did not result in an increase in respiratory rates (not shown). This is in contrast to results shown in Fig. 2 indicating that mitochondria prepared in mannitol/sucrose buffer do not allow exit or entry of cytochrome c. Nevertheless, the decline in state 3 respiratory rates in mitochondria isolated in either KCl or mannitol/sucrose-based buffers indicates ischemia results in mitochondrial alterations that prevent cytochrome c from carrying out efficient electron transport and promote cytochrome c release in KCl buffers.

*Ischemia-induced Alterations in the Interaction(s) of Cytochrome c with the Inner Mitochondrial Membrane—Evidence indicates that mitochondrial cytochrome c can be associated with the inner membrane in both loosely and tightly bound states and can also be present as a soluble protein in the interfibrillar space (15, 16). These forms appear to exist in dynamic equilibrium, the relative distributions of which depend on factors such as ionic strength and redox status (15, 16). Thus, the observed ischemia-induced release of cytochrome c and decline in the rate of state 3 respiration could be due in part to ischemia-induced alterations in the associations of cytochrome c with the inner membrane followed by KCl-induced extrusion of labile cytochrome c from the mitochondria. To explore this possibility, mitochondria isolated in mannitol/sucrose-based homogenization buffer were subsequently diluted into KCl buffer, and release of cytochrome c from the mitochondria was monitored. Consistent with results shown in Fig. 3, total mitochondrial cytochrome c content was unaltered by ischemia (Fig. 5). Following incubation in KCl buffer, mitochondria were centrifuged, and cytochrome c released from (supernatant) and retained within (pellet) the mitochondria was assessed by Western blot analysis. As shown in Fig. 5, KCl induced a significant release of cytochrome c from mitochondria. KCl-induced release was enhanced in mitochondria isolated from ischemic relative to perfused myocardial tissue (Fig. 5). As determined by densitometric analysis, 42.8 ± 9.1 and 71.0 ± 2.2% (p ≤ 0.004 for a two-tailed t test) of total cytochrome c was released from mitochondria isolated from hearts exposed to perfusion and ischemia, respectively. Thus, following treatment with KCl, mitochondria from ischemic myocardial tissue contain 50.7% of the cytochrome c content relative to...
Mitochondria at a concentration of 0.5 mg/ml were incubated and the respiratory activities were assessed using a Clark-type oxygen electrode. Mitochondria isolated in mannitol/sucrose homogenization buffer. Hearts obtained from adult rats were exposed to perfusion (P, 60 min) or no flow ischemia (I, 30 min) (n = 3). Mitochondria were then isolated, and the respiratory activities were assessed using a Clark-type oxygen electrode. Mitochondria at a concentration of 0.5 mg/ml were incubated and the respiratory activities were assessed using a Clark-type oxygen electrode. Mitochondria isolated in either KCl or mannitol/sucrose-based homogenization buffers and subsequently fixed for electron microscopic analysis as described under “Materials and Methods.”

Assay Buffer: Mannitol/Sucrose

KCl

FIG. 4. Effect of ischemia on state 3 respiration of mitochondria isolated in mannitol/sucrose homogenization buffer. Hearts obtained from adult rats were exposed to perfusion (P, 60 min) or no flow ischemia (I, 30 min) (n = 3). Mitochondria were then isolated, and the respiratory activities were assessed using a Clark-type oxygen electrode. Mitochondria at a concentration of 0.5 mg/ml were incubated with 15 mM glutamate for 2.0 min at 25°C. State 3 respiration was then initiated by the addition of 0.5 mM ADP. Assay buffer contained either 120 mM KCl, 5.0 mM KH₂PO₄, pH 7.25, or 210 mM mannitol, 70 mM succrose, 5.0 mM KH₂PO₄, 1.0 mM EDTA, pH 7.4, as indicated.

FIG. 5. Effect of ischemia on the susceptibility of mitochondria isolated in mannitol/sucrose to KCl-dependent release of cytochrome c. Hearts obtained from adult rats were exposed to perfusion (P, 60 min) or no flow ischemia (I, 30 min). Mitochondria were isolated in buffer containing 210 mM mannitol, 70 mM succrose, 5.0 mM MOPS, 1.0 mM EDTA, pH 7.25. Mitochondria were diluted to 1.0 mg/ml in oxidative phosphorylation assay buffer composed of 120 mM KCl, 5.0 mM KH₂PO₄, pH 7.25, and allowed to respire with 15.0 mM glutamate for 5.0 min. The samples were then centrifuged at 10,000 × g for 5.0 min. Total mitochondrial cytochrome c content (total, aliquot of mitochondria obtained just prior to centrifugation) as well as levels of cytochrome c retained within (pellet) and extruded from (supernatant) the mitochondria were determined by Western blot analysis as described under “Materials and Methods.” The blot shown is representative of three separate rats for each experimental protocol.

perfused values (Fig. 5), in good agreement with the results presented in Fig. 1. No release was observed in mannitol/sucrose (not shown), indicating that these effects were not simply the result of mechanical disruption. Mitochondria isolated in KCl-based homogenization buffer subjected to the same experimental protocol failed to release any of the remaining cytochrome c (not shown). Thus, ischemia-induced alterations in the associations of cytochrome c with the inner mitochondrial membrane appear to be retained upon isolation, allowing for enhanced KCl-induced release from the mitochondria.

To provide further support for decreased association of cytochrome c with the inner mitochondrial membrane during ischemia, mitochondria isolated in mannitol/sucrose buffer were incubated in hypotonic buffer and isolation buffer containing 0.025% Tween 20. These conditions are well known to favor the disruption of the outer mitochondrial membrane and thus can be used to assess relative differences in the labile pools of cytochrome c. As shown in Fig. 6, mitochondria isolated from ischemic relative to perfused tissue exhibited enhanced release of cytochrome c upon exposure to either condition. As determined by densitometric analyses, the fraction of total cytochrome c released from mitochondria from perfused and ischemic hearts was 10.6 ± 3.2 and 23.5 ± 4.9%, respectively (p < 0.001 for a two-tailed t-test, n = 3). These values are less than those observed upon incubation of mitochondria with KCl (Fig. 4.1).

DISCUSSION

Cytochrome c is an essential component of the mitochondrial electron transport chain. Located on the outer surface of the inner mitochondrial membrane, cytochrome c passes electrons between complexes III and IV. In this study, we provide evidence that ischemia-induced declines in the rate of mitochondrial respiration and ATP synthesis are due, at least in part, to decreased association of cytochrome c with the inner mitochondrial membrane. Inhibition of NADH-linked ADP-dependent respiratory rates resulting from ischemia was observed in mitochondria isolated in either KCl or mannitol/sucrose-based

FIG. 6. Effect of ischemia on the susceptibility of mitochondria isolated in mannitol/sucrose to release of cytochrome c upon outer membrane disruption. Hearts obtained from adult rats were exposed to perfusion (P, 60 min) or no flow ischemia (I, 30 min). Mitochondria were isolated in buffer containing 210 mM mannitol, 70 mM succrose, 5.0 mM MOPS, 1.0 mM EDTA, pH 7.25. Mitochondria were diluted to 1.0 mg/ml in either 10 mM MOPS, 0.5 mM EDTA, pH 7.25 (Hypotonic) or mannitol/sucrose-based mitochondrial isolation buffer containing 0.025% Tween 20 (Tween). The samples were incubated at 25°C for 20 min followed by centrifugation at 10,000 × g for 5.0 min. Cytochrome c released upon outer membrane rupture was assessed in supernatants by Western blot analysis as described under “Materials and Methods.”

FIG. 7. Structural analysis of mitochondria isolated from perfused and ischemic myocardial tissue. Following isolation in mannitol/sucrose buffer, mitochondria were processed for electron microscopic analysis as described under “Materials and Methods.” The electron micrographs are at a magnification of 10,000×.
homogenization buffers. Enhanced release of cytochrome c and restoration of respiratory activity upon incubation with exogenous cytochrome c were evident in mitochondria isolated from ischemic cardiac tissue in KCl medium. Although ADP-dependent respiratory rates were depressed, cytochrome c release caused by ischemia was not observed when mitochondria were isolated in mannitol/sucrose buffer. Nevertheless, enhanced ischemia-dependent cytochrome c release was observed upon exposure of mannitol/sucrose mitochondrial preparations to KCl containing buffer, hypotonic solution, or detergent. These observations are consistent with the view that events that occur during ischemia result in decreased association of cytochrome c with the inner mitochondrial membrane, thereby slowing electron transport and eliciting declines in the rate of ADP-dependent mitochondrial respiration. Thus, ischemia increases the pool of cytochrome c available for release upon isolation or incubation of mitochondria in KCl medium.

The results of this study have important implications for investigations into the mechanism(s) by which mitochondrial structure/function are altered during cardiac ischemia/reperfusion and the progression of other pathological conditions. Standard protocols for isolation of mitochondria utilize either KCl or mannitol/sucrose containing buffers. Although the composition of KCl medium typically reflects physiological K+ and strong ionic strength (17, 18), the use of this buffer could promote occurrences not directly related to experimental conditions. Moreover, certain structural properties may differ in mitochondria isolated in KCl versus mannitol/sucrose buffers, with further alterations possible upon evaluation of mitochondrial respiratory function in a variety of possible oxidative phosphorylation buffers. In particular, cytochrome c exit from and entry into the mitochondria appears dependent on factors related to medium composition through processes not clearly understood. The mechanisms currently under investigation include KCl-dependent insertion of certain Bcl-2 family proteins (e.g. Bax, Bak, Bad, and tBid) into the outer membrane and induction of the mitochondrial permeability transition (13–15, 19–22). Clearly, translocation of cytochrome c is likely dependent on the presence of various components, many of which may be differentially retained under numerous isolation and assay conditions. For example, in earlier work utilizing an isolated buffer-perfused guinea pig heart model (3) and an in vivo dog model of coronary occlusion (1), the addition of cytochrome c to mitochondria isolated from ischemic myocardial tissue resulted in variable restoration of mitochondrial respiration. Murfitt et al. (1) reported that diminished rates of respiration were not abolished by cytochrome c addition, whereas Piper et al. (3) observed partial restoration of mitochondrial respiratory function. We found that at concentrations greater than optimum (14 nmol/mg mitochondrial protein), cytochrome c exerted inhibitory effects on state 3 respiratory rates (not shown). Thus, apparent discrepancies in the effects of cytochrome c are likely due to the concentrations utilized and/or the potential contribution(s) of a wide variety of other factors.

Previous findings and information on the nature of the interaction of cytochrome c with the inner mitochondrial membrane suggest a plausible mechanism by which the associations of cytochrome c with the inner mitochondrial membrane are altered during cardiac ischemia. In numerous models of ischemia, cardiolipin content in the mitochondrial membrane has been shown to decrease (23–25). Positively charged residues on the surface of cytochrome c complement negatively charged residues of electron transport chain proteins, thereby conferring specific interactions (18, 26). Similar electrostatic interactions are thought to anchor cytochrome c to cardiolipin within the membrane. Orrenius and co-workers (15) have shown that peroxidation of cardiolipin is an efficient means for promoting cytochrome c detachment and can be used to distinguish between loosely and tightly bound forms of the protein. It is therefore likely that the loss of cardiolipin during ischemia results in a disruption of electrostatic interactions and an increase in the dissociable pool of cytochrome c. This would also explain the fact that the latency of cytochrome c and the deficit in state 3 respiratory rates were largely preserved when mitochondria were isolated in a low ionic strength mannitol/sucrose buffer. Isolation of mitochondria in high ionic strength buffer serves to enhance detachment even in control mitochondria but also, by mechanisms not fully understood, allows exit and entry of cytochrome c from the mitochondria.

Alterations in the subcellular distribution of cytochrome c can have profound effects on cellular viability. In addition to a decline in the rate of ATP synthesis, disruption of the interaction(s) of cytochrome c with the inner mitochondrial membrane would result in an increase in the ratio of reduced to oxidized components of the electron transport chain preceding complex IV. This would be expected to promote mitochondrial generation of oxygen radicals during reoxygenation. Interestingly, it has been recently shown that cytochrome c-depleted rat brain mitochondria produce elevated levels of reactive oxygen species (27). Cardiac ischemia/reperfusion has also been associated with the induction and propagation of the apoptotic process (28–33). Cytochrome c may play a central role in this pathway. Once released into the cytosol, cytochrome c can form a complex with apoptotic-protease-activating factor-1 and pro-caspase 9. This results in caspase 9-dependent activation of caspase 3, nuclear DNA fragmentation, and cell death (21, 34–36). Increases in the labile pool of cytochrome c during cardiac ischemia are therefore likely to prime mitochondria for subsequent release of the protein in response to certain physiologic and pathophysiologic conditions. Potential mechanism(s) for reperfusion-induced cytochrome c extrusion from the mitochondria include the formation of cytochrome c-permeable pores derived from pro-apoptotic members of the Bcl-2 family such as Bax and/or induction of the mitochondrial permeability transition (3, 13–15, 19–22, 37). Clearly, events initiated during ischemia predispose the myocardium to detrimental effects of reperfusion. Decreased association of cytochrome c with the inner mitochondrial membrane is likely to be one such event. Future studies must address the consequences of this event during cardiac reperfusion.

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