Modulation of electron transport protects cardiac mitochondria and decreases myocardial injury during ischemia and reperfusion

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Chen Q, Camara AK, Stowe DF, Hoppel CL, Lesnefsky EJ. Modulation of electron transport protects cardiac mitochondria and decreases myocardial injury during ischemia and reperfusion. Am J Physiol Cell Physiol 292: C137–C147, 2007. First published September 13, 2006; doi:10.1152/ajpcell.00270.2006.—Mitochondria are increasingly recognized as lynchpins in the evolution of cardiac injury during ischemia and reperfusion. This review addresses the emerging concept that modulation of mitochondrial respiration during and immediately following an episode of ischemia can attenuate the extent of myocardial injury. The blockade of electron transport and the partial uncoupling of respiration are two mechanisms whereby manipulation of mitochondrial metabolism during ischemia decreases cardiac injury. Although protection by inhibition of electron transport or uncoupling of respiration initially appears to be counterintuitive, the continuation of mitochondrial oxidative phosphorylation in the pathological milieu of ischemia generates reactive oxygen species, mitochondrial calcium overload, and the release of cytochrome c. The initial target of these deleterious mitochondrial-driven processes is the mitochondria themselves. Consequences to the cardiomyocyte, in turn, include oxidative damage, the onset of mitochondrial permeability transition, and activation of apoptotic cascades, all favoring cardiomyocyte death. Ischemia-induced mitochondrial damage carried forward into reperfusion further amplifies these mechanisms of mitochondrial-driven myocardial injury. Interruption of mitochondrial respiration during early reperfusion by pharmacologic blockade of electron transport or even recurrent hypoxia or brief ischemia paradoxically decreases cardiac injury. It increasingly appears that the cardioprotective paradigms of ischemic preconditioning and postconditioning utilize modulation of mitochondrial oxidative metabolism as a key effector mechanism. The initially counterintuitive approach to inhibit mitochondrial respiration provides a new cardioprotective paradigm to decrease cellular injury during both ischemia and reperfusion.

cardioliopin; cytochrome c; complex I; cytochrome oxidase

MITOCHONDRIA are both targets and sources of injury during cardiac ischemia and reperfusion. This review addresses the emerging concept that modulation of mitochondrial oxidative metabolism during ischemia or early reperfusion protects mitochondrial function and decreases myocardial cell death. Mitochondria contribute to their own damage during ischemia, since blockade of electron transport immediately before ischemia dramatically attenuates damage to mitochondrial electron transport, with preserved oxidative phosphorylation, retention of cytochrome c, and decreased release of reactive oxygen species (ROS). Ischemic preconditioning (IPC) leads to a partial uncoupling of mitochondrial respiration, resulting in decreased mitochondrial injury following subsequent sustained ischemia. A substantial portion of damage to mitochondrial electron transport and oxidative phosphorylation surprisingly occurs during ischemia, rather than during reperfusion. Ischemic mitochondrial damage appears to be a major mechanism of cardiac injury, since reperfusion of myocardium that contain mitochondria with preserved oxidative function in fact markedly decreases myocardial injury. During reperfusion, ischemically damaged mitochondria further augment oxidative damage, calcium-driven myocyte injury, and the activation of apoptotic programs. Transient blockade of mitochondria during early reperfusion, by ischemia, hypoxia, or pharmacological inhibition paradoxically decreases myocardial injury, presumably by blunting the deleterious consequences of respiration in mitochondria that suffered ischemic damage. Thus the restriction of mitochondrial oxidative metabolism by therapeutic intervention via the activation of signaling cascades is a key mechanism of cardiac protection during ischemia and reperfusion. This conceptual approach should continue to promote the continued development of mitochondrial-based treatments to limit cardiac injury during ischemia and during reperfusion.

MITOCHONDRIA AS TARGETS OF DAMAGE DURING ISCHEMIA

Mitochondria sustain progressive damage during myocardial ischemia (45, 47, 69, 79, 90, 113, 120, 126, 140), including to the electron transport chain (10, 45, 47, 83, 90, 113, 120, 143). Ten to twenty minutes of ischemia decreases complex I activity (47, 120). Damage to the phosphorylation apparatus, including complex V (120) and the adenine nucleotide transporter (10, 45), also occurs relatively early in ischemia. As ischemic periods are lengthened to 30 and 45 min, oxidative phosphorylation through complex III (83) and cytochrome oxidase decreases (87, 90, 113, 140). Damage to the phosphorylation apparatus does not explain the decrease in state 3 respiration observed as ischemia progresses since dinitrophenol-uncoupled respiration is decreased, localizing the functionally significant site of damage to the electron transport chain (ETC)
DURING ISCHEMIA

Cardiac mitochondria exist in two functionally distinct populations, subsarcolemmal mitochondria (SSM) that reside beneath the plasma membrane and interfibrillar mitochondria (IFM) located between the myofibrils (87, 107). SSM have a decreased capacity for calcium accumulation compared with IFM (108). Calcium loading in SSM led to the release of cytochrome c, whereas a similar extent of calcium loading in IFM did not result in cytochrome c release, even when the capacity of IFM to retain calcium was exceeded (108). Thus SSM are more susceptible to calcium overload-mediated cytochrome c release and mitochondrial damage compared with IFM (108). Progression of ischemic damage is more rapid in SSM than in IFM (45, 69, 90, 126, 140).

Complex I activity decreases during ischemia due to a decrease in the NADH dehydrogenase component (104, 120), likely due to the loss of the flavin mononucleotide coenzyme (120). Damage to the NADH dehydrogenase component of complex I can increase electron leak and the production of ROS (139). Complex I activity is also modulated by posttranslational modifications, including S-nitrosylation (22) and phosphorylation (31). Ischemia damages complex III by functional inactivation of the iron-sulfur protein (ISP) subunit (83). The 22 kDa ISP contains a 2 Fe-2 S redox-active iron-sulfur cluster. Ischemic damage to the iron-sulfur protein decreases the intensity of the electron paramagnetic resonance signal of the iron-sulfur cluster without the loss of the ISP peptide, suggesting that ischemia disrupts the cluster without degradation of the ISP subunit (83). Cytochrome oxidase, composed of 13 peptide subunits, requires the integrity of catalytic subunits (13) and of regulatory and structural subunits (8), as well as an intact inner mitochondrial membrane environment enriched in the phospholipid cardiolipin for optimal activity (119, 144). Ischemia does not lead to the functional inactivation of a subunit peptide (90). Decreased respiration through cytochrome oxidase occurs due to a selective decrease in cardiolipin content (89).

MITOCHONDRIA AS SOURCES OF CARDIAC INJURY DURING ISCHEMIA

Because of the limitation of oxygen that is present during ischemia, the initial focus on oxidative damage to myocardium historically involved reperfusion. A detectable burst of ROS is generated early in reperfusion (4, 16, 71, 96, 97, 112). Early research led to the belief that during cardiac ischemia, oxygen content would rapidly decrease to complete anoxia, with all oxygen consumed by cytochrome oxidase. However, during the initial progression of myocardial ischemia, oxygen remains available (68). During simulated ischemia in cardiomyocytes, under conditions of oxygen depletion, the ROS generation actually increases (15, 142). In the setting of low-flow ischemia, ROS production occurs continuously (97). Even during stop flow ischemia in the isolated perfused heart, tissue oxygen remains detectable by sensitive electron paramagnetic resonance measurement at least during the initial 10 min of ischemia (68). The production of ROS monitored online increases during global ischemia, which is consistent with the presence of oxygen during the evolution of ischemic injury (71, 77, 116, 129).

Mitochondria are a primary source for ROS production during ischemia (15, 39, 142). Superoxide production during simulated ischemia in cardiac myocytes is decreased by blockade of electron transfer into complex III (15, 142). In contrast, antimycin A blockade distal within complex III or inhibition of cytochrome oxidase increased ROS generation (15). These observations are in line with complex III as the dominant site for extra mitochondrial release of ROS (30, 59). Complex III is emerging as the major site for the production of ROS that are detected within the cardiomyocyte during ischemia.

Ischemia leads to release of cytochrome c from mitochondria. Cytochrome c content decreases in SSM at 30 min of ischemia, concomitant with cardiolipin loss (89, 90). Cardiolipin interacts with cytochrome c via nonionic (121, 122, 128) and electrostatic (106) mechanisms that localize cytochrome c at the inner membrane (106). Cardiolipin depletion delocalizes cytochrome c from the inner membrane (106, 125), the first step in cytochrome c release from mitochondria (106). A subsequent increase in permeability of the mitochondrial outer membrane then allows the loss of delocalized cytochrome c (106). Outer membrane permeability increases due to disruption of the outer membrane lipid bilayer, as a result of the activation of cytosolic and perhaps mitochondrial phospholipases (50), or the insertion of proapoptotic peptides into the mitochondrial outer membrane (73).

Cytochrome c release into cytosol occurs during ischemia in isolated rat and rabbit hearts (18, 20, 21, 89, 90). Release of cytochrome c during ischemia activates downstream caspase 3 leading to an increase in the number of cardiomyocytes displaying apoptotic markers (21) that becomes increasingly evident during reperfusion (21, 25, 53, 63). In the isolated perfused rabbit heart, the decrease in mitochondrial cytochrome c content occurs during ischemia, without additional decreases during reperfusion (81). Thus injury to mitochondria during ischemia is sufficient to release cytochrome c for activation of apoptotic pathways.

BLOCKADE OF ELECTRON TRANSPORT PROTECTS MITOCHONDRIA AND DECREASES ROS PRODUCTION DURING ISCHEMIA

Mitochondria generate cytotoxic ROS during ischemia (15, 71, 142). Cardiolipin, required for cytochrome oxidase activity (119, 144), is enriched in oxidatively sensitive linoleic acyl-groups (65). We propose that during ischemia, oxidative damage produced by the ETC, most likely complex III, leads to the decrease in cardiolipin content, in turn leading to the loss of cytochrome c and decreased rates of oxidation through cytochrome oxidase. In this event, the blockade of electron flow during ischemia should interrupt this pathogenic sequence.

To address this proposed scheme during in situ ischemia in the intact heart, the ETC was inhibited with rotenone, an irreversible inhibitor of complex I. The contents of cardiolipin, cytochrome c, and the rate of oxidation through cytochrome oxidase were measured after 45 min of global ischemia in the isolated rabbit heart (80). In untreated controls, ischemia decreased the contents of cardiolipin and cytochrome c, and decreased respiration through cytochrome oxidase as described above. In the presence of rotenone-mediated blockade of electron transport, cardiolipin content was preserved in SSM even after 45 min of ischemia (80) (Fig. 1). Rotenone treatment
favored the retention of cytochrome c by SSM (80) (Fig. 1). ADP-stimulated respiration through cytochrome oxidase in SSM was higher in rotenone-treated hearts (Fig. 1). Thus, blockade of the proximal ETC with rotenone, a high-affinity and irreversible inhibitor of complex I, markedly attenuated ischemic damage to cardiolipin, cytochrome c, and cytochrome oxidase, even following a relatively prolonged period of 45 min. On the basis of the critical role of cardiolipin in retaining cytochrome c within the mitochondria, it is not surprising that preservation of cardiolipin content during ischemia by rotenone treatment led to preserved cytochrome c content. Thus ischemic damage to mitochondria is mediated by the mitochondria themselves (80). Limitation of electron flow during ischemia is a new concept to limit mitochondrial damage during ischemia. Although the irreversible inhibitor rotenone was useful to test experimentally this concept, a reversible inhibitor of electron transport was required to carry the concept forward into reperfusion. In this way, the relationship between preservation of the ETC during ischemia and the reduction of myocardial injury after ischemia and reperfusion could be tested.

Amobarbital (Amytal) is a short-acting barbiturate that inhibits complex I at the rotenone site (40, 67). Amobarbital inhibits respiration with glutamate at 1–3 mM concentrations, whereas succinate respiration is not impaired (61). At ~5 mM, amobarbital also inhibits succinate respiration and complex V (24). Inhibition of respiration through complex I by amobarbital is rapidly reversible (127). Amobarbital treatment of the isolated rat heart immediately before ischemia protects oxidative phosphorylation and preserves cytochrome c content in SSM and IFM during ischemia (26). This protection is concentration dependent with 2–2.5 mM providing the optimal range (26). These concentrations are consistent with those previously found to selectively inhibit complex I (61).

Amobarbital treatment also preserves oxidative phosphorylation through cytochrome oxidase, previously observed with rotenone treatment. Respiration with glutamate, a complex I substrate, is also preserved (26) (Fig. 2). Thus, amobarbital must have washed out of the mitochondria during the isolation process, confirming the rapidly reversible nature of inhibition. Amobarbital avidly binds to albumin (24), and the use of isolation buffers containing bovine serum albumin during mitochondrial isolation (46, 107) favors the redistribution of amobarbital out of the mitochondria. In contrast, when rotenone was administered to the heart, glutamate respiration in isolated mitochondria was blocked (80). These findings indicate that amobarbital treatment immediately before ischemia leads to a reversible inhibition of respiration, in line with previous findings in isolated mitochondria (127) and in the isolated heart (4, 110).

Amobarbital treatment protects against ischemia-induced decreases in glutamate respiration (Fig. 2). Reversible blockade of respiration at complex I protected against ischemic damage (120, 143) to complex I (26). Preservation of respiration with duroquinol and tetramethylpentadecane-ascorbate as substrates indicates that amobarbital treatment also protects against damage to the distal ETC. Amobarbital treatment markedly attenuated cytochrome c loss during ischemia (26). Thus blockade of electron transport at complex I immediately before ischemia using two chemically dissimilar compounds protects against ischemic damage to the ETC.

Studies at the level of the intact heart provide additional support for the specificity of amobarbital treatment and provide insights into mechanisms of protection (2, 3). Isolated buffer-perfused guinea pig hearts were treated with either 2.5 mM amobarbital or vehicle for 1 min immediately before 30 min of ischemia, followed by 60 min of reperfusion. Mitochondrial NADH, FAD, calcium, and intracellular ROS were measured by fluorescence spectrophotometry with a fiber optic probe placed at the left ventricular free wall. Tissue autofluorescence
was used to measure NADH and FAD (5–7, 115, 116, 118, 129). Mitochondrial Ca$^{2+}$ concentration was measured in indo-1-AM-loaded hearts after quenching cytosolic Ca$^{2+}$ with MnCl$_2$ (116, 117, 129). ROS, primarily superoxide, was monitored in dihydroethidium (DHE) loaded hearts (23, 71, 116, 129, 146) by measuring the fluorescence of a DHE intermediate that results primarily from the reaction with superoxide ($\lambda_{ex}$, 540 nm; $\lambda_{em}$, 590 nm) (146). NADH increased in both groups during early ischemia; during late ischemia, NADH declined in the vehicle group but remained elevated in the amobarbital group, consistent with in situ inhibition of complex I (Fig. 3). During late ischemia, DHE fluorescence was higher in the vehicle group than in the amobarbital group, supporting a decrease in the previously observed ROS generation during ischemia in this model (71) by blockade of electron transport with amobarbital (2, 3). Mitochondrial Ca$^{2+}$ loading during ischemia was attenuated in the amobarbital-treated hearts compared with vehicle controls. Thus blockade of electron transport during ischemia preserves respiratory function in isolated mitochondria (26) accompanied by decreased production of ROS and reduced Ca$^{2+}$ accumulation as evident in the intact heart (2, 3). Protection of mitochondria by blockade of electron transport at the rotenone site of complex I localizes the site of ROS production distal to complex I, most likely at complex III.

Blockade of electron transport is protective during ischemia, when mitochondria are sources of likely oxidative damage in this setting. Protection by blockade of electron transport during pathological processes is in stark contrast to the blockade of electron transport during aerobic metabolism. Inhibition of respiration at complex I under aerobic conditions leads to cellular injury (91) and appears to be an effector of mitochondrial-derived oxidative injury following the release of cytochrome $c$ in settings of apoptosis or ischemia (29, 75). Thus, in pathological settings, such as ischemia or early reperfusion, modulation of mitochondrial metabolism can be beneficial, when the result of mitochondrial respiration is cellular injury. Reperfusion in the setting of preserved mitochondrial function can now be achieved. Protection of mitochondrial respiration during ischemia by reversible blockade of electron transport allows assessment of the contribution from ischemic mitochondrial damage to myocardial injury during reperfusion.

**IMPROVED MITOCHONDRIAL FUNCTION DURING ISCHEMIA PRESERVES MITOCHONDRIAL FUNCTION AND DECREASES CARDIAC INJURY ON REPERFUSION**

The ability to protect mitochondrial respiration during ischemia allows a critical test of the contribution of ischemic mitochondrial damage to myocardial injury observed after
reperfusion. Isolated, buffer-perfused rat hearts treated with either 2.5 mM amobarbital or vehicle for 1 min immediately before ischemia underwent 25 min of ischemia, followed by 30 min of reperfusion. Amobarbital treatment preserved the rate and coupling of oxidative phosphorylation in both SSM and IFM following reperfusion compared with vehicle-treated controls (28). Thus, protection of the ETC by reversible blockade of electron transport during ischemia was carried forward into reperfusion. Reversible blockade of electron transport during ischemia protected complex I itself, as shown by preserved rates of glutamate respiration (Fig. 4). The distal ETC was also protected, with preserved respiration through complex III and cytochrome oxidase (complex IV) as well as improved retention of cytochrome c by mitochondria (28) (Fig. 4). In addition, amobarbital-mediated blockade of respiration during ischemia preserved the integrity of the inner and outer mitochondrial membranes assessed after reperfusion, consistent with the retention of cytochrome c (28). In isolated guinea pig hearts, reperfusion after amobarbital treatment was associated with less mitochondrial ROS and Ca²⁺ loading and higher NADH than the vehicle controls (2, 3) (Fig. 3). Thus protection of cardiac mitochondria during ischemia is carried forward into the early reperfusion period and supports the hypothesis that mitochondrial damage occurs predominantly during ischemia.

Support for the concept that mitochondrial function can be protected during global ischemia as well as on reperfusion was shown using fluorescence spectrophotometry in isolated hearts by lower levels of ROS and mitochondrial [Ca²⁺] and improved redox state after IPC (5, 71, 115), pharmacological preconditioning (6, 7, 115, 117, 118, 129), and hypothermia (116). In all of these studies, contractile function was improved and infarct size reduced on reperfusion.

The role of ischemic mitochondrial damage in myocardial injury was then addressed. Reperfusion of myocardium with reduced ischemic damage to the ETC led to improved contractile recovery, decreased marker enzyme release, and decreased infarct size following reperfusion in the isolated perfused rat heart (28) (Fig. 4). Treatment of isolated guinea pig hearts with amobarbital just before 30 min of ischemia resulted in a 87 ± 5% higher left ventricular developed pressure at 60 min of reperfusion and a 33 ± 7% smaller infarct size compared with the vehicle control group (2). Thus reperfusion of the heart in the setting of preserved mitochondrial function improved contractile recovery and decreased infarct size. The reduction of ischemic damage to cardiac mitochondria preserves mitochondrial function during reperfusion and decreases myocardial injury.

The preservation of mitochondrial function during ischemia and reperfusion by blockade of electron transport attenuates cardiac injury during reperfusion by at least two potential mechanisms. First, the capacity of cardiac mitochondria to release H₂O₂ during reperfusion is substantially attenuated when oxidative phosphorylation is preserved by amobarbital treatment (27) (Fig. 4). Protection of the ETC chain during ischemia by amobarbital markedly decreased net H₂O₂ production with both complex I and complex II substrates in isolated SSM and IFM compared with those from vehicle-treated control hearts (27). The decrease in H₂O₂ production with succinate as substrate in the presence of rotenone to block reverse electron flow indicates that protection of mitochondria decreases ROS production most likely from complex III. Thus preservation of respiratory function decreases mitochondrial production and release of ROS during reperfusion.

Second, blockade of electron transport during ischemia enhanced the retention of cytochrome c by cardiac mitochondria following reperfusion. Amobarbital treatment decreased state 4 respiratory rates after ischemia and reperfusion, an indicator of decreased damage to the mitochondrial inner membrane (28), protection that favors the retention of cytochrome c (89, 106). Normally the outer mitochondrial membrane is impermeable to cytochrome c (18, 145). Delocalized cytochrome c is released from the outer mitochondrial membrane when outer-membrane permeability increases (106, 145). Blockade of electron transport during ischemia preserved outer-membrane integrity measured after reperfusion (28), unlike the increased outer-membrane permeability present in mitochondria from vehicle-treated hearts. Preserved outer-membrane integrity also favors the retention of cytochrome c by mitochondria. The mechanism leading to preserved outer-membrane integrity achieved by the reversible blockade of electron transport during ischemia is the subject of ongoing work.

The cardiac protection observed when the heart is reperfused in the setting of preserved mitochondrial function provides strong support for the working hypothesis that ischemic damage to mitochondria is a key mechanism of myocardial injury during reperfusion. Blockade of electron transport during ischemia has provided a mechanistic link regarding the key role of mitochondrial damage during ischemia in the genesis of cardiac injury during reperfusion.

MODULATION OF MITOCHONDRIAL RESPIRATION DURING REPERFUSION PROTECTS AGAINST CONSEQUENCES OF ISCHEMIA-INDUCED MITOCHONDRIAL DAMAGE

Ischemia and reperfusion result in mitochondrial dysfunction with decreases in oxidative capacity, loss of cytochrome c,
and the generation of ROS. During ischemia of the isolated perfused rabbit heart, SSM sustain a decrease in cardiolipin, cytochrome c, and oxidation through cytochrome oxidase (89). Reperfusion did not lead to additional damage in the distal electron transport chain in this model (81). Oxidation through cytochrome oxidase and the content of cytochrome c did not decrease further during reperfusion. Ischemic injury led to persistent defects in oxidative phosphorylation during the early reperfusion period (81, 88). The decrease in cardiolipin content accompanied by persistent decrements in the content of cytochrome c and oxidation through cytochrome oxidase is a potential mechanism of additional myocyte injury during reperfusion. Relative blockade at cytochrome oxidase can increase ROS production from the electron transport chain (4, 30, 114). Mechanisms of mitochondrial-derived myocyte damage include ROS production (4, 14, 15, 137, 138), onset of mitochondrial permeability transition (21, 145), and the activation of programmed cell death pathways that become more evident during reperfusion (25, 53). Ischemic damage to the distal electron transport chain emerges as a potential link between ischemia and the mitochondrial-driven myocyte injury that occurs during reperfusion.

Pioneering studies by Ganote and colleagues (51, 52) showed that inhibition of mitochondrial respiration can decrease contraction band formation and attenuate enzyme release during reoxygenation, suggesting that resumption of mitochondrial metabolism during reoxygenation can initially lead to deleterious consequences. Reperfusion-induced declines in cardiac function and the accumulation of oxidatively damaged lipids are diminished when mitochondrial respiration was reversibly inhibited during early reperfusion using amobarbital (4), underlining the physiologic significance of mitochondrial ROS production during reperfusion to cardiac injury during reperfusion. In contrast, inhibition of cytochrome oxidase did not confer protection (4). The observed decrease in ROS production and improved myocardial protection when amobarbital was given during reperfusion strongly suggest that the site of ROS production is distal to the site of amobarbital block and proximal to cytochrome oxidase, namely complex III.

Mitochondrial permeability transition (MPT) appears to contribute to myocardial injury during ischemia (21) and especially during reperfusion (36). A component of MPT observed in situ in the heart likely occurs secondary to transient, reversible opening of the transition pore (145). Increased calcium availability during reperfusion enhances mitochondrial calcium loading (11, 55, 57). Enhanced mitochondrial oxidative damage and calcium loading predispose to the onset of MPT during reperfusion (36, 145). On the basis of their greater sensitivity to calcium-mediated damage (108), MPT during reperfusion may largely involve SSM. MPT appears to be reversible during reperfusion, with closure of the permeability transition pore enhanced by pyruvate oxidation during early reperfusion (35, 37, 58, 145). Inhibitors of MPT decrease myocardial damage during ischemia and reperfusion (36, 43, 54). Protection of mitochondrial function during ischemia by reversible inhibition of electron transport (26) led to decreased ROS production and mitochondrial calcium loading during reperfusion (2), which would be expected to decrease the probability of opening of permeability transition pore during reperfusion. Pharmacological inhibition of mitochondrial respiration during early reperfusion by amobarbital decreases mitochondrial-driven myocardial injury during reperfusion (4). Restriction of oxidative metabolism during early reperfusion using hypoxic reperfusate also attenuates mitochondrial and cardiac damage (111, 124). Transient blockade of mitochondrial oxidative metabolism by the repetitive, brief reinitiation of intermittent stop flow ischemia, postconditioning, decreases cardiac injury (9, 147). Postconditioning decreases the generation of ROS from mitochondria (62, 124, 130). Mitochondrial calcium overload and the probability of MPT are also attenuated (9, 17, 62, 130). Thus a variety of approaches can be used to modulate the aberrant oxidative metabolism of ischemically damaged mitochondria to interrupt the link between ischemic damage to mitochondria and mitochondrial-driven cardiac injury during reperfusion.

**IPC MODULATES MITOCHONDRIAL FUNCTION BEFORE INDEX ISCHEMIA**

Myocardial protection mediated by IPC is evoked by transient episodes of brief, nonlethal ischemia interspersed with periods of reperfusion (102). IPC decreases mitochondrial damage from a subsequent prolonged index ischemia (95, 131). The myocardial and mitochondrial protection from IPC involves the coordinated interplay of trigger and effector mechanisms. IPC activates endogenous agonists that bind to cell surface receptors, in turn activating intracellular kinase cascades, especially PKC (70) and PKG (34, 60). These signal transduction networks converge on mitochondria and modulate respiration (34, 136). Modulation of mitochondrial oxidative metabolism before a prolonged ischemic insult is a likely mechanism of cardioprotection.

Isolation of cardiac mitochondria following the IPC stimulus, before the index ischemia, decreased mitochondrial oxidative phosphorylation in one study (38), but not another (72). As a consequence of the observation of the robust mitochondrial and myocardial protection observed after blocking electron transport during ischemia, we suggested that the IPC stimulus would decrease complex I activity and attenuate state 3 respiration through complex I as a potential mechanism of cardioprotection. IPC did not decrease state 3 respiration nor complex I activity in SSM or IFM (132). IPC increased state 4 respiration and decreased the respiratory control rate (RCR) in IFM, indicating decreased coupling of respiration, in line with the primary observations of other investigators as discussed below. Thus, protection of IPC does not occur via the relative blockade of electron transport at complex I at the onset of the sustained index ischemia. The potential of complex I to sustain post-translational modification via phosphorylation (123), nitrosylation (19, 22), or glutathionylation (32), with the potential to modulate complex I activity and respiration with complex I substrates was not observed, even when mitochondria were isolated in the presence of phosphatase inhibitors to preserve phosphorylation status (132). Thus, the cardioprotection achieved with pharmacological blockade of electron transport does not overlap with the mechanisms of IPC. The attenuation of electron transport during ischemia represents an alternative approach to cardioprotection when the triggers or effectors of IPC are impaired.

A high mitochondrial membrane potential favors ROS generation. Uncoupling of mitochondrial respiration decreases membrane potential and consequent electron leak, thereby...
reducing ROS production from the ETC (99). Thus, mild uncoupling of mitochondrial respiration may lead to cardiac protection. Opening of mitochondrial ATP-sensitive K⁺ (K_{ATP}) channel (34, 44) either by IPC or by pharmacological treatment decreases cardiac ischemia and reperfusion injury (56). The mitochondrial K_{ATP} channel is located in the inner membrane, and opening of mitochondrial K_{ATP} channels may lead to mild uncoupling (34, 74, 103). However, experimental results remain inconsistent regarding the uncoupling effect of mitochondrial K_{ATP} channel opening (105). IPC does, however, decrease ROS during simulated ischemia in cardiac myocytes (141) and during the index global ischemia in the isolated heart (71), most likely as a consequence of the uncoupling of respiration. IPC leads to the uncoupling of respiration observed in isolated mitochondria (103). Interestingly, overexpression of uncoupling protein 2, located in the mitochondrial inner membrane, protects cardiac myocytes against ischemia and reperfusion injury by reducing ROS generation and decreasing mitochondrial Ca²⁺ overload (135). Dinitrophenol, a chemical uncoupler of respiration protects isolated heart against ischemia and reperfusion injury when given immediately before ischemia (66, 98). Uncoupling of respiration allows the dissipation of membrane potential and circumvents the accumulation of reducing equivalents at cytochrome b of complex III (41, 42) and perhaps the iron-sulfur centers of complex I (76). The presence of reducing equivalents at these redox centers favors “electron leak” to form ROS from complex I (76). The presence of reducing equivalents at these complexes III (41, 42) and perhaps the iron-sulfur centers of complex I (76) may be especially important to decrease cytotoxic ROS production during the period of ischemia when low amounts of residual oxygen (68) are still present.

POSSIBLE CARDIOPROTECTION BY MILD PROTON LEAK

Most protons reenter the mitochondrial matrix through complex V generating ATP in the process. A small proton leak directly through the inner mitochondrial membrane bypasses complex V and has the effect to partially uncouple respiration from phosphorylation. This additional proton leak partially dissipates the inner membrane potential and stimulates respiration (electron flow) at a given ATP synthetic rate. The results of a recent study have suggested that a mild proton leak occurs in mitochondria during IPC (103). This was assessed indirectly by a faster respiratory rate for a given membrane potential. Interestingly, this could be blocked by the uncoupling protein inhibitor guanosine diphosphate, suggesting that a small proton leak in IPC is mediated by an uncoupling protein (103). The pharmacological preconditioning that occurs with K_{ATP} (44) or K_{Ca} channel openers (129) may indicate that matrix K⁺ influx is the key to initiating cardioprotection against ischemia. It is possible that opening of the mitochondrial K_{ATP} or K_{Ca} channel also may allow matrix H⁺ influx in exchange for matrix K⁺ efflux to induce a mild uncoupling effect, but the activity of the K⁺/H⁺ antiporter may be insignificant to induce sufficient H⁺ leak (103). Because the other critical component of IPC and pharmacological preconditioning appears to be the generation of “signaling” ROS, it is difficult to reconcile how mild uncoupling might also increase ROS generation unless the proton gradient and/or the membrane gradient remain fully maintained. However, Heinen et al. (64) demonstrated recently that the putative K_{Ca} channel opener NS1619 not only increased state 2 and 4 respiration but also enhanced ROS release while maintaining mitochondrial membrane potential in guinea pig isolated cardiac mitochondria. This release occurred only at low concentrations of NS1619 and the effect was blocked by paxilline, an inhibitor of these channels. They postulated that NS1619 promoted a small H⁺ “leak” by matrix entry of H⁺ for K⁺ efflux via nonsaturated K⁺/H⁺ exchange to maintain the transmembrane H⁺ gradient.

INCREASED INJURY IN THE AGED HEART DURING ISCHEMIA AND REPERFUSION: REVERSAL BY RESTORATION OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION BEFORE ISCHEMIA

The aged heart sustains increased damage during ischemia and reperfusion (11, 12, 48, 82, 92). Isolated, buffer-perfused hearts from 24-mo-old Fischer 344 rats sustain greater myocardial injury after ischemia and reperfusion than hearts from 6-mo-old adult controls (82, 93, 94, 134). Unfortunately, IPC provides minimal cardioprotection in the aged rat (1, 133, 134) and human hearts (78). Thus, a potentially useful approach to protect aged myocardium is ineffective, and highlights the importance of considering other approaches to modulate mitochondrial oxidative metabolism to protect the aged heart.

In the aged heart, ischemic damage to mitochondria is superimposed upon aging-induced defects in mitochondrial oxidative metabolism (83, 84, 86, 100). We evaluated whether an intervention with the potential to improve mitochondrial function in the aged heart before ischemia could decrease myocardial injury during subsequent ischemia and reperfusion. Aging decreases oxidative function only in IFM, whereas SSM are unaffected (46). Treatment with acetylcarnitine was reported (109) to restore aging-induced decreases in cytochrome oxidase activity in the heart. We applied acetylcarnitine treatment to test whether aging-induced decreases in mitochondrial respiration could be improved.

Treatment of aged rats with acetylcarnitine increased the maximal rate of oxidative phosphorylation in IFM to rates observed in the adult heart (85). Respiration in acetylcarnitine-treated aged hearts remained well coupled (85). Adult and aged hearts next underwent 25 min of ischemia, followed by 30 min of reperfusion. In the aged heart, acetylcarnitine improved functional recovery, which was similar to the recovery of treated or untreated adult hearts (85). Acetylcarnitine markedly reduced the release of lactate dehydrogenase in the aged heart, indicating less myocyte necrosis. When the aged heart sustained ischemia and reperfusion following restoration of mitochondrial function, there was less myocardial damage and improved contractile recovery. In contrast, acetylcarnitine treatment had no effect upon the extent of myocardial damage or contractile recovery in the adult heart. These observations provide strong support for the contribution of aging-related defects in mitochondrial metabolism to the enhanced cardiac damage observed following ischemia and reperfusion.

Acetylcarnitine could improve aging defects in electron transport by two mechanisms. First, acetylcarnitine was proposed to increase the content of cardiolipin (109), required for optimal activity of cytochrome oxidase (119, 144). However, aging did not decrease the content of cardiolipin in the aged Fischer 344 rat heart (101). Second, treatment with acetylcarni-
nitine increases transcription of mitochondrial DNA in the aged heart leading to increased mitochondrial protein synthesis and an increased content of mitochondrial-encoded electron transport subunits (49). Regardless of the mechanism, the option to modulate mitochondrial oxidative metabolism by treating age-related, rather than ischemia-induced, damage in a preemptive fashion represents a novel cardioprotective strategy for the aged heart.

Our working model for increased injury in the aged heart is that addition of ischemia-induced defects upon preexisting aging defects would enhance mitochondrial-derived myocyte injury in the aged heart (86). Consistent with previous observations in the adult heart, mitochondrial damage occurred mainly during ischemia, rather than during reperfusion, in the aged heart (86). The contribution of the ETC to damage during ischemia in the aged heart was tested by using rotenone to block electron flow. Rotenone treatment of aged hearts immediately before the onset of ischemia attenuated ischemic damage to IFM (86). Thus, as in the adult heart, the ETC contributes to mitochondrial damage during ischemia in the aged heart. Amobarbital treatment immediately before ischemia (26) protects oxidative phosphorylation in the aged heart following reperfusion (C. Tanaka- Esposito, Q. Chen, and E. J. Lesnfsky, unpublished observations). Thus reversible blockade of electron transport can attenuate the ischemic component of mitochondrial damage in the aged as well as in the adult rat heart.

These studies demonstrate the potential to independently modulate both the age-related and ischemia-induced components of mitochondrial oxidative metabolism that contribute to injury to the aged heart by directly targeting the mitochondrion. Relief of aging-related defects in oxidative metabolism can decrease ischemic myocardial injury in the aged heart (85). In addition, blockade of electron transport attenuates the ischemic mitochondrial damage in the adult and senescent hearts. Future work will uncover the potential interactive benefit of these complimentary approaches to reduce synergistically injury to the aged heart during ischemia and reperfusion.

In summary, the blockade of electron transport or the partial uncoupling of respiration lessens cardiac injury during ischemia and during early reperfusion. Although the concept that disrupting effective oxidative metabolism to protect the heart initially appears counterintuitive, both of these interventions decrease the accumulation of reducing equivalents at key centers in the electron transport chain, namely complex I and complex III, under conditions that favor ROS generation. The protection achieved is mediated via the decreased production of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. J Biol Chem 268: 18532–18541, 1993.


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