Mitochondrial dysfunction in cardiac ischemia–reperfusion injury: ROS from complex I, without inhibition

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Abstract

A key pathologic event in cardiac ischemia reperfusion (I–R) injury is mitochondrial energetic dysfunction, and several studies have attributed this to complex I (CxI) inhibition. In isolated perfused rat hearts, following I–R, we found that CxI-linked respiration was inhibited, but isolated CxI enzymatic activity was not. Using the mitochondrial thiol probe iodobutyl-triphenylphosphonium in conjunction with proteomic tools, thiol modifications were identified in several subunits of the matrix-facing 1α sub-complex of CxI. These thiol modifications were accompanied by enhanced ROS generation from CxI, but not complex III. Implications for the pathology of cardiac I–R injury are discussed.

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1. Introduction

Cardiovascular disease (CVD) is the number 1 cause of death in the United States, with a large proportion of these deaths due to myocardial infarction (MI). Despite recent progress in understanding the origins of CVD, there is still a lack of understanding on the mechanisms of cell injury in MI. The amount of injury depends on the length of the ischemic period and the reperfusion conditions, with short periods of ischemia being relatively harmless and even eliciting cardioprotection (vis-à-vis ischemic preconditioning). Longer ischemic periods result in irreversible injury, often due to hypercontraction. The reperfusion phase is also harmful, whereupon injury is thought to be mediated in part by both reactive oxygen species (ROS) and reactive nitrogen species (RNS), although the sources and targets of ROS and RNS are poorly understood [1–5].

It has been known for some time that mitochondrial perturbations contribute to cardiac dysfunction in I–R injury (reviewed in [6]). Most of the energetic (ATP) requirements of the cardiomyocyte are met by mitochondria, and in addition the organelle plays important roles in Ca2+ homeostasis [7] and apoptosis [8]. The precise molecular changes that occur within mitochondria during I–R injury remain unclear, and have been the focus of much research. It has been reported that respiratory complexes I, III, IV and V, and many Krebs cycle enzymes are all affected by I–R injury [6,9–18]. In particular, complex I (CxI) has been identified as a target for oxidative damage in I–R injury and related pathologies such as autolysis [10–14,18]. In contrast, it was recently shown that in isolated cardiac mitochondria the activities of CxI, III, and IV were not inhibited following exposure to 50 μM H2O2, but the Krebs cycle enzymes aconitase, α-KGDH, and succinate dehydrogenase were inhibited [19]. Thus, the precise type and context of the oxidative insult appears to play an important role in the pattern of mitochondrial injury.

Complex I is a 900 kDa, >46 subunit enzyme that oxidizes NADH, passing electrons to ubiquinone, and pumping protons across the inner membrane. The resulting proton gradient is then utilized for the production of ATP, and since the heart relies almost entirely on the CxI-linked oxidation of fatty acids and glucose [20,21], perturbations to CxI can have a large impact on cardiac energetics. Early studies in canine myocardial...
dium suggested that CxI activity was decreased following I–R injury through the loss of a noncovalently bound FMN [18]. Notably, the FMN site of CxI has been proposed as a site of ROS generation [22,23], although this remains controversial [24]. In contrast, others have attributed the decrease in CxI activity following I–R to ROS-mediated oxidation of cardiolipin, the latter being critical for CxI function [10]. Consistently, several laboratories report that ROS or RNS can inhibit CxI [10,19,25–31]. In addition, it has been shown that CxI inhibition following I–R depends on the entry of Ca2+ into the mitochondrial matrix [12]. Unifying both the Ca2+ and ROS/RNS data, it has recently been shown that a combination of Ca2+ and NO· is required to inhibit CxI in vitro [25]. Such Ca2+ dependent CxI dysfunction in I–R may proceed via the known ability of Ca2+ to enhance mitochondrial ROS generation [32,33], leading to secondary generation of ONOO− from NO· and O2·-. Alternatively, several studies have suggested that CxI modification may occur via s-nitrosation [25,29,31], although direct molecular evidence for this is lacking.

Despite these studies, following I–R injury both the molecular defect in CxI and the source of ROS are not well understood. In order to probe the role of CxI in I–R injury both as a source and target of ROS, we investigated the molecular nature of the CxI lesion. Surprisingly, we found that 25 min ischemia plus 30 min reperfusion did not inhibit CxI activity in rat hearts, but did lead to thiol modifications within the complex, and an increase in ROS generation from CxI but not Cx III. Overall, these data suggest that in I–R injury, CxI thiols may be altered sufficiently to increase ROS production while not affecting overall enzymatic activity. The implications of these findings for mitochondrial and cardiac function in I–R injury are discussed.

2. Materials and methods

Male Sprague–Dawley rats (200–250 g) were obtained from Harlan and handled in accordance with the procedures of the University Committee on Animal Research (UCAR). All chemicals were from Sigma (St. Louis MO) unless otherwise stated. Statistical differences between the control and I–R groups were determined by Student’s t test, with significance set at P < 0.05.

2.1. Heart perfusions and mitochondrial isolation

Isolated rat hearts were retrograde (Langendorff) perfused essentially as described [15] in constant flow mode (12 ml/min/gain tissue) with oxygenated (95% O2, 5% CO2) Krebs–Henseleit (KH) buffer. Hearts were immersed in KH in a chamber maintained at 37 °C, and cardiac functionality was monitored by a water-filled left-ventricular latex balloon linked to a pressure transducer with digital recording (Dataq, Akron OH). In addition, diastolic stiffness was quantified as the slope of the relationship between diastolic pressure and balloon digital recording (Dataq, Akron OH). In addition, diastolic stiffness was quantified as the slope of the relationship between diastolic pressure and balloon digital recording (Dataq, Akron OH). In addition, diastolic stiffness was quantified as the slope of the relationship between diastolic pressure and balloon digital recording (Dataq, Akron OH). In addition, diastolic stiffness was quantified as the slope of the relationship between diastolic pressure and balloon digital recording (Dataq, Akron OH). In addition, diastolic stiffness was quantified as the slope of the relationship between diastolic pressure and balloon digital recording (Dataq, Akron OH).

At the end of all perfusion protocols, hearts were removed onto ice-cold buffer and mitochondria prepared as described [15]. Protein concentrations were determined using the Folin-phenol reagent against a standard curve of bovine serum albumin [36]. The mitochondrial marker enzyme citrate synthase was measured (see below) to determine mitochondrial yield and purity.

2.2. Mitochondrial respiration and complex assays

Mitochondrial O2 consumption was measured at 37 °C using a Clark-type O2 electrode in a magnetically stirred 0.25 ml chamber. Mitochondria were incubated at 0.5 mg protein/ml in respiration buffer (RB) comprising sucrose (300 mM), KCl (50 mM), KH2PO4 (5 mM), MgCl2 (1 mM), EGTA (5 mM), and Tris–HCl (20 mM), pH 7.35. Complex I linked state 4 respiration was initiated by addition of glutamate (10 mM) plus malate (2.5 mM), followed shortly thereafter by addition of ADP (100 μM) to initiate state 3 respiration. The enzymatic activities of CxI and citrate synthase were measured spectrophotometrically as previously described [37].

2.3. 4-iodobutyl-triphenylphosphonium (IBTP) labeling

One mg of mitochondrial protein was incubated in 1 ml of RB with the respiratory substrates glutamate (10 mM), malate (2.5 mM), and succinate (10 mM). After a 1-min equilibration, IBTP (25 μM) was added and mitochondria incubated for an additional 5 min with mixing to ensure adequate oxygenation. Mitochondria were then pelleted by centrifugation (5 min at 10,000 × g), snap frozen in liquid N2, and stored at −80 °C until proteomic analysis [38,39]. Samples (1 mg mitochondrial protein) were also prepared without IBTP, for biotin-maleimide labeling (see below).

2.4. Blue-native electrophoresis, and biotin-maleimide labeling

Blue-native (BN) gels were run essentially as described [39]. One mg mitochondrial pellets from IBTP labeling (above) were resuspended in 225 μl of BN-extraction buffer. After 30 min extraction on ice, samples were centrifuged at 14,000 × g for 5 min, and 200 μl of supernatant mixed with 12.5 μl of 5% Coomassie blue G in aminocaproate (0.5 M). Forty μl of sample (200 μg protein) was loaded into each lane, and gels were run as described [39]. Biotin-maleimide labeling was performed on non-IBTP labeled samples during the protein extraction step for BN gels. After 10 min on ice, 220 μM maleimide-PEO-biotin (Pierce, Rockford IL) was added, and samples incubated for an additional 20 min in the dark at 25 °C. Remaining sample extraction and BN gel procedures were as described above.

2.5. 2D BN gels and Western blotting

Complex I bands cut from the BN gel were placed between the plates of a 15% SDS-PAGE gel with 5% stacker. A solution of melted agarose (1%), SDS (1%), and TBB (0.5%) was poured into the remaining headspace of the plates. After the agarose had set, a solution of SDS (1%) and β-ME (0.01%) was poured over the top edge of the gel and left for 10 min Gels were run at standard voltages, then either fixed and silver-stained (SilverQuest, Invitrogen, Carlsbad CA), or transferred to nitrocellulose using semi-dry apparatus. Transfer of protein was examined by Ponceau S staining, and membranes were then blocked during the protein extraction step for BN gels. After 10 min on ice, 220 μM maleimide-PEO-biotin (Pierce, Rockford IL) was added, and samples incubated for an additional 20 min in the dark at 25 °C. Remaining sample extraction and BN gel procedures were as described above.

2.6. 3D gels (blue-native, IEF, SDS-PAGE)

Complex I bands cut from the BN gel, homogenized, and serially extracted in buffer containing urea (7 M), thiourea (2 M), CHAPS (2%), lauryl-maltoside (0.5%), diithiothreitol (30 mM), tributylphosphine (10 μM), and BioLyte™ ampholytes, at 25 °C. Polycrylamide was removed by centrifuga-
tion, and supernatants were loaded into “IPG-zoom” cassettes (Invitrogen) containing IPG strips (linear pH 3–10, BioRad, Hercules CA), sealed and incubated overnight at 25 °C. Strips were focused at 200 V for 20 min, 450 V for 20 min, 750 V for 20 min, 2000 V for 1 h. After IEF, strips were equilibrated in buffer containing urea (6 M), SDS (2%), Tris (0.375 M), glycerol (20%) and DTT (0.2 mg) for 15 min, then rinsed in SDS-PAGE running buffer and placed between the plates of a 15% SDS-PAGE gel with 5% stacker. A solution of melted agarose (1%) was poured into the remaining headspace of the plates, and gels and Western blots run as described above.

2.7. Peptide mass fingerprinting

Gel spots were cut and de-stained (using reagents in the Invitrogen SilverQuest kit), then equilibrated in NH₄HCO₃ (100 mM), reduced with DTT, alkylated with IAA, and washed with NH₄HCO₃ (100 mM) in acetonitrile (50%). Trypsin digestion, extraction, and MALDI-TOF peptide mass fingerprinting were performed as described [39,40].

2.8. Measurement of ROS

Mitochondrial ROS production was measured spectrofluorimetrically at 37 °C using the Amplex Red reagent (Molecular Probes, Eugene OR). Cuvets contained RB (see above), Amplex Red (10 μM), type II horseradish peroxidase (1 U/ml), Cu/Zn superoxide dismutase (80 U/ml), and mitochondria (0.5 mg/ml). Glutamate (2.5 mM) and malate (1.25 mM) were added to obtain a basal rate of CxI ROS production, followed by rotenone (2.5 μM) to maximize ROS from CxI. In separate incubations, succinate (2.5 mM) and rotenone (2.5 μM) were added to obtain a basal rate of ROS production from complex II to III (rottenone being present to prevent electron back-flow through CxI), followed by antimycin A (0.5 μM) to maximize generation at complex III. Rates of ROS generation were calibrated by adding known amounts of H₂O₂ at the end of each run.

3. Results

Cardiac functional damage caused by I–R injury is shown in Fig. 1. The sample trace of left-ventricular pressure in Fig. 1A shows the development of hyper-contracture during ischemia (arrow). Following reperfusion, diastolic pressure was increased, systolic pressure decreased, and thus developed pressure (systolic minus diastolic) was depressed (Fig. 1B). Diastolic stiffness was also elevated following I–R (Fig. 1C). Previously, elevation in this parameter has been correlated to mitochondrial dysfunction [15].

3.1. Mitochondrial dysfunction following I–R

Inhibition of complex I (NADH)-linked state 3 respiration has previously been reported in mitochondria isolated from hearts subject to I–R [18], and this finding is confirmed in Fig. 2A. This inhibition has previously been attributed to direct inhibition of complex I itself [9–13]. However, when CxI activity (normalized to protein) was measured in this study, no difference was found between control and I–R samples (Fig. 2B).

Since the heart undergoes edema during I–R injury, I–R tissue is less dense than control tissue, and thus may yield different amounts of mitochondria. However, mitochondrial yields were not different between control and I–R samples (16.8 ± 1.3 mg protein per heart respectively). Furthermore, the purity/enrichment of the mitochondrial fraction was not different, since the activity of the mitochondrial matrix marker enzyme citrate synthase (CS) was the same in control and I–R groups (Fig. 2C). Thus, even normalizing CxI activity to CS activity does not reveal an enzymatic defect at the CxI level (Fig. 2D).

These results are contrary to studies that have demonstrated CxI inhibition following I–R injury and associated pathologies [9–13]. Such a variance may originate from differences in the models of I–R injury used (e.g. animal species and age, I–R protocol), as evidenced by a greater than 10-fold variation in the reported baseline activities of CxI. Notably, it was reported cardiac I–R injury led to a significant decrease CxI activity in

Fig. 1. Langendorff functional data. (A) Typical left ventricular pressure trace (mm Hg) of I–R heart, with x axis compressed. (a) and (b) denote the onset of ischemia and reperfusion respectively, and the arrow indicates ischemic hypercontracture. (B) Left ventricular developed pressure (LVDP) in control perfusion (white bars) and I–R (black bars), both pre- and post- the perfusion protocol. (C) Diastolic stiffness constant in control perfusion (white bars) and I–R (black bars). All data are means ± S.E.M. from 7 independent experiments. *P < 0.01 between control perfusion and I–R groups.
old rats (28 months), but was without effect in young rats (8 months) [13]. The rats used in this study were relatively young (2 months). Regarding the I–R protocol, we found that extending the duration of ischemia to 40 min (vs. the 25 min shown in Fig. 2) elicited no further effect on CxI activity (control 298 ± 27 vs. I–R 363 ± 16 nmol NADH/min/unit CS).

Notably, none of the previous studies of CxI in I–R normalized CxI activity to CS, although it is unlikely that this accounts for the different findings because we did not find differences in the mitochondrial yields or specific activities of CS (see above). It is also possible that variances in CxI assay method may account for these contrary results. The current assay uses Co–Q1 as electron acceptor (vs. ferricyanide in some other studies [41]) and would not be expected to detect perturbations in the pathway of electron flux through the enzyme, or defects in the coupling of H+ pumping to e− flux. A lack of knowledge regarding the precise electron flux pathways within mammalian CxI currently precludes a full explanation.

Since it was found that CxI-linked respiration was depressed following I–R (Fig. 2A), we hypothesized that the enzymatic defect may lie upstream of CxI in the TCA cycle. It has previously been reported that several TCA cycle enzymes are sensitive to oxidative stress [13,19,42,43] and as shown in Fig. 2E, α-KGDH is indeed inhibited following I–R injury. While the decrease in α-KGDH activity (~22%) is alone not sufficient to account for the loss of respiratory activity, inhibition of other TCA cycle enzymes such as aconitase and pyruvate dehydrogenase [13,19,42,43] would be sufficient to elicit such respiratory inhibition. However, the focus of this investigation was CxI, and owing to the limited amounts of mitochondria available, we did not pursue this possibility further by assaying other TCA cycle enzymes.

3.2. Proteomic studies of CxI modification

While it may appear unusual to examine CxI modifications in a situation where no enzymatic inhibition was found, the
proteomic examination of CxI was performed concurrently with the enzymatic studies. Thus, the proteomic studies had yielded interesting results well before the conclusion regarding no CxI inhibition was reached.

3.3. Nitrotyrosine modification of CxI

Complex I has previously been identified as a target for tyrosine nitration [44], and therefore mitochondrial samples were resolved on BN gels, the CxI band subjected to SDS-PAGE to resolve the subunits, and Western blotting for nitrotyrosine performed. Fig. 3A shows significant nitration of various subunits in CxI following treatment of isolated mitochondria with ONOO−. Interestingly however, tyrosine nitration was not detected in mitochondria isolated from I−R hearts (Fig. 3B), despite over-exposure of the blot membrane (>24 h ECL development, vs. 20 min for the ONOO−-treated mitochondrial blot). Furthermore, no modification of CxI by the lipid oxidation product 4-HNE was detected (Western blot, Calbiochem monoclonal anti-4-HNE Ab, data not shown). In addition, as shown in Fig. 3C and D, the loss of CxI activity did not correlate with the degree of nitration, the latter occurring only at higher levels of ONOO−, beyond those at which the complex was already inhibited. Owing to limitations in the antibody detection of nitrotyrosine (e.g., epitope specificity), quantitative interpretation of such data may be restricted. Nevertheless, these results suggest that nitration of CxI can occur with ONOO− treatment, but is probably an epiphenomenon and not the primary mechanism of enzymatic inhibition. The other reactivities of ONOO− (including but not limited to oxidation of thiols, tryptophan nitration, or protein oxidation via secondary OH formation, [45]) probably account for the loss of CxI activity in this case.

3.4. Thiol modifications in CxI

Since it was suspected that cysteine thiols in CxI may be modified in I−R injury, mitochondria were labeled with the mitochondria-specific thiol probe IBTP, and separated on BN gels. Fig. 4A shows a typical BN gel, indicating that the physical amount of CxI is not different between control and I−R samples, and this is confirmed by a Western blot for the CxI 39 kDa subunit (Fig. 4B).

The results of an IBTP Western blot on CxI subunits are shown in Fig. 4C (i.e. CxI cut from the BN gel and resolved into subunits by SDS-PAGE), with a corresponding Coomassie stained gel in Fig. 4D. The presence of a band on the blot indicates that a protein at that molecular weight contains a free thiol capable of reacting with IBTP. Thus, a diminished signal in the I−R samples indicates that thiols are no longer able to react with the IBTP probe either due to oxidation, disulfide formation, s-nitrosation, or other such modifications.

Since the resolution of 1D SDS-PAGE gels is somewhat limited, a 3D gel method was also developed in which CxI bands cut from BN gels were subjected to IEF and then SDS-PAGE, thereby constituting 3 dimensions of separation. The 3D gels were Western blotted for IBTP, with the enhanced resolution of the 3D system affording less overlap between bands/spots, and thus greater confidence in mass spectrometric identifications (Fig. 4E and F).

The 3D separation resulted in fewer CxI subunits being visible on the gel, probably owing to their hydrophobic/basic nature and thus incompatibility with IEF. Furthermore, the separation revealed that fewer CxI subunits were present in the I−R samples, and numerous subunits exhibited small changes in isoelectric point following I−R. The corresponding 3D IBTP gels also showed significantly fewer spots than the 1D IBTP gels shown in panel C, but nevertheless, excision of proteins from either the 2D or 3D gels permitted identification of several proteins with diminished IBTP labeling in the I−R samples, and these data are shown in Table 1. Notably, all of the modified proteins were located in the matrix-facing 1α sub-complex of CxI [46].

3.5. Mitochondrial ROS generation in I−R

Complex I has previously been reported as a site of mitochondrial ROS generation [22–24,33], and since the modified subunits in the 1α sub-complex contain several electron-transporting moieties, including Fe−S centers and the FMN binding site [46] (Fig. 5A), it was hypothesized that thiol modifications in this sub-complex might enhance ROS.
generation. This appears to be the case, since the data in Fig. 5B show that mitochondria from I–R hearts exhibited elevated ROS generation when respiring on CxI-linked substrate, but a decreased generation of ROS when respiring on CxII-linked substrates. Notably, no change was seen in the maximally stimulated rate of ROS generation from CxI (Fig. 5C) in the presence of rotenone, again suggesting no change in the overall enzymatic activity of the complex. A faster rate of ROS generation from Cx III in I–R mitochondria vs. controls was revealed upon addition of antimycin A, which maximizes ROS from this complex. However, such a supra-physiological ROS generation condition may not be extrapolated to the in situ condition.

3.6. Pharmacologic cardioprotection and CxI thiol modifications

As part of a series of ongoing studies on cardioprotection by diazoxide (Dz) and cariporide (Cp), mitochondria were harvested from hearts subject to I–R with or without Dz/Cp treatment [34,35]. These compounds are proposed to elicit cardioprotection by convergent mechanisms, whereby Dz opens mitochondrial K\textsubscript{ATP} channels, possibly preventing mitochondrial Ca\textsuperscript{2+} overload, and Cp inhibits NHE-1, preventing cytosolic Na\textsuperscript{+} overload which may subsequently prevent Ca\textsuperscript{2+} overload [34,35,47]. As shown in Fig. 6A, administration of Dz/Cp to hearts elicited a substantial improvement in post-I–R functional recovery. In addition, this recovery was associated with prevention of CxI thiol modifications (Fig. 6B).

4. Discussion

The main findings of this study are as follows: (i) Mitochondrial CxI activity is not inhibited in I–R injury, despite a substantial decrease in CxI-linked respiration. (ii) Tyrosine nitration does not occur at CxI in I–R. (iii) Modification of thiol s of the 1\textalpha sub-complex elicits an elevation in CxI-linked ROS generation.

Previously, multiple studies have reported damage to the mitochondrial oxidative phosphorylation machinery in I–R injury and associated pathologies [6,9–18], with most of these studies concentrating on respiratory complexes I–V. In addition, a large body of literature exists on the susceptibility of mitochondria to inhibition by ROS and RNS in a variety of model systems [19,25–32,41–44]. Studies have also shown that free radical scavengers, especially those targeted to mitochondria, can ameliorate I–R injury [5,48,49]. Free radicals have also been shown to cause contractile dysfunction and arrhythmias in perfused heart models [50]. Despite these findings, the molecular events underlying oxidative inhibition of the respiratory chain and ROS generation in I–R are poorly understood. This is especially true for CxI, since it contains at least 46 different subunits, and its enzymatic mechanism is still poorly understood.
under debate [46]. Thus, we sought to apply proteomic and other tools to investigate CxI in I–R injury.

Surprisingly, despite observing a decrease in CxI-linked respiration following I–R, no decrease was seen in the enzymatic activity of CxI itself. One reason for this lack of effect may be that only “healthy” mitochondria were isolated, thereby removing any differences present in the intact tissue. However, two pieces of evidence suggest this was not the case: firstly, respiration was inhibited, suggesting mitochondria isolated from I–R hearts were damaged (Fig. 2A). Secondly, the IBTP labeling data (Fig. 4C) clearly show modification of mitochondrial proteins was occurring. Thus, it was unlikely that we missed the inhibition of CxI due to poor experimental design or assay methods.

If CxI activity was not inhibited in I–R injury, why was CxI-linked respiration inhibited? The data in Fig. 2E showing inhibition of the Krebs cycle enzyme α-KGDH, coupled with several reports that Krebs cycle enzymes are sensitive to oxidative stress [13,19,42,43], suggests that the Krebs cycle and not the respiratory chain is a major site for oxidative damage during I–R injury. While the degree of inhibition of α-KGDH is not sufficient to account for all of the inhibition of CxI-linked respiration, it is expected that inhibition of the other Krebs cycle enzymes is also occurring, leading to a cumulative effect on respiration.

Regarding the disparity of our data with the many previous reports of CxI inhibition in I–R and associated pathologies [6,9–18], notable differences between previous and present investigations could include: (i) the experimental model (perfused hearts vs. cardiomyocytes), (ii) the animal species and the age of animals used, (iii) normalization of data to mitochondrial marker enzymes such as CS, (iv) the duration of ischemia and reperfusion, (v) the use of volatile anesthetics that precondition the heart [9], (vi) gender differences, it can be summarized that further investigation is beyond the scope of this discussion to elucidate each of these differences, it can be summarized that further investigation is required, especially in humans, to determine whether enzymatic inhibition of CxI is an important factor in cardiac I–R injury. Most notably, age appears to be a critical factor since it has previously been reported that following I–R, CxI inhibition occurs only in old rats, not in young rats like those used in the current study [13].

Temporally, despite the lack of inhibition of CxI in the current I–R model, investigations into the post-translational modification of CxI were already underway, and yielded some interesting results. While ONOO⁻-treated mitochondria exhibited nitrization of CxI subunits (Fig. 3A and C), no such modification was seen in I–R mitochondria (Fig. 3B), suggesting that CxI nitration is not significant in I–R. Furthermore, the mismatch between CxI nitration and loss of activity in ONOO⁻ treatment (Fig. 3D) suggests that ONOO⁻ may inhibit CxI by mechanisms other than nitration.

One possible mechanism of CxI inhibition that can be mediated by ONOO⁻ and other ROS/RNS is the modification of cysteine thiols, including but not limited to oxidation, nitrosation, nitrination, glutathionylation, or disulfide formation [45]. To investigate thiol modifications, we used the mitochondrial thiol probe IBTP, and demonstrated that thiol groups in the 1α sub-complex are modified following I–R injury. The
precise molecular nature of the thiol modification leading to loss of IBTP labeling is not yet known.

Since the accumulation of IBTP into mitochondria depends on $\Delta \psi$, and it is known that $\Delta \psi$ is lower in mitochondria from I–R hearts [51,52], it could be argued that the loss of IBTP labeling in certain CxI subunits in I–R mitochondria may simply be due to less uptake of IBTP in these mitochondria. However, several factors suggest this is not the case: Firstly, IBTP labeling is only lost in certain subunits of CxI, not all subunits as would occur if IBTP uptake was diminished. Secondly, even if $\Delta \psi$ was diminished significantly, the Nernstian accumulation of IBTP would not drastically affect its intra-mitochondrial level. For example a drop of $\Delta \psi$ from 160 mV down to 130 mV would cause the intra-mitochondrial concentration of IBTP (at an external concentration of 25 \ensuremath{\mu}M) to fall from 21 mM to 7 mM, which is still above the threshold needed for adequate labeling over a time course of minutes [38]. Thirdly, experiments performed with biotin-maleimide labeling, which is not sensitive to $\Delta \psi$, gave similar results (not shown). Furthermore it should be noted that several CxI sub-units that are not in the 1\ensuremath{\alpha} sub-complex were labeled by IBTP (Fig. 4), and that such labeling was not lost following I–R, thereby suggesting specific modifications to the 1\ensuremath{\alpha} sub-complex.

The 1\ensuremath{\alpha} sub-complex of CxI consists of the NADH-binding 51 kDa subunit, the FMN-binding site, the 24 and 10 kDa subunits, the tightly bound 75 kDa subunit, and numerous Fe– S centers [46]. The presence of so many electron carriers and the documented generation of ROS by CxI [22– 24], led us to hypothesize that thiol modifications within the 1\ensuremath{\alpha} sub-complex may elevate ROS generation by the complex. Indeed, as Fig. 5B shows, CxI-linked but not CxII-III-linked ROS generation was significantly elevated in I–R mitochondria. Notably, no difference between control and I–R mitochondria was seen when CxI was maximally stimulated to generate ROS by adding rotenone (Fig. 5C). This suggests that the generation of ROS by CxI is not due to different amounts of CxI, which is in agreement with the data in Figs. 2D and 4A. Interestingly, when antimycin A was added to maximally stimulate CxIII-linked ROS generation, I–R mitochondria generated significantly more ROS than those from control hearts. This artificially high ROS rate may be more representative of a diminished antioxidant status in I–R hearts than due to any enzymatic differences in complex III, although the latter has been demonstrated in I–R [6].

Comparison of CxI activity (nmols NADH/min) with rates of ROS generation (pmols H$_2$O$_2$/min) reveals that the percentage of electron flux through CxI diverted to ROS generation increases from ~0.01% in control conditions to ~0.015% following I–R. Thus, while CxI enzymatic activity is not inhibited by I–R injury, modification of thiol groups may result in diversion of more electrons towards ROS generation. It remains unknown whether such a small increase in ROS generation can cause pathologic effects within the mitochondrion, but notably a recent hypothesis paper has suggested that inhibition of TCA cycle enzymes by ROS may be a protective strategy, since it would diminish NADH generation by the TCA cycle, thereby slowing electron flow into the respiratory chain, and limiting downstream ROS production [53]. Thus, ROS generation at CxI and subsequent inhibition of the Krebs cycle by ROS, would appear to form a feed-back loop mechanism. The proximity of the 1\ensuremath{\alpha} sub-complex of CxI to the matrix TCA cycle enzymes would greatly facilitate such a system.

In summary, the current investigation further implicates mitochondrial ROS generation and ROS-mediated post-translational modifications in the pathology of I–R, providing a framework for understanding the molecular events at CxI. It is expected that targeted therapeutics which can prevent such modifications may be beneficial in I–R injury.

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