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Electron flow into cytochrome *c* coupled with reactive oxygen species from the electron transport chain converts cytochrome *c* to a cardiolipin peroxidase: role during ischemia–reperfusion

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ABSTRACT

Background: Cytochrome *c* (Cyt *c*) is a mobile component of the electron transport chain (ETC.) which contains a tightly coordinated heme iron. In pathologic settings, a key ligand of the cyt *c*'s heme iron, methionine (Met_{80}), is oxidized allowing cyt *c* to participate in reactions as a peroxidase with cardiolipin as a target. Myocardial ischemia (ISC) results in ETC. blockade and increased production of reactive oxygen species (ROS). We hypothesized that during ischemia–reperfusion (ISC-REP); ROS generation coupled with electron flow into cyt *c* would oxidize Met_{80} and contribute to mitochondrial-mediated ETC. damage.
Methods: Mitochondria were incubated with specific substrates and inhibitors to test the contributions of ROS and electron flow into cyt *c*. Subsequently, cyt *c* and cardiolipin were analyzed. To test the pathophysiologic relevance, mouse hearts that underwent ISC-REP were tested for methionine oxidation in cyt *c*.
Results: The combination of substrate/inhibitor showed that ROS production and electron flux through cyt *c* are essential for the oxidation of methionine residues that lead to cardiolipin depletion. The content of cyt *c* methionine oxidation increases following ISC-REP in the intact heart.
Conclusions: Increase in intra-mitochondrial ROS coupled with electron flow into cyt *c*, oxidizes cyt *c* followed by depletion of cardiolipin. ISC-REP increases methionine oxidation, supporting that cyt *c* peroxidase activity can form in the intact heart.
General significance: This study identifies a new site in the ETC. that is damaged during cardiac ISC-REP. Generation of a neoperoxidase activity of cyt *c* favors the formation of a defective ETC. that activates signaling for cell death.

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1. Introduction

Mitochondria are the cellular powerhouses that generate ATP for cell survival. Unfortunately, during cardiac ischemia, they are both targets of injury and sources of cellular damage [1]. The sequence of events leading to ischemic mitochondrial damage involves direct damage to the electron transport chain (ETC.), increased generation of reactive oxygen species (ROS) [2], mitochondrial membrane permeabilization [3], depletion of cytochrome *c* (cyt *c*) and activation of cell death pathways [4]. Proximal blockade of electron transport during ischemia decreases mitochondrial damage and cardiac injury whereas distal blockade of the ETC. does not protect [5]. Thus, not only does the ETC. itself contribute to the ischemic damage to mitochondria [6], the segment located between the quinol oxidation site of complex III and the heme aa_3 site of complex IV appears responsible for the damage [5]. Key components of this site are cyt *c* and cardiolipin, and they can potentially contribute an important role in ischemia-mediated cardiac injury [7].

Cyt *c*, localized at the inner membrane of mitochondria, is a mobile component of the ETC. and contains a tightly coordinated heme iron. It is a positively charged protein (iso-electric point pH 10) with approximately 30% of the protein surface containing binding sites for anionic lipids such as cardiolipin [8–11]. Complex interactions of cardiolipin (pKa 3.8) with cyt *c*, including electrostatic, hydrogen bonding and hydrophobic interactions, are responsible for localizing cyt *c* at the inner mitochondrial membrane [11–14]. In general, heme-containing redox

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proteins, including cyt *c*, can induce oxidative stress by single electron leak reactions that produce free radicals. They can also act as peroxidases via the catalysis of the two-electron reduction of H_2O_2 to H_2O. Exceptionally, cyt *c* reacts very slowly with H_2O_2 [15] since the heme iron is occupied by ligands in all the six coordination positions [16]. The heme iron in cyt *c* has two axial bonds, one with His_{18} and the other with Met_{80}. Under normal conditions, it is the Met_{80} co-ordination of the heme that blocks the interaction of iron with potential ligands including NO, O_2, CO and H_2O_2 [17]. Oxidation of the cyt *c* methionine residue to methionine sulfoxide [Met(O)] opens this iron coordination site, providing access to the heme catalytic center for small molecules such as H_2O_2 [13,18,19]. As a result, cyt *c* converts to a peroxidase thereby reducing H_2O_2 and simultaneously oxidizing tightly associated cardiolipin.

Several in vitro studies that included oxidation by HOCl [20], nitration of Tyr_{67} by $ONOO^-$ [21] or carboxy-methylation of Met_{80} [22] were shown to disrupt the Fe – Met_{80} bond of cyt *c* and thereby facilitate reactivity of the heme iron. Nitration of Tyr_{67} by $ONOO^-$ may result in secondary oxidation of Met_{80} [21]. Structural changes, along with the loss of axial ligand (Met_{80}), alter the redox catalytic reactivity of cyt *c* by enhancing its peroxidase activity in the presence of negatively charged phospholipid membranes [14,17,23]. In vitro radiolysis of cyt *c* found that Met_{80} was the prime target for oxidation [24]. Exposure of cyt *c* to radiolysis found Met_{80}, along with Phe_{36} and Phe_{46}, were the amino acids most susceptible to hydroxyl radical-mediated oxidation [25]. Oxidation of cyt *c* with HOCl showed that Met_{80} oxidation was more favored than Met_{65} and led to an increase in peroxidase activity [20].

The peroxidase activity of cyt *c* utilizes cardiolipin, a phospholipid unique to the mitochondrial inner membrane, as the target. Cardiolipin interacts with ETC. complexes and is required for their optimal activity [26,27]. The peroxidation and depletion of cardiolipin generate key signals for the activation of cell death programs [14,28], including the release of cyt *c* and Smac/Diablo from mitochondria into the cytosol [28]. Inhibition of the peroxidase activity of cyt *c* by nitric oxide prevents cardiolipin oxidation and cyt *c* loss [29], suggesting that the peroxidase is a potential mechanism that contributes to mitochondrial damage. However, the molecular switch that disrupts the electron shuttling function of cyt *c* and leads to conversion into a peroxidase has not been identified.

Cyt *c* peroxidase activity was measured using chemiluminescence, fluorescence and electron paramagnetic resonance-based assays in in vitro liposomal systems [17,19,28,29] or cell stress models [28]. However, these approaches to characterize cyt *c* peroxidase are difficult to employ at the tissue level due to the complex mixture of proteins present. The relevance of cyt *c* peroxidase formation to ischemia–reperfusion injury in the heart is unknown. In the present study, we demonstrate that an increase in intra-mitochondrial ROS generation, coupled with electron flow through the cyt *c* segment of the ETC., oxidizes cyt *c* at the Met_{80} residue, followed by depletion of cardiolipin. Moreover, ischemia and reperfusion increase Met(O) formation in cyt *c*, indicative of in situ peroxidase formation in the heart.

2. Materials and methods

2.1. Materials

Chemicals used in the mitochondrial isolation procedure were supplied by Sigma-Aldrich (Saint Louis, MO). Unless otherwise stated, all other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Langendorff mouse heart perfusion

All animal experiments were conducted under the Guidelines on Humane Use and Care of Laboratory Animals for Biomedical Research published by the National Institutes of Health (revised 2011). The Institutional Animal Care and Use Committees of Virginia Commonwealth University and the McGuire Veterans Affairs Medical Center approved the study. 8–10 week old male C57BL/6 mice (24.6 ± 0.9 g) were anesthetized with pentobarbital sodium (0.1 mg/g i.p.) and anticoagulated with heparin (1000 U/g i.p.). Hearts were excised and retrograde perfused via the aorta in the Langendorff mode with modified Krebs–Henseleit buffer (115 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.1 mM $MgSO_4 \cdot H_2O$, 0.9 mM KH_2PO_4, and 5.5 mM glucose; pH 7.4) oxygenated with 95% O_2/5% CO_2 [30]. Cardiac function was monitored with a balloon inserted into the left ventricle and data were recorded digitally using the Powerlab recording system (AD Instruments, Colorado Springs, CO). After 15 min of stabilization by buffer perfusion, experimental hearts were either subjected to 45 min control perfusion (TC), or 25 min global stop-flow ISC followed by 30 min REP. Hearts were harvested for mitochondrial isolation [5,30,31] at the end of experiment.

2.3. Isolation of mitochondria, cytosol, and heart tissue homogenates

The heart was washed and placed in pre-chilled modified Chappell-Perry 1 (CP1) buffer (pH 7.4) with the following composition: 100 mM KCl, 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 5 mM $MgSO_4 \cdot 7H_2O$, and 1 mM adenosine 5′-triphosphate disodium (ATP). Next, the heart tissue was dried with Whatman filter paper, weighed, and then thoroughly minced in a chilled glass beaker. The minced heart tissue was transferred to a chilled glass tube for homogenization. The tissue was homogenized in 3 mL of CP1 buffer using a polytron tissue blender (Kinematica, Bohemia, NY) for 2.5 s at a rheostat setting of 10,000 rpm. 50 μL of the homogenate was saved as the heart tissue extract. The remaining polytron homogenate was centrifuged at 6000 ×*g* for 10 min at 4 °C and the supernatant was saved as a crude cytosol for further purification. The homogenate pellet was re-suspended in 3 ml of CP1 buffer and incubated with 5 mg/g (wet weight) trypsin (Sigma-Aldrich, Saint Louis, MO) for 15 min at 4 °C. Next, 3 mL of CP2 buffer [CP1 buffer containing 0.2% bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, MO)] was added to block the trypsin digestion. Digested tissue was then homogenized (two strokes) with a tight Teflon pestle/glass tube homogenizer set at steady stirring speed of 600 rpm. The remaining homogenate was centrifuged at low speed 500 ×*g* for 10 min and the supernatant was again centrifuged at 3000 ×*g* for 10 min at 4 °C. The pellet containing the mitochondria was washed with 2 mL of KME buffer [100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA] and centrifuged at 3000 ×*g* for 10 min. Lastly, the mitochondrial pellet was re-suspended in 80–100 μL of KME and the protein concentration was measured using the Lowry method [31].

2.4. Measurement of oxidative phosphorylation in intact mitochondria

Oxidative phosphorylation in freshly isolated mitochondria was measured using an oxygen electrode at 30 °C (Strathkelvin Instruments, Glasgow, Scotland) as previously described [32] with minor modifications [5,30,31]. Glutamate (20 mM) + malate (5 mM) (complex I substrate) or succinate (20 mM) plus 7.5 μM rotenone (complex II substrate) stimulated respiration was performed using 150 μg mitochondrial protein. TMPD (N,N,N′,N′ tetramethyl p-phenylenediamine, 1 mM)-ascorbate (10 mM, complex IV substrate via cytochrome *c*) + 7.5 μM rotenone was used to measure respiration selectively through cyt *c* and cytochrome oxidase using 50 μg of mitochondrial protein. State 3 respiration was measured by the rate of oxygen consumption following the addition of 0.2 mM ADP (final concentration) followed by state 4 respiration. Finally, the maximal rate of ADP-stimulated respiration was measured in the presence of 2 mM ADP. Uncoupled respiration was measured using 0.3 mM dinitrophenol (DNP).