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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).

2.5. In-vitro modulation of electron transport chain

One mg of isolated mitochondria was incubated with 20 mM succinate + 7.5 μM rotenone with or without 10 mM TTFA, 10 mM antimycin A or 2 mM azide. Incubation studies of mitochondria were carried out in MSM/EDTA buffer, pH 7.4 (220 mM mannitol, 70 mM sucrose, 5 mM MOPS, 2 mM EDTA) for 50 min at 37 °C with gentle rotation. Then, the mitochondrial protein was spun down and the pellet was re-suspended in immunoprecipitation buffer provided with the kit (Invitrogen, Carlsbad, CA). The production and release of H_2O_2 from mitochondria were measured using Amplex Red as previously described [33]. Oxidative stress in the mitochondrial matrix was assessed by measurement of the oxidatively sensitive enzyme aconitase [34,35].

2.6. Immunoprecipitation of cytochrome c

Cyt *c* was immunoprecipitated from isolated mitochondria using Dynabeads® Protein G immunoprecipitation (IP) kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 50 μL (1.5 mg) of Dynabeads® and 8 μg of anti-mouse cyt *c* antibody (BD Biosciences San Jose, CA), diluted in antibody binding and washing buffer [PBS buffer + 0.02% Tween-20], were mixed and incubated at RT for 120 min. 0.5 mg of mitochondrial protein suspended in IP buffer (Invitrogen, Carlsbad, CA) was added to the Dynabeads®-antibody complex and incubated with rotation for 120 min. at RT. The elution buffer [50 mM glycine, pH 2.8] was then added to resuspend the Dynabeads®-antibody–antigen complex and incubated with rotation for 5 min at RT. Finally, the supernatant containing eluted antibody and antigen was transferred to a clean tube. For the eluted protein to be used for Western blotting, the pH of the eluate was adjusted by adding 1 μL of 2.8 M Tris pH 8.5 for 45 μL of eluate.

2.7. Western blot analysis

Equal amounts of protein were loaded and separated using 4–20% gradient Tris-glycine pre-cast gels (Invitrogen, Carlsbad, CA) and transferred to Immobilon-P PVDF (polyvinyldifluoridine) membranes (Millipore, Billerica, MA). The blots were then incubated for 1 h at RT with 5% (w/v) non-fat dry milk (Bio-Rad, Hercules, CA) in TBS-Tween buffer (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween-20). Next, the membranes were incubated overnight at 4 °C with a 1:2500 dilution of mouse anti-cytochrome c (BD biosciences, San Jose, CA; Cat. #556433) or 1:1000 of polyclonal anti-methionine sulfoxide antibody (Cayman chemicals, Ann Arbor, MI; Cat. # 600161) prepared either in 5% BSA/TBS-T or 0.5% (w/v) non-fat dried milk/TBS. Next day, the blots were incubated with a 1:50,000 dilution of anti-mouse or 1:10,000 anti-rabbit IgG $F(ab)_2$ fragments conjugated with Horse Radish Peroxidase (HRP) in 5% BSA/TBS-Tween buffer for 1 h. The immunoreactive proteins were visualized using Amersham ECL Plus western blotting detection reagents (GE Healthcare Lifesciences, Piscataway, NJ) or SuperSignal Femto maximum sensitivity substrate kit (Thermo Fisher Scientific Inc., Rockford, IL). Protein bands on western blot were quantified by densitometric analysis using ImageJ software (NIH, Bethesda, MD).

2.8. In-solution digestion and mass spectrometry analysis

In-solution digestion of immunoprecipitated cyt *c* was initiated by incubating 40 μL of the isolated protein solution with 5 μl of 0.5 M tris (2-carboxyethyl) phosphine (TCEP) for 1 h at 37 °C, followed by incubation with 15 μL of 55 mM iodoacetamide for 1 h at 37 °C. 100 μl of digestion buffer [Chymotrypsin: 500 mM Tris·HCl (pH 8.0), 10 mM calcium chloride] was added and the reaction was allowed to proceed at 37 °C for 1 day with gentle shaking. 200 μL of 10% TFA and 50 μL of acetonitrile were added to stop the reaction and the solution was subjected to reversed-phase chromatography for solid-phase extraction of peptides. The resulting peptide mixture was evaporated to dryness and resuspended in 30 μL of 95/5 0.1% TFA (aq)/acetonitrile before being subjected to LC-MS/MS analysis on a system centered around a Thermo LTQ XL linear ion trap instrument.

Peptide separation was performed on a CTICAP5150100 reversed-phase C18 column (5 μm particles, 150 μm × 100 mm, 300 Å pores) (Column Technology, Fremont, CA) at a flow rate of ~0.5 μL min^{-1}. The injection volume was 20 μL, with a CapTrap (Michrom, Auburn, CA) trapping column being used to reduce sample-loading time. Mobile phase A was 0.1% formic acid in water while B was 0.1% formic acid in methanol. The gradient program was initiated with a rapid increase from the equilibration composition of 5% B to 15% B over 5 min followed by slower increases to 80% B over 70 min and then to 95% B over 15 min. Electrospray ionization was used for mass spectrometry interfacing. The instrument was programmed to cycle between determining the ten most intense incoming ions and recording tandem mass spectra for each of these ions, and ions picked for fragmentation twice in a 30 s window were excluded for the next 180 s. Peptides were identified from fragmentation patterns using the version of X! Tandem (2011.12.01) integrated into version 4.6.3 of the Trans-Proteomic Pipeline software package (Institute for Systems Biology, Seattle, WA). The protein sequence database used in the search consisted of the NCBI Reference Sequence version of the complete mouse proteome (29958 proteins; downloaded from ftp://ftp.ncbi.nlm.nih.gov/refseq/M_musculus/mRNA_Prot on October 16, 2013) and 115 common contaminant proteins (Global Proteome Machine, common Repository of Adventitious Proteins; downloaded from ftp://ftp. thegpm.org/fasta/cRAP on October 16, 2013).

2.9. LC-MS/MS analysis of cardiolipin

Isolated mitochondria (1 mg) in 50 μL of 100 mM potassium phosphate buffer were mixed with 1,2-dipalmitoyl-sn-glycero-3-phospho-N-methylethanolamine (internal standard) and extracted with 1 mL of chloroform:methanol, 2:1 containing 2 mM butylated hydroxytoluene as an antioxidant according to the procedure [36]. The lipids were separated by reversed-phase LC using a Supelco 2.1 (i.d.) × 150 mm Ascentis C18 column (Sigma, St. Louis, MO) and a binary solvent system at a flow rate of 0.3 mL/min with a column temperature of 60 °C. Prior to injection of the sample, the column was equilibrated for 0.5 min with a solvent mixture of 30% mobile phase A (CH_3OH/H_2O, 50/50, v/v, with 0.25% of triethylamine and glacial acetic acid) and 70% mobile phase B (IPA/H_2O, 90/10, v/v, with 0.25% of triethylamine and glacial acetic acid). After sample injection (typically 40 μL), the A/B ratio was maintained at 30/70 for 2 min, followed by a linear gradient to 100% B over 7 min, which was held at 100% B for 4.3 min, followed by a 0.6 min gradient return to 30/70 A/B. Cardiolipin peaks were quantified during each run by multiple reaction monitoring (MRM) analysis, and the structure was confirmed via a MS2 scan within the linear ion trap during the elution of each peak [36]. Cardiolipin peaks were summed to yield the total cardiolipin measurement.

2.10. Statistical analysis

Parameters were expressed as mean ± standard error of the mean (SEM). The data consisting of two groups were analyzed by two-tailed Student's t-test. Differences among multiple groups were compared by one-way ANOVA analysis with post hoc comparisons performed using the Student–Newman–Keuls test. All analyses were executed using SigmaPlot ver.11 software (Systat Software Inc., Chicago, IL). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Mass spectrometric analysis of cytochrome c containing oxidized methionine residue

As an initial control, isolated mitochondria from non-ischemic control mouse hearts were exposed to the oxidant hypochlorous acid (10 mM HOCl for 15 min). Following exposure, cyt *c* was immunocaptured and analyzed for oxidation of the Met_{80} residue using western blotting (Supplemental Fig. 1) and mass spectrometry (Fig. 1). LC-MS/MS analysis of the chymotryptic digest of murine cyt *c*, using collision-induced dissociation as the fragmentation method, yielded a short peptide IPGTKMIF containing oxidized Met_{80} (AA 75–82; calculated MH^+ *m/z* with Met(O) = 922.51) with good coverage of fragment ions around the oxidation site. As shown in Fig. 1, b-ions flank the modification site for unambiguous localization. Identification was further supported by a characteristic peak for the neutral loss of methane sulfenic acid, which is common in peptides containing oxidized methionine residues [37]. The numerous water losses from the b-ions are consistent with the presence of threonine. Similar tandem mass spectra for oxidized IPGTKMIF were obtained on digestion of commercial cyt *c* preparations that had been treated with HOCl and, for immunoprecipitated cyt *c*, Met(O) at Met_{80} was also observed in a longer peptide, LENPKKYIPGTKMIF (Supplemental Fig. 2); in this case, an intense methane sulfenic acid neutral loss peak was observed and the most intense b- and y-ions were consistent with the proline effect [38] Since mass spectrometry confirms Met(O) formation, immunoblotting cyt *c* for Met(O) can also be utilized for detecting Met_{80} oxidation thereby confirming cyt *c* peroxidase formation.

3.2. Distal blockade of electron transport chain increases methionine oxidation of cytochrome c

We hypothesize that during ISC-REP, an increase in intramitochondrial ROS generation coupled with the electron flow into the cyt *c* segment of the ETC. is required to oxidize the Met-80, leading to conversion to the peroxidase form. To test this hypothesis, isolated mitochondria from non-ischemic hearts were incubated with the following combinations of substrates and inhibitors in order to test the importance of ROS and electron flux into cyt *c* in generating cyt *c* methionine sulfoxidation. As shown in Fig. 2, (A) succinate + rotenone [39]: Rotenone inhibits reverse electron transfer to complex I; (B) succinate + rotenone + TTFA: TTFA blocks distal within complex II [40], leading to an increase in ROS from complex II and blockade of electron flux into complex III; (C) succinate + rotenone + antimycin A [41,42]: antimycin A inhibits the Q_i center of complex III, leading to an increase in ROS from complex III and markedly attenuates electron flux into cyt *c*; (D) succinate + rotenone + azide [5]: Azide inhibits electron transport at the aa_3 heme center of complex IV, resulting in an increase in intramitochondrial ROS from proximal sites and electron flux into cyt *c*.

Following incubations, cyt *c* was immunocaptured and immunoblotted for cyt *c* and Met(O). As shown in Fig. 2, distal blockade of complex IV with azide showed a significant increase in the signal as a result of Met(O) formation as an indicator of peroxidase formation, compared to TTFA or antimycin A [$p < 0.05$ vs. succ + rot + TTFA or succ + rot + antimycin A].

ROS production was assessed as the net extramitochondrial release of H_2O_2 [33] and the inactivation of the oxidatively sensitive matrix enzyme aconitase [35]. H_2O_2 release is an index of oxidants directed toward the intermembrane space, whereas aconitase provides and indication of oxidants directed toward the matrix [34]. Using succinate plus rotenone as substrates, inhibition of complex II with TTFA or inhibition of the Q_i site of complex III with antimycin A each increased the release of H_2O_2 from mitochondria (succinate + rotenone 51 ± 4; plus TTFA 67 ± 16; plus antimycin A 86 ± 16; pmol/min/mg, ± SE, n = 3). On the other hand, in line with previous observations [33], the addition of azide to succinate + rotenone did not increase the release of H_2O_2 from mitochondria (42 ± 3 pmol/min/mg, ± SE, n = 3, p = NS vs. succinate plus rotenone). In contrast to the results with ROS directed toward the intermembrane space, incubation with succinate + rotentone (similar to the approach to assess the methione oxidation of cyt *c*), resulted in a marked loss of aconitase activity which is consistent with a substantial release of oxidants directed toward the matrix, in the presence of either TTFA, antimycin A or azide (aconitase activity: mouse mitochondria incubated for 10 min 147 ± 58; succinate + rotenone 7 ± 1; plus TTFA 7 ± 4; plus antimycin A 5 ± 1; plus azide 6 ± 1, nmol/min/mg, ± SE, n = 3, all $p < 0.01$ vs. succinate + rotenone alone).

3.3. Cardiolipin content was decreased following distal blockade of electron transport

Next, we asked whether the formation of cyt *c* peroxidase, as a result of distal blockade of ETC. in isolated mitochondria, would consequently

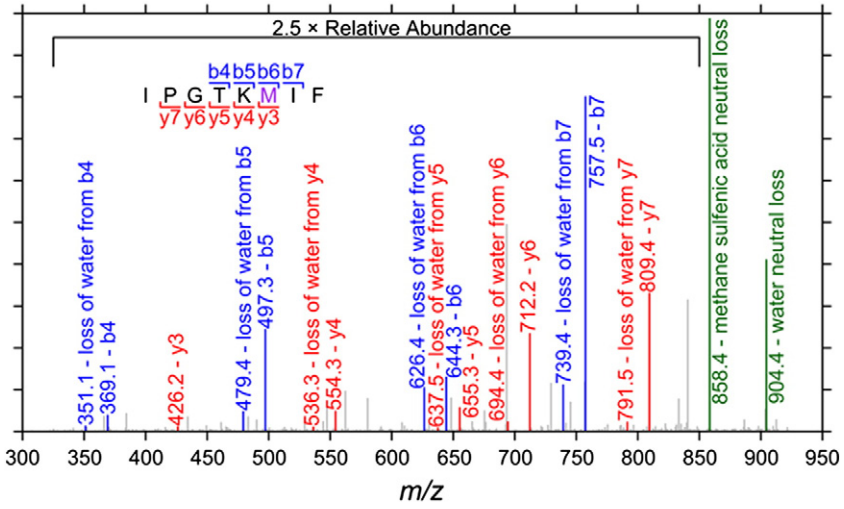

Fig. 1. Detection of chymotryptic peptide IPGTKMIF oxidized at methionine from mouse mitochondria treated with HOCl. Annotated tandem mass spectrum, recorded using the linear ion trap mass spectrometer, for IPGTKMIF oxidized at methionine. The fragmentation method was collision-induced dissociation and the precursor ion (*m/z* = 922.5) was singly charged; therefore, all displayed ions are singly charged. The spectrum is dominated by a peak at *m/z* = 858.4 that is consistent with the neutral loss of methane sulfenic acid from the precursor ion, which is a characteristic of peptides containing Met(O). Good coverage is achieved for the heavier b- and y-ions, with the b-ions providing comprehensive fragment ion coverage around Met_{80}.

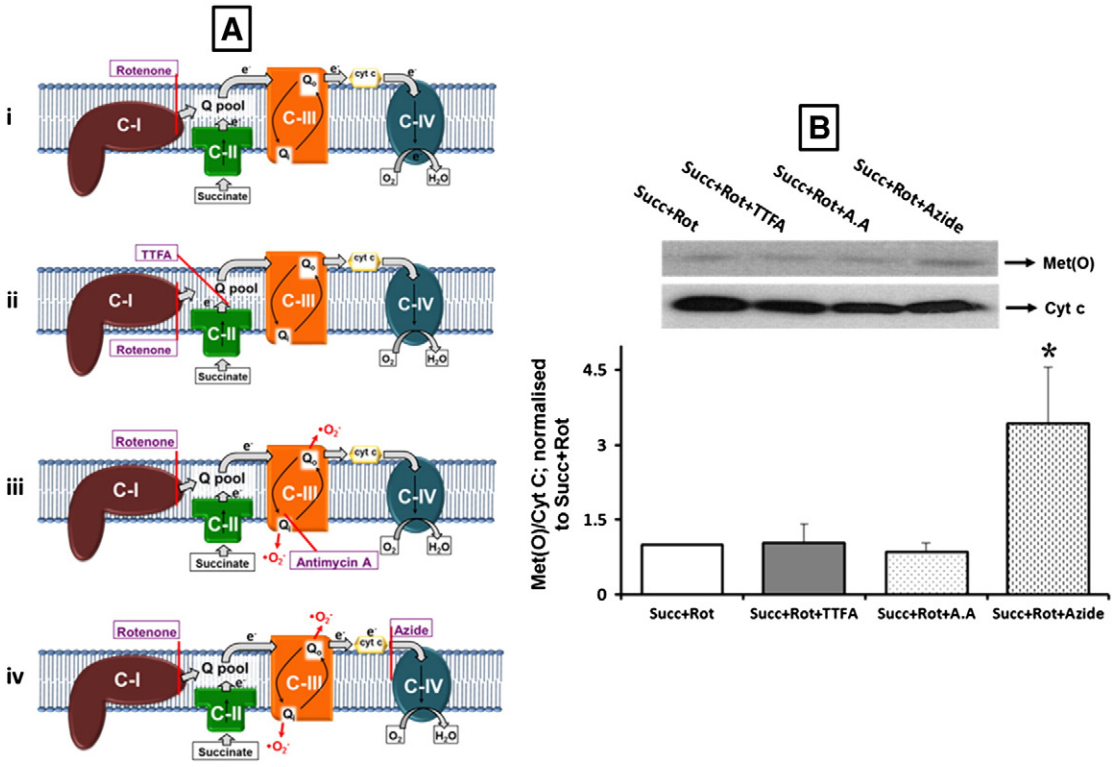

Fig. 2. ROS and electron flow into cytochrome *c* are essential for its modification. (A) Schematic of mitochondrial incubations with substrates and inhibitors designed to test the contribution of ROS and electron flow in oxidation of cyt *c*. (B) Western blot showing detection of Met(O) and cyt *c* at 12 kDa. Isolated mitochondria were incubated for 50 min with succinate + rotenone (i), succinate + rotenone + TTFA (ii), succinate + rotenone + A.A (iii), succinate + rotenone + azide (iv) and then subjected to IP. Results are expressed as mean ± SEM, n = 5–7 for each group. *$p < 0.05$ vs. Succ + Rot. One representative blot out of seven independent experiments is shown. Succ, succinate; Rot, rotenone; TTFA, Thenoyltrifluoroacetone; A.A, Antimycin A; cyt *c*, cytochrome *c*; Met(O), methionine sulfoxide.

alter the cardiolipin content. Isolated mitochondria were incubated in KME buffer containing succinate and rotenone with or without azide for 90 min. Phospholipids from the mitochondrial incubations were extracted and quantified using LC-MS/MS analysis. As shown in Fig. 3, the total content of cardiolipin was significantly decreased in incubations containing succinate + rotenone + azide that led to enhanced Met(O) formation (Fig. 2) when compared to incubations containing succinate + rotenone alone ($p < 0.05$ vs. succ + rot).

3.4. Cyt c from ISC-REP hearts exhibit increased methionine modification

Next, we tested the relevance of potential cyt *c* peroxidase formation during the oxidative stress event of cardiac ischemia and reperfusion. The protein yield of mitochondria isolated following 25 min of ischemia followed by 30 min reperfusion was similar to the protein yield in the time control (TC) perfusion group (Fig. 4A). Glutamate + malate (complex I) and TMPD + ascorbate-dependent (complex IV) respiration was significantly decreased following ischemia–reperfusion (Table 1), which was in line with previously published reports [1,43–45]. Uncoupled respiration (with DNP) measured using TMPD + ascorbate as substrate (data not shown), was also decreased, confirming that ischemia–reperfusion damaged the electron transport chain.

Isolated mitochondria from hearts that underwent ischemia and reperfusion or non-ischemic control perfusions were subjected to immunoprecipitation to capture cyt *c*. The immunocaptured cyt *c* was blotted for Met(O) and cyt *c* (control for equal mitochondrial protein loading). As shown in Fig. 4C, the densitometry ratio of Met(O) to cyt *c* in ischemic-reperfused hearts showed an increase in the Met(O) formation when compared to TC perfusion ($p < 0.05$).

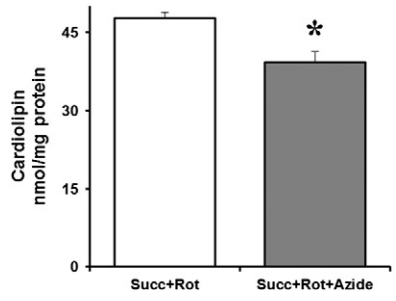

Fig. 3. Content of cardiolipin is decreased following incubation of mitochondria with succinate + rotenone + azide. Quantification of total cardiolipin content per mg mitochondrial protein (nmol/mg protein) in isolated mitochondria incubated with succinate (Succ) + rotenone (Rot) or succinate + rotenone + azide for 90 min. Results are expressed as mean ± SEM, n = 3 for each group. All results are compared using Student's t-test. *$p < 0.05$ vs. Succ + Rot.

4. Discussion

In the present study, we have shown that electron flow through the cyt *c* segment of the ETC., coupled with excessive ROS production, is required for the oxidation of cyt *c* resulting in the depletion of cardiolipin from the mitochondria. In addition, the physiological relevance was shown for the first time during cardiac ischemia and reperfusion. During oxidative stress, cyt *c* converts to a peroxidase and specifically oxidizes the linoleic acyl-groups of cardiolipin [14,28,46]. Two methionines are found in murine cyt *c*, located at positions 65 and 80. Oxidation of Met_{80} results in the formation of cyt *c* peroxidase, which reduces H_2O_2 to H_2O and simultaneously generates oxidized cardiolipin. Hence, Met(O) formation is the mechanistic indicator for cyt *c* peroxidase formation. Using a mass spectrometric approach to analyze immunoprecipitated cyt *c* from mouse mitochondria, we have shown that Met_{80} is readily oxidized to Met(O). While not detecting Met(O) at

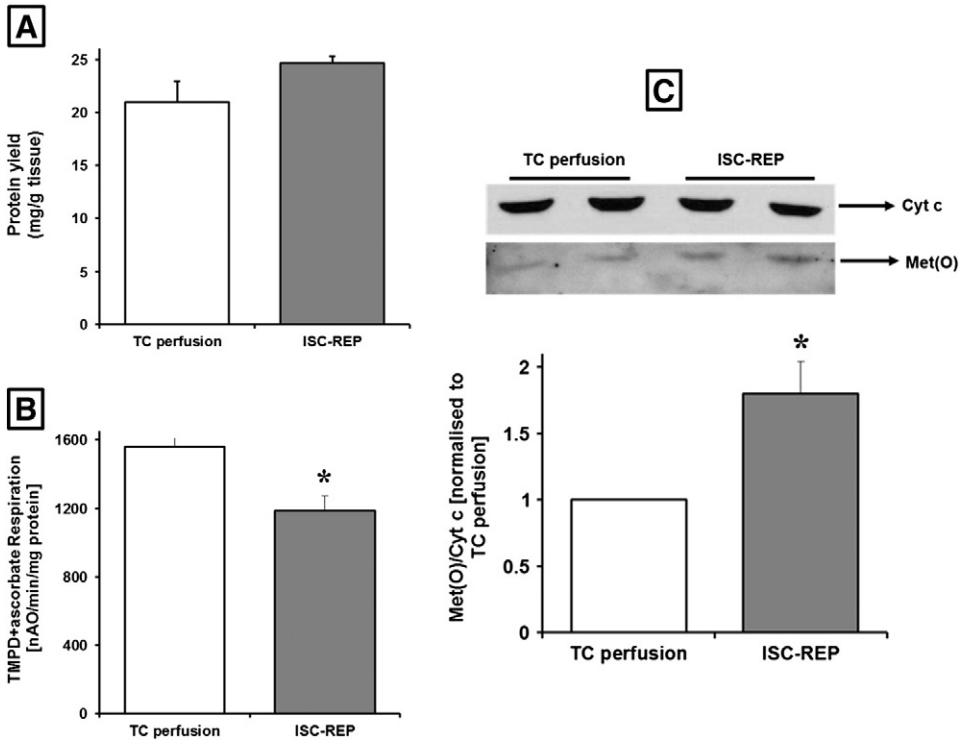

Fig. 4. Ischemic-reperfused hearts show increased Met(O) compared to buffer perfused hearts. (A) The recovery of cardiac mitochondria, assessed by protein yield, was unaltered by ischemia and reperfusion. (B) TMPD + ascorbate dependent oxidative phosphorylation was decreased following ischemia–reperfusion. For TMPD + ascorbate-dependent respiration, only azide-sensitive data are depicted. Results are expressed as nanoatoms of atomic oxygen/min/mg of mitochondrial protein (C) Western blot showing detection of Met(O) and cyt *c* at 12 kDa in buffer perfused time control (TC) and ischemic-reperfused hearts. Results are expressed as mean ± SEM. *$p < 0.05$ vs. TC. All results are compared using one-way ANOVA test. n = 5 in all groups. Met(O), methionine sulfoxide.

Met_{65} does not preclude oxidation at this site, the combination of readily oxidized Met_{80} with previous reports that Met_{80} is the most reactive site in cyt *c* toward superoxide radicals [20,24] strongly support that the majority of Met(O) in cyt *c* is located at Met_{80}.

The contribution of ROS and electron flow through cyt *c* in Met_{80} oxidation forming cyt *c* peroxidase was studied by incubating mitochondria with specific substrates and inhibitors to isolate particular segments of the ETC. Incubation with succinate + rotenone + TTFA does not increase methionine sulfoxidation which suggests that the complex II-generated ROS do not contribute to the peroxidase formation. Mitochondrial incubation with succinate + rotenone + antimycin A also does not increase Met(O) formation, which suggests that the increase in ROS due to complex III blockade is not sufficient to convert cyt *c* to a peroxidase. Antimycin A, via binding to the b heme, disrupts the bifurcation of electron flow within the complex in tandem with increasing the production of reactive oxygen species from the Qo site that are directed toward the intermembrane space [33,42,47,48]. Interestingly, incubation of mitochondria with succinate + rotenone + azide significantly increases the Met(O) signal, consistent with peroxidase formation. This observation suggests that, along with the increase in intramitochondrial ROS, electron flow into cyt *c* is essential for oxidizing cyt *c* at the methionine residue, resulting in the formation of cyt *c* peroxidase. This demonstrates that distal blockade of the ETC. at complex IV with azide results in cyt *c* peroxidase formation when compared to proximal blockade of ETC. with TTFA or Antimycin A (Fig. 2). These data highlight the importance of the availability of electron flow through the cyt *c* segment as a key factor that influences peroxidase formation along with increased ROS during ISC-REP.

In this study, incubation of isolated mitochondria for 50 min with succinate + rotenone + azide enhanced peroxidase formation as detected by increased Met(O) signal but did not yet alter cardiolipin content (data not shown). Longer mitochondrial incubation with succinate + rotenone + azide for a total of 90 min resulted in the depletion of cardiolipin (Fig. 3). This finding suggests that peroxidase formation occurs earlier than the depletion of cardiolipin, as expected. In addition, the result confirms that the increase in ROS generation along with electron flow through cyt *c* depletes cardiolipin from the mitochondria. Cardiolipin can undergo oxidation at the unsaturated *cis* double bonds of the linoleic (C18:2) fatty acyl chains that will result in the formation of peroxy-groups [49]. The hydrolysis of the peroxidized cardiolipin can form monolysocardiolipin [50]. Alternatively, the peroxy-acyl groups can interact directly with proteins, leading to covalent cardiolipin–protein complexes. Also, inter-molecular dimerization of cardiolipin by peroxyl bond formation undergoes decomposition and finally generates a secondary peroxidation product, 4-hydroxy-2-nonenal

Table 1
Ischemia–reperfusion affects mitochondrial oxidative phosphorylation. Mitochondria were isolated and rates of respiration were recorded. Based on state 3 and state 4 rates, respiratory control ratios (RCRs) were calculated. During recording of 2 mM ADP-dependent maximal state 3 respiration, uncoupler 2,4-dinitrophenol (DNP) was added to establish the relative contribution of the phosphorylation apparatus to the observed defects in integrated respiration. Results are presented as mean ± SEM. Values of state 3, state 4, 2 mM ADP and DNP rates are expressed in nanoatoms of atomic oxygen/min/mg of mitochondrial protein. n = 5 in all groups. ADP, adenosine di-phosphate; TMPD, N,N,N′,N′-tetramethyl-p phenylenediamine; RCR, respiratory control ratio; DNP, 2,4-dinitrophenol.

	TC perfusion n = 5	ISC-REP n = 5
Glutamate + malate		
State 3	346 ± 20	198 ± 9*
State 4	61 ± 6	75 ± 3
RCR	5.7 ± 0.9	2.7 ± 0.2*
2 mM ADP	438 ± 36	221 ± 17*
DNP	418 ± 28	192 ± 15*
TMDP/ascorbate		
2 mM ADP	1560 ± 62	1184 ± 88*

* $p < 0.05$ vs. TC perfusion.

(HNE) [51]. HNE forms Michael adducts by interacting with the nucleophilic groups in cysteine, histidine or lysine residues of the proteins [52]. HNE can interact with ε-NH_2 groups of Lys residues forming Schiff-bases [53]. The decomposed cardiolipin or cardiolipin interacting with proteins is no longer extractable by organic solvents and is difficult to detect [54]. Thus, peroxidation of cardiolipin can result in the depletion of cardiolipin without an increase in the content of the less stable peroxidation products, as observed following ischemia or reperfusion in cardiac models [45,55].

The relevance of the cyt *c* peroxidase during cardiac ischemia and reperfusion is unknown. Myocardial ischemia damages the ETC., resulting in decreased rates of respiration [1,2,7,43,45] as shown in this study by decreases in glutamate + malate- and TMPD-dependent respiration (Table 1, Fig. 5). To identify potential formation of cyt *c* peroxidase during ischemia–reperfusion in the heart, the immunocapture approach was used to specifically separate cyt *c* from other mitochondrial proteins (Fig. 4) and to assess Met(O) formation. As shown in Fig. 4C, our study for the first time demonstrates a significant increase in in situ Met(O) formation in reperfused hearts. Previous studies of cardiac ischemia and reperfusion in multiple animal models showed a selective decrease in cardiolipin content whereas the content of other phospholipids was preserved [45,55]. Ischemia did not alter the composition of cardiolipin that remained in the mitochondrial membrane [45]. As a result of cardiolipin oxidation and depletion, pro-apoptotic proteins including cyt *c* and Smac/Diablo are released into the cytosol resulting in cell death [28]. Observations from the ex-vivo ischemia–reperfusion studies strongly support the notion that the blockade of electron flow into cyt *c* protects mitochondria and preserves cardiolipin content [5,6,39,41,56–59].

In summary, an increase in ROS during ischemia, as a consequence of ETC.-driven ETC. damage, oxidizes cyt *c* Met_{80} that favors conversion of cyt *c* to a peroxidase (Fig. 5). Apart from ROS, electron flow through the cyt *c* segment of the ETC. is required for the genesis of peroxidase activity, which in turn depletes cardiolipin and favors the release of pro-apoptotic proteins into the cytosol resulting in cell death.

5. Translational relevance

Current therapeutic interventions to pre-condition and post-condition the heart during ischemia rely on cytoprotective signaling pathways. However, these therapeutic interventions are relatively ineffective in aging and diabetes due to defects in the signaling cascades [2–4]. Hence, direct therapeutic targeting of dysfunctional mitochondria will provide the potential to bypass the upstream signaling defects and intervene directly with the effector organelle. Proximal blockade of ETC. during ischemia or at the onset of reperfusion leads to the preservation of cyt *c* and cardiolipin contents with less cardiac injury [41,56]. Recently, the mitochondria-targeted peptide drug, SS-31, given during renal ischemia reduced reperfusion injury by specifically targeting mitochondria [60]. Here, we have identified a therapeutic target in the mitochondria, cyt *c* peroxidase, which can provide new site-specific strategies to protect mitochondria and thus the heart during ischemia and reperfusion. Recent work suggests that SS-31 inhibits the cyt *c* peroxidase as a likely mechanism of tissue protection [61]. This peptide decreases injury during ischemia and reperfusion in the heart [62], consistent with the formation of cyt *c* peroxidase during ISC and REP observed in the current study.

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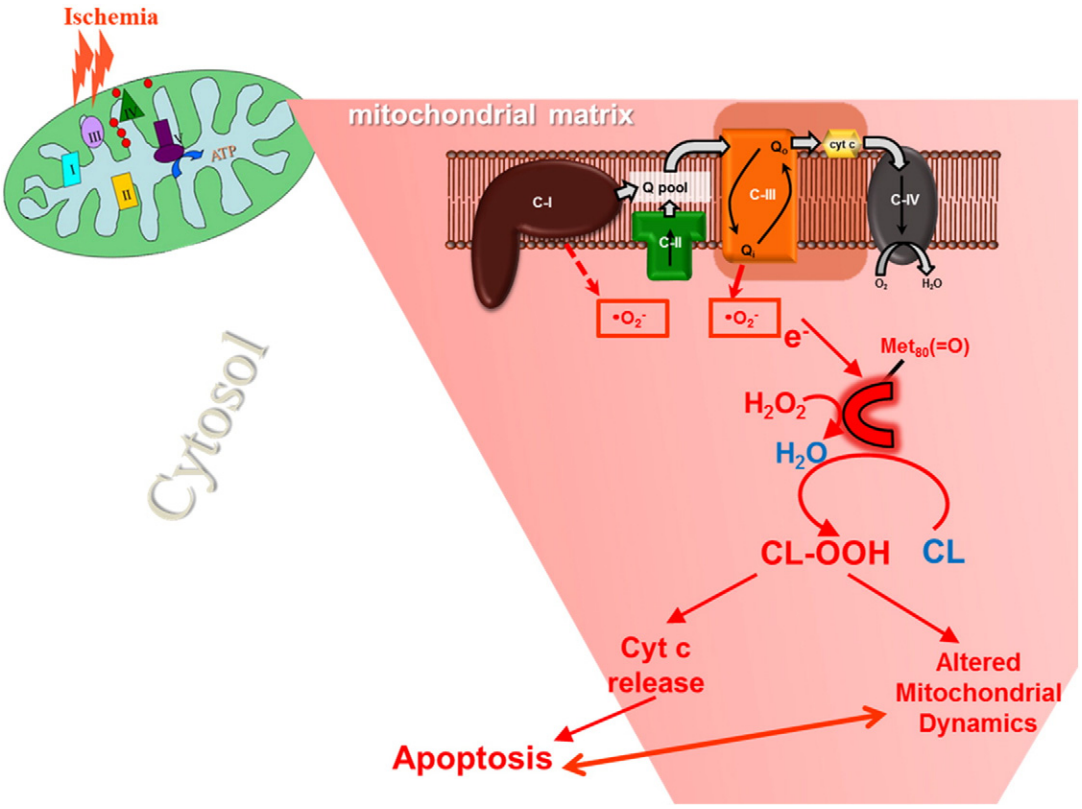

Fig. 5. Schematic representation showing the formation and role of cytochrome *c* peroxidase during ISC-REP. Electron flow into cyt *c* and ROS from the ETC. oxidize cyt *c* during ischemia and reperfusion converting cyt c into a peroxidase. Cyt *c* peroxidase utilizes H_2O_2 as a substrate, peroxidizes cardiolipin, and thereby depletes cardiolipin from the mitochondrial membrane. The peroxidation and depletion of cardiolipin alter the mitochondrial outer membrane permeation thereby triggering cell death.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.07.017.

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