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Mitochondrial Complex I in the Post-ischemic Heart:

REPERFUSION-MEDIATED OXIDATIVE INJURY AND PROTEIN CYSTEINE SULFONATION

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Abstract

A serious consequence of ischemia-reperfusion injury (I/R) is oxidative damage leading to mitochondrial dysfunction. Such I/R-induced mitochondrial dysfunction is observed as impaired state 3 respiration and overproduction of $\cdot\text{O}_2^-$. The cascading ROS can propagate cysteine oxidation on mitochondrial complex I and add insult to injury. Herein we employed LC-MS/MS to identify protein sulfonation of complex I in mitochondria from the infarct region of rat hearts subjected to 30-min of coronary ligation and 24-h of reperfusion in vivo as well as the mitochondria of sham controls. Mitochondrial preparations from the I/R regions had enhanced sulfonation levels on the cysteine ligands of iron-sulfur clusters, including N3 (C₄₂₅), N1b (C₉₂), N4 (C₂₂₆), N2 (C₁₅₈/C₁₈₈), and N1a (C₁₃₄/C₁₃₉). The 4Fe-4S centers of N3, N1b, N4, and N2 are key redox-active components of complex I, thus sulfonation of metal-binding sites impaired the main electron transfer pathway. The binuclear N1a has a very low redox potential and an antioxidative function. Increased C₁₃₄/C₁₃₉ sulfonation by I/R impaired the N1a cluster, potentially contributing to overall $\cdot\text{O}_2^-$ generation by the FMN moiety of complex I. MS analysis also revealed I/R-mediated increased sulfonation at the core subunits of 51 kDa (C₁₂₅, C₁₈₇, C₂₀₆, C₂₃₈, C₂₅₅, C₂₈₆), 75 kDa (C₃₆₇, C₅₅₄, C₅₆₄, C₇₂₇), 49 kDa (C₁₄₆, C₃₂₆, C₃₄₇), and PSST (C₁₈₈). These results were consistent with the consensus indicating that 51 kDa and 75 kDa are two of major subunits hosting regulatory thiols, and their enhanced sulfonation by I/R predisposed the myocardium to further oxidant stress with impaired ubiquinone reduction. MS analysis further showed I/R-mediated enhanced sulfonation at the supernumerary subunits of 42 kDa (C₆₇, C₁₁₂, C₁₈₃, C₂₅₃), 15 kDa (C₄₃), and 13 kDa (C₇₉). The 42 kDa protein is metazoan-specific, which was reported to stabilize mammalian complex I. C₄₃ of the 15 kDa subunit forms an intramolecular

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AUTHOR CONTRIBUTIONS: PTK and CLC conducted most of the experiments and analyzed the results. PL conducted experiments on the respiratory analysis. LZ conducted the LC-MS/MS experiments at OSU. JLZ provides the inputs of expertise in disease model of in vivo myocardial I/R, data interpretation, and discussion. YRC conceived the idea for the project, conducted the enzymatic assay of complex I activity, and wrote the paper.

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disulfide bond with C₅₆, which was reported to stabilize complex I structure. C₇₉ of the 13 kDa subunit is involved in Zn²⁺-binding, which was reported functionally important for complex I assembly. C₇₉ sulfonation by I/R was found to impair Zn²⁺-binding. No significant enhancement of protein sulfonation was observed in mitochondrial complex I from the rat heart subjected to 30 min ischemia alone in vivo despite a decreased state 3 respiration, suggesting that the physiologic conditions of hyperoxygenation during reperfusion mediated an increase in complex I sulfonation and oxidative injury. In conclusion, sulfonation of specific cysteines of complex I mediates I/R-induced mitochondrial dysfunction via impaired ETC activity, increasing $\bullet\text{O}_2^-$ production and mediating redox dysfunction of complex I.

Keywords

Complex I; protein cysteine sulfonation; protein structure; mitochondrial dysfunction; myocardial ischemia and reperfusion

1. Introduction

Mitochondrial dysfunction in myocardial ischemia-reperfusion injury is caused by overproduction of oxygen free radical(s) and their derived reactive oxygen species (ROS) [1–6]. With myocardial ischemia and reperfusion (I/R), oxygen delivery to the myocardium during ischemia is not sufficient to meet the need for mitochondrial oxidation under the physiological conditions of hypoxia, leaving the mitochondrial electron transport chain (ETC) in a more reductive state. In the ischemic heart, reperfusion is a “double-edged sword.” Re-establishment of reperfusion is mandatory to salvage the ischemic myocardium from infarction, but reperfusion *per se* can contribute to injury and ultimate infarct size.

Reperfusion with high oxygen tension (pO₂) results in increased electron leakage from the ETC that, in turn, reacts with residual molecular oxygen to give $\bullet\text{O}_2^-$ [6]. Because of the lack of ADP along with a decrease in antioxidant scavenging capacity, re-introduction of oxygen with reperfusion will result in pO₂ overshoot in the myocardium and induce physiological conditions of hyperoxygenation, as has been determined previously by EPR oximetry [7, 8]. Reperfusion thus greatly increases electron leakage, leading to ROS overproduction in the mitochondria. The overproduction of ROS initiates oxidative damage to the ETC [4, 9].

Complex I (EC 1.6.5.3. NADH:ubiquinone oxidoreductase) is a key membrane protein complex in mitochondria that catalyzes the oxidation of NADH in the mitochondrial matrix. NADH oxidation is coupled to reduction of ubiquinone at the mitochondrial inner membrane as one part of the respiratory electron transport chain. Complex I mediates electron transfer from NADH to ubiquinone through the prosthetic groups of flavin mononucleotide (FMN), [2Fe-2S] (N1a and N1b), [4Fe-4S] (N3, N4, N5, N6a, N6b, and N2), and ubiquinone. The main electron transfer pathway within complex I is proposed as NADH → FMN → N3 → N1b → N4 → N5 → N6a → N6b → N2 → Q [10]. The catalysis of complex I further contributes to $\bullet\text{O}_2^-$ generation in mitochondria [11, 12]. In the mitochondria of the post-ischemic myocardium, the overproduction of $\bullet\text{O}_2^-$ and its derived oxidants can impair the ETC. A decrease in the enzymatic activity of mitochondrial complex I has been reported in

the post-ischemic heart [4, 7, 11]. Oxidative injury to complex I and the vicious cycle of $\bullet\text{O}_2^-$ overproduction by impaired complex I are thus postulated to mediate the critical disease mechanism of myocardial ischemia and reperfusion [4, 5, 9].

Complex I has been further reported to be the major component of the ETC that hosts protein regulatory thiols that are thought to function in antioxidant defense and redox signaling [13, 14]. Physiologically, the complex I-derived regulatory thiols have been implicated in respiratory regulation, nitric oxide utilization [15, 16], and the redox status of mitochondria [17–19]. It has been well documented that the subunits of Ndufv1 (51 kDa) and Ndus1 (75 kDa) from the hydrophilic domain of complex I are two of major polypeptides hosting regulatory thiols in mitochondria [13, 14, 20, 21]. Both the 51 kDa and 75 kDa subunits are involved in the redox modification of S-glutathionylation and protein thiol radical formation in vitro and in vivo [13, 14, 20–22]. In the animal disease model of myocardial ischemia and reperfusion injury, we have demonstrated that intrinsic and reversible S-glutathionylation of the complex I 51 kDa and 75 kDa subunits can be detected in the normal rat myocardium, and enhanced in the post-ischemic myocardium [11]. In vitro oxidant exposure of mitochondria also resulted in impaired complex I activity, but reversal of glutathionylation by dithiothreitol (DTT) failed to restore complex I activity, suggesting a non-essential role for reversible S-glutathionylation and a possible essential role of irreversible cysteine oxidation in oxidant-induced damage to complex I activity [23]. Rationally, a reperfusion-enhanced irreversible sulfonation (conversion of protein thiol to protein sulfonic acid) of complex I is thus postulated in the post-ischemic heart, playing a seminal role in promoting oxidative injury and the disease process of infarction.

This study was undertaken to address fundamental questions regarding the molecular mechanism of oxidative injury in the post-ischemic heart. Here we have focused on the oxidative post-translational modification of mitochondrial complex I. We have demonstrated reperfusion-dependent augmented cysteine sulfonation and sulfinylation (conversion of protein thiol to protein sulfinic acid) of the hydrophilic domain of complex I in the mitochondria of the post-ischemic heart in vivo. We have further identified the specific cysteine residues involved in complex I-derived sulfonation and sulfinylation. The results of the current study provide deep insights into the disease mechanism: how I/R-induced site-specific cysteine oxidation of complex I mediates impairment of enzymatic catalysis, enhancing $\bullet\text{O}_2^-$ production, redox dysfunction, and consequent mitochondrial dysfunction.

2. EXPERIMENTAL PROCEDURES

2.1. Animals –

Male Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN). The SOD2-tg mice (strain: FVB-Tg (Myh6-SOD2, Tyr) 3Pne/J) were obtained from the Jackson Laboratory. All procedures were performed with the approval (protocol no. 15–028) of the Institutional Animal Care and Use Committee (IACUC) at Northeast Ohio Medical University (Rootstown, OH) and conformed to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the NIH.

2.2. Reagents –

NADH (β -Nicotinamide adenine dinucleotide, reduced disodium salt hydrate), diethylenetriaminepentaacetic acid (DTPA), ubiquinone-1 (Q_1), ADP (Adenosine 5'-diphosphate monopotassium salt dihydrate), oligomycin A, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), rotenone, DTT (1,4-Dithiothreitol), and polyethylene glycol superoxide dismutase (PEG-SOD) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO) and used as received. The DMPO spin trap was purchased from Dojindo Molecular Technologies, Inc. (Rockville, MD), and stored under argon at -80°C until needed. The spin probe, 1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine.HCl (CM-H) was purchased from Enzo Life Science (Farmingdale, NY). The proteases of a Trypsin/LysC mix (Mass Spec Grade, catalog no. V5073) and chymotrypsin (sequencing grade, catalog no. V1062) were purchased from Promega Corporation (Madison, WI), and stored at -20°C until needed.

2.3. Myocardial infarction model, echocardiography, infarct size, and mitochondrial preparation –

The procedure to produce a myocardial infarct involved an in vivo ischemia (30-min) and reperfusion (24-h) rat heart model (FIGURE S1A). Sprague-Dawley rats (~ 300 – 350 g and 9–10 weeks old) were anesthetized with isoflurane ((2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane)) via inhalation, and subjected to 30 min LAD (left anterior descending coronary artery) ligation, followed by a 24 h reperfusion according to our recently published protocol [4]. Sham controls were created by a similar procedure except that the suture placed around the LAD was not occluded. An ischemic heart region was created by a similar approach except that the myocardial tissue was immediately excised right after a 30 min LAD coronary ligation without the procedure of blood reflow. Impairment of cardiac function by I/R was analyzed and confirmed by two-dimensional echocardiography (M-mode) using the Vevo 770/3.0 system and software (VisualSonics) as described previously [24].

At 24 hours after reperfusion, the rat was re-intubated and ventilated, the left coronary artery was re-occluded, and 2.0% Evans blue (w/v, Sigma-Aldrich) was injected from the inferior vena cava to delineate the non-ischemic myocardial tissue. The rat heart was then cut into transverse slices. The slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) to determine the infarct area and photographed under a dissecting microscope according to the method published previously [8]. Left ventricle area (LV), area at risk (AAR), and infarct area (IF) were determined by computerized planimetry. The area of myocardial tissue showing white color was determined as IF. Infarct size was expressed as percentage of the IF in AAR.

Risk regions of myocardial tissues from the sham control, ischemic heart, and post-ischemic heart were excised and subjected to mitochondrial preparation and respiratory analysis according to published protocols [4, 25].

To avoid any artifacts arising from air oxidation and post-isolation oxidation, the buffer used for mitochondrial preparation was prebubbled with argon for 1-h by following the approach

reported previously in studying hypochlorite-induced methionine oxidation of cytochrome *c* [26].

2.4. Analytical Methods –

Optical spectra were measured on a Shimadzu 2600 UV/VIS recording spectrophotometer. The protein concentrations of mitochondrial preparations were determined by the Lowry method using BSA (bovine serum albumin) as a standard. The concentration of Q₁ was determined by absorbance spectra from NaBH₄ reduction using a millimolar extinction coefficient of $\epsilon_{(275\text{nm}-290\text{nm})} = 12.25 \text{ mM}^{-1}\text{cm}^{-1}$.

Measurements of mitochondrial NADH-linked $\cdot\text{O}_2^-$ production and redox activity of converting cyclic hydroxylamine (CM-H) to stable nitroxide by EPR spin trapping and direct EPR were carried out on a Bruker EMX Micro spectrometer operating at 9.43 GHz with 100 kHz modulation frequency at room temperature by following the established protocol [24]. The spectral simulations for spin quantitation were performed using the WinSim program developed at NIEHS by Duling [27].

2.5. Analysis of the ETC complexes from the mitochondrial preparations of sham control and post-ischemic rat hearts by native polyacrylamide gel electrophoresis (native PAGE) –

Preparations of mitochondria (80 μg of protein samples) were mixed with sample buffer (50 mM Bis-Tris-HCl, pH 7.2 containing 50 mM NaCl, 10% glycerol, 0.1 mg Ponceau S, and 0.01% dodecyl- β -D-maltoside), and then subjected to blue native polyacrylamide gel electrophoresis (3–15% Bis-Tris gel, NativePAGE™, Life Technologies, Carlsbad, CA) at 4 °C for 5.5 h (150 volts) using a running buffer of 50 mM Bis-Tris/Tricine containing 0.01% dodecyl- β -D-maltoside, pH 7.4–7.6, and then stained with coomassie blue R-250. Note: the native polyacrylamide gel was pre-run at 4 °C (150 volts) for 4 h prior to sample loading. Cathode buffer used: 50 mM Bis-Tris/Tricine-NaOH containing 0.01% dodecyl- β -D-maltoside and 0.05% sodium deoxycholate, pH 8.8, without addition of coomassie blue G-250 dye. Anode buffer used: 50 mM Bis-Tris/Tricine, pH 7.4–7.6. The clear gel was then subjected to *in-gel* activity staining for mitochondrial complex I [28, 29].

2.6. In-gel proteolytic digestion with trypsin/Lys C mixture or chymotrypsin, LC-MS/MS analysis, and quantitation –

Gel bands containing intact complex I were alkylated with iodoacetamide (1 mM used, carbamidomethylation) and then digested with sequencing-grade trypsin/lysC mixture or chymotrypsin from Promega (Madison WI) *under conditions of argon saturation* as described in the previous publication [21, 26]. Digested samples were analyzed via nanoflow liquid chromatography interfaced with an Orbitrap Fusion mass spectrometer (Thermo Scientific). The Orbitrap Fusion was coupled to a Dionex UltiMate 3000 nanoflow LC system. The analytical column was a Dionex Acclaim PepMap RSLC 75 $\mu\text{m} \times 15 \text{ cm}$ with a trapping column (C18 PepMap 100, 5 μm). Mobile phase A was water with 0.1% formic acid, and mobile phase B contained 80% acetonitrile with 0.1% formic acid. Samples were loaded onto the trap column, washed with a solution containing 2% acetonitrile with 0.1% formic acid for 5 min, and back-flushed to the analytical column with 5% mobile phase B.

For peptide separation, a 75-min gradient from 5% to 35% mobile phase B was followed by increasing the mobile B phase from 35% B to 55% B within 15 min. The gradient was increased again to 90% B in 5 min, and the column was washed with 90% mobile phase B for another 4 min before being equilibrated using 2% B for another 16 min. The flow rate was set at 300 nL/min. MS/MS data was acquired with a spray voltage of 1.7 KV and a capillary temperature of 275 °C; the S-Lens RF level was set at 60%. The scan sequence of the mass spectrometer was based on the preview mode data dependent TopSpeed™ method with CID and ETD as fragmentation methods: the analysis was programmed for a full scan recorded between m/z 400 – 1600 and an MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive scans of the five most abundant peaks in the spectrum.

To achieve high mass accuracy MS determination, the full scan was performed in FT mode and the resolution was set at 120,000. The AGC Target ion number for the FT full scan was set at 4×10^5 ions, the maximum ion injection time was set at 50 ms, and the micro scan number was set at 1. MSn was performed using ion trap mode to ensure the highest signal intensity of MSn spectra. The AGC Target ion number for the ion trap MSn scan was set at 100 ions, the maximum ion injection time was set at 250 ms, and the micro scan number was set at 1. The CID fragmentation energy was set to 35%. Dynamic exclusion was enabled with a repeat count of 1, an exclusion duration of 60s, and a low mass width and high mass width of 10ppm.

Sequence information from the MS/MS data was processed by converting the raw files into a merged file (mgf) using an in-house program, RAW2MZXML_n_MGF_batch (merge.pl, a Perl script). Isotope distributions for the precursor ions of the MS/MS spectra were deconvoluted to obtain the charge states and monoisotopic m/z values of the precursor ions during the data conversion. The resulting mgf files were searched using Mascot Daemon by Matrix Science, version 2.3.2 (Boston, MA), and the database was searched against the SwissProt rat database. The mass accuracy of the precursor ions was set to 10 ppm, with an accidental pick of one ^{13}C peak also included in the search. The fragment mass tolerance was 0.5 Da. The variable modifications considered were methionine oxidation [M(ox)], deamidation of asparagine or glutamine [N(de) and Q(de)], cysteine carbamidomethylation [C(cam)], cysteine dioxidation (CSO_2H), and cysteine trioxidation (CSO_3H). Four missed cleavages for the enzyme were permitted. A decoy database was also searched to determine the false discovery rate (FDR), and peptides were filtered according to the FDR. The significance threshold was set at $p < 0.05$, and bold red peptides were required for valid peptide identification. Any modifications or low-score peptide/protein identifications were manually checked for validation.

The label-free quantification (LFQ min. ratio count: 2, Fast LFQ, LFQ min. number of neighbors: 3, LFQ average number of neighbors: 6) was performed by the software MaxQuant with the false discovery rate (FDR) set to 0.01 according to published approaches [30, 31]. The MS/MS of the peptide sequence was visualized by the MaxQuant viewer with advanced annotation.

The S-sulfonated or S-sulfinated tryptic or chymotryptic peptides identified by LC-MS/MS analysis are presented in Tables 1–4 based on the amino acid sequences of the precursors containing the transient signaling peptides of complex I, including the core subunits of 51 kDa (Ndufv1), 75 kDa (Ndus1), 24 kDa (Ndufv2), PSST (Ndufs7), 49 kDa (Ndufs2), and the supernumerary subunits of 42 kDa (Ndufa10), 15 kDa (Ndufs5), and 13 kDa (Ndufs6).

2.7. Data analysis –

Statistical analysis was performed using Origin 9.1 data analysis software. Results were presented as mean $\pm$ SE, followed by *p* value. A probability value $*p < 0.05$ was used to establish statistical significance.

2.8. Molecular structure and modeling of mammalian complex I –

In the presentation of Figs. 4–7, the three-dimensional structure of mammalian complex I was adopted using the published atomic structure of ovine respiratory complex I solved by cryo-electron microscopy (protein data bank code 5LNK, resolution: 3.9 Å) [32].

3. RESULTS

3.1a. Cardiac function and infarct size in the post-ischemic rat heart –

Echocardiographic measurement indicated that I/R has no effect on heart rate (in bpm, 363.9 ± 29.7 vs 365.9 ± 29.9 , $n = 11$, sham control vs I/R). However, I/R significantly decreased the parameters of ejection fraction (in %, 77.2 ± 3.8 vs 56.5 ± 8.5 , $n = 11$, sham control vs I/R), fractional shortening (in %, 48.9 ± 5.3 vs 30.3 ± 6.5 , $n = 11$, sham control vs I/R), and cardiac output index (in ml/min/kg, 0.28 ± 0.07 vs 0.17 ± 0.04 , $n = 11$, sham control vs I/R). The ratio of infarct area to area of at risk (IF/AAR) is $27.5 \pm 8.7\%$ ($n = 19$). The ratio of risk region tissue to whole heart is $26.3 \pm 6.9\%$ ($n = 19$).

3.1b. NADH-linked state 3 oxygen consumption rate in the post-ischemic heart –

It has been hypothesized that overproduction of oxygen free radicals during myocardial I/R can induce Ca^{2+} overloading, depolarize the mitochondrial membrane, and uncouple mitochondrial respiration [33–35]. Therefore, the NADH-mediated oxygen consumption rates (OCR) of mitochondria from the sham control, ischemic, and post-ischemic myocardia were induced by adding the substrates glutamate plus malate, and evaluated under state 3 conditions (ADP-induced). As indicated in Figures 1A and 1B, NADH-linked, ADP-stimulated respiration was decreased in the mitochondria from the ischemic and post-ischemic myocardia, from 209.9 ± 11.1 to 150.1 ± 12.9 (in nmol O_2 /min/mg protein, sham control vs ischemic mitochondria, $p < 0.01$, $n = 7-10$) and to 95.6 ± 8.7 (sham control vs I/R mitochondria, $p < 0.001$, $n = 10$), thus confirming impairment of the ADP-dependent electron transfer from NADH to oxygen due to ischemic injury and ischemia/reperfusion injury. ADP-stimulated OCR was further decreased from 150.1 ± 12.9 to 95.6 ± 8.7 (ischemic mitochondria vs I/R mitochondria, $p < 0.01$, $n = 7-10$), due to reperfusion-induced injury during the physiological hyperoxygenation conditions.

3.1c. Oxidative injury of complex I, superoxide generation, and redox activity in the mitochondria of the post-ischemic rat heart –

To support the role of complex I in the oxidative impairment of NADH-dependent OCR mediated by post-ischemic mitochondria (Figure 1A–1B), mitochondrial preparations were subjected to analysis of complex I activity catalyzing the electron transfer rate from NADH to Q₁. The specific activity of complex I in normal mitochondria isolated from the sham control rat heart was 447.5±35.1 nmol NADH oxidized/min/mg protein (*n* = 6). No significant difference in the complex I activity was detected in the ischemic mitochondria (Figure 1C). However, a significant decrease in the activity of complex I was detected in post-ischemic mitochondria compared to the sham control and ischemic mitochondria (*n* = 6 and *p* < 0.01 in Figure 1C), implying that increased ROS production induced by hyperoxygenation during reperfusion mediated post-ischemic injury in complex I. Treatment of the isolated mitochondria of sham control and the post-ischemic hearts with a thiol reducing agent, DTT, failed to reverse the impaired activity of complex I, indicating that I/R induces an irreversible oxidative injury in complex I (Figure 1C).

The production of •O₂⁻ mediated by isolated mitochondria at state 2 and state 3 respiratory states was induced by glutamate/malate (NADH-linked), and measured by EPR spin-trapping with DMPO. As shown in the Figure 2A–2B), an SOD-dependent four-line spectrum of DMPO/•OH was detected, indicating that •O₂⁻ generation was mediated by the mitochondria of sham control under the conditions of state 2 respiration. Addition of ADP (state 3 respiration) diminished mitochondria-mediated •O₂⁻ generation by 66.1±3.6%, indicating coupling of enhanced OCR with oxidative phosphorylation decreased e⁻ leakage for •O₂⁻ production. The measured NADH-linked •O₂⁻ generation by post-ischemic mitochondria increased by 35.8±7.2% and 182.8±43.5% under state 2 and state 3 respiratory conditions.

X-band EPR was further employed to measure the redox status of mitochondria during the oxidation of CM-H hydroxylamine to a stable nitroxide. It was determined that post-ischemic mitochondria exhibited a higher redox activity in mediating CM-H oxidation than sham mitochondria under NADH-linked energized conditions (Figure 2C–2D), suggesting a more oxidized physiological setting in the post-ischemic mitochondria.

3.2. Sulfonation of the cysteinyl residues involved in the metal binding of complex I iron-sulfur clusters in the post-ischemic heart –

The iron sulfur (Fe/S) clusters together with a non-covalently bound FMN are the main redox-active components of complex I. Mammalian mitochondrial complex I hosts eight Fe/S clusters. The Fe/S centers of N1a (24 kDa, Ndufv2), N3 (51 kDa, Ndufv1), N1b, N4 (75 kDa, Ndus1), N6a, N6b (TYKY, Ndufs8) and N2 (PSST, Ndufs7) are EPR detectable, while the Fe/S cluster of N5 (75 kDa, Ndus1) has been so far invisible to EPR [12, 36–38]. Specific cysteine residues have been identified as the metal binding sites of Fe/S clusters in complex I [10]. It is hypothesized that in the post-ischemic heart, the central mechanism of I/R-induced oxidative injury of complex I is impairment of the Fe/S clusters by redox modifications of metal binding sites. To gain a deeper insight into the complex I-derived irreversible cysteine oxidations mediated by I/R, we subjected the mitochondrial

preparations of sham control, ischemic, and post-ischemic rat hearts to native PAGE, and confirmed the PAGE results with in-gel NADH NTB (nitrotetrazolium blue) reductase activity staining of complex I [28, 29] (see FIGURE S1B). The protein band of complex I from the native gel was subjected to in-gel digestion with trypsin/Lys C mixture or chymotrypsin, followed by nano-LC-MS/MS analysis. To understand the complex I-derived cysteine oxidations mediated by I/R, we further examined the oxidative modification with cysteine sulfonation or cysteine sulfination (conversion of SH to SO₃H or SO₂H) of complex I [21]. The mass spectra were scrutinized for a mass shift of 48 Da or 32 kDa caused by sulfonation or sulfination. Mass spectrometric analysis showed a 48 Da mass increase in the tryptic sulfonated peptides accommodating metal-binding cysteines of the 51 kDa, 24 kDa, 75 kDa, and PSST subunits in complex I from the post-ischemic myocardium. These sulfonated or sulfinated peptides containing complex I metal-binding sites in post-ischemic mitochondria were detected and significantly enhanced by I/R (see Figure 3A, Table 1, and MS/MS spectra of FIGURE S2A-S2G), including N3_C425 (m/z 818.4101³⁺ and m/z 818.7462³⁺, N3 center of 51 kDa subunit), N1a_C134' and N1a_C134 (m/z 885.9027²⁺ and 893.8997²⁺, N1a center of 24 kDa subunit), N1a_C139 (m/z 894.3970²⁺, N1a center of 24 kDa subunit), N1b_C92 (m/z 600.2777²⁺, N1b center of 75 kDa subunit), N4_C226a (m/z 1295.2929³⁺, N4 center of 75 kDa subunit) and N4_C226b (m/z 1204.0669²⁺), N2_C158 and N2_C188 (m/z 1292.0502²⁺ and 996.2049³⁺, N2 center of PSST subunit). These sulfonated/sulfinated peptides were either nonexistent or observed at very low intensity in ischemic and sham control mitochondria (Figure 3A).

3.3. Cysteine sulfonation of the redox thiols from the 51 kDa FMN-binding subunit (Ndufv1) of complex I in the post-ischemic heart –

To gain a deeper insight into the complex I-derived irreversible cysteine oxidations mediated by I/R, LC-MS/MS analysis has identified 86.7% of the amino acid sequence of the complex I 51 kDa subunit (FIGURE S3). MS analysis showed a 48 Da mass increase in the tryptic sulfonated peptides of the complex I 51 kDa subunit from the post-ischemic mitochondria (Figure 3B and Table 2). The intensities of these sulfonated peptides of the complex I 51 kDa subunit in the post-ischemic mitochondria were significantly enhanced (Figure 3B, MS/MS spectra of FIGURE S2H-S2N), including 51kDa_C125 (m/z 957.4329²⁺), 51kDa_C187 (m/z 906.8730²⁺), 51kDa_C206 (m/z 1029.9814²⁺), 51kDa_C238 (m/z 1130.9068³⁺), 51kDa_C255 (m/z 1130.9079³⁺), and 51kDa_C286 (m/z 882.4191³⁺). In complex I of the sham control mitochondria and ischemic mitochondria, these sulfonated peptides were either nonexistent or observed at very low intensity (Figure 3B). MS analysis also identified the tryptic peptide with m/z 898.8759²⁺ corresponding to ¹⁸⁵NAC(SO₂H)¹⁸⁷DSDYDFDVFFVR¹⁹⁹ (51kDa_C187'), indicating addition of sulfinic acid (SO₂H) at C₁₈₇ of the 51 kDa subunit (Table 2). These results suggest that oxidation at the specific cysteine sites C₁₂₅, C₁₈₇, C₂₀₆, C₂₃₈, C₂₅₅, and C₂₈₆ of the 51 kDa subunit was enhanced by IR injury.

3.4. Cysteine sulfonation of the 75 kDa subunit (Ndus1) of complex I in the post-ischemic heart –

MS analysis further revealed the profile of cysteine oxidation in the complex I 75 kDa subunit of the post-ischemic rat heart, indicating that 95.1% of the 75 kDa polypeptide was

identified in the MS/MS spectra (FIGURE S3). The mass spectra were examined for enhanced sulfonation and sulfination as indicated in Figure 3C and Table 3 (MS/MS spectra of FIGURE S2O-S2R). A mass shift of 48 Da was seen for doubly charged ions at m/z 1215.5327²⁺, m/z 748.8933²⁺, and m/z 776.3456²⁺ corresponding to the modified tryptic peptides of 75kDa_C367c, 75kDa_C354, and 75kDa_C727; triply charged ions at m/z 1005.4692³⁺, m/z 1005.1365³⁺, and m/z 1068.8497³⁺ corresponded to the modified peptides of 75kDa_C367a, 75kDa_C367b and 75kDa_C564. Detailed MS/MS analysis provided additional sequential information for the localization of sulfonation on C₃₆₇, C₅₅₄, C₅₆₄, and C₇₂₇ of the 75 kDa subunit. Likewise, a mass shift of 32 Da due to incomplete cysteine oxidation was detected in the MS/MS spectra of doubly charged ions of the tryptic peptide 75kDa_C367' (C₃₆₇ sulfination, m/z 1207.5371²⁺ in Table 3).

3.5. Cysteine sulfonation of the subunits from the PSST, 49 kDa, and other supernumerary subunits of complex I in the post-ischemic heart –

MS analysis also showed the profile of enhanced cysteine sulfonation in other nuclear-encoded subunits of complex I from the post-ischemic rat heart, but not existing in the ischemic heart (Table 4 and Figure 3D). They are classified as belonging to subcomplex 1 α of mammalian complex I [39]. MS analysis revealed amino acid sequence coverage indicating that 66.9% of the PSST, 91.9% of the 49 kDa, 81.6% of the 42 kDa, 72.4% of 15 kDa, and 79.2% of 13 kDa polypeptides were identified in the MS/MS spectra (FIGURE S3). These subunits with I/R-induced enhancement of cysteine oxidation include the PSST subunit (Ndufs7) at C₁₇₅ (PSST_C175, m/z 869.9533²⁺); the 49 kDa subunit (Ndufs2) at C₁₄₆ (49kDa_C146, m/z 1137.4858²⁺), C₃₂₆ (49kDa_C326, m/z 832.3545²⁺) and C₃₄₇ (49kDa_C347, m/z 504.7558²⁺); the 42 kDa subunit (Ndufa10) at C₆₇ (42kDa_C67, m/z 748.3747²⁺), C₁₁₂ (42kDa_C112, m/z 1160.5258³⁺), C₁₈₃ (42kDa_C183, m/z 712.8274²⁺ and 42kDa_C183', m/z 522.2561³⁺), and C₂₅₃ (42kDa_C253, m/z 1186.0051²⁺); the 15 kDa subunit (Ndufs5) at C₄₃ (15kDa_C43, m/z 745.8391²⁺); and the 13 kDa subunit (Ndufs6) at C₇₉ (13kDa_C79, m/z 707.3430²⁺) (Table 4, Figure 3D, and MS/MS spectra of FIGURE S2S-S2W).

3.6. Protein sulfonation of complex I was not observed in the mitochondria of the ischemic rat heart –

MS analysis of mitochondrial complex I from the rat heart subjected to 30-min ischemia *in vivo* and then processed under anaerobic conditions with argon gas purging of the mitochondrial preparation buffers indicated that most specific cysteines were not sulfonated, except that a relatively low level of C₁₈₈ sulfonation (0.2% of carbamidomethylated peptide) was detected at the PSST subunit of the ischemic heart (Figure 3). Alkylation of most cysteine residues with carbamidomethylation from complex I were exclusively detected in the ischemic mitochondria (Tables S1-S4). Therefore, the physiological conditions of hypoxia during ischemia were highly reductive, which might suppress the basal level of complex I sulfonation detected in the sham control mitochondria (Figure 3). As reported previously using a mouse model with *in vivo* EPR oximetry, ischemia decreases pO₂ in the myocardium from 8–10 Torr to < 2 Torr, and reperfusion markedly increases pO₂ up to 60 Torr in the post-ischemic myocardium [7, 8]. Hyperoxygenation induced by reperfusion thus

drastically enhanced complex I cysteine sulfonation, leading to the consequence of oxidative injury of complex I in the post-ischemic heart.

3.7. Overexpression of SOD2 in myocytes alleviates complex I sulfonation enhanced in the post-ischemic heart –

Reperfusion-mediated overproduction of $\bullet\text{O}_2^-$ clearly served as the major causative factor to enhance cysteine sulfonation/sulfination of complex I in the post-ischemic heart. Overexpression of SOD2 in myocytes protects mitochondrial function from post-ischemic injury [40]. Study was conducted using the mouse model of SOD2-tg [41] with the Langendorff protocol reported previously [42, 43], indicating cardiac-specific overexpression of SOD2 significantly diminished the complex I sulfonation/sulfination enhanced by global ischemia (30-min) and reperfusion (45-min). Specific cysteinyl residues identified protected from I/R-enhanced sulfonation/sulfination in the complex I of SOD2-tg murine heart include C₇₈ (cysteine ligand of cluster N1b), C₁₇₆ (cysteine ligand of cluster N4), C₃₆₇/C₅₅₄ of the 75 kDa subunit (ndufs1), C₄₂₅ (cysteine ligand of cluster N3) of 51 kDa subunit (ndufv1); C₁₈₃ of the 42 kDa subunit (ndufa10), C₁₂₇/C₁₂₈ of the 24 kDa subunit (ndufv2), C₆₆ of PGIV subunit (19 kDa, ndufa8) as shown in the Table S5.

4. DISCUSSIONS

4.1. Irreversibly oxidative injury and mitochondrial dysfunction in the post-ischemic heart –

Current results confirmed the impairment of electron transfer from NADH to O₂ due to ischemia and I/R under the coupling conditions in the presence of ADP. These results further suggest that a defect in complex I of the respiratory chain weakened NADH-mediated OCR and impaired mitochondrial function in the ischemic and post-ischemic hearts. Following ischemia, blood reflow caused the tissue pO₂ of the myocardium to overshoot [7, 8]. The physiological hyperoxygenation and ROS overproduction thus mediated a reperfusion-induced respiratory defect in the mitochondria. As shown in previous work using the mouse model with in vivo EPR oximetry, a marked reperfusion-induced hyperoxygenation state was observed in the post-ischemic myocardium with a maximum value of 61.7±2.7 Torr at 24-h reperfusion [8]. Moreover, the 24-h timepoint is of translational interest with implication for eventual recovery, ultimate myocardial salvage, as well as ensured survival of post-surgery. The burst of ROS is thought to occur within the first few minutes of reperfusion. However, no significant impairment of complex I catalytic activity with enhanced cysteine sulfonation was detected in the post-ischemic mitochondria after 10-min reperfusion, although state 3 OCR by mitochondria was decreased by 40% (Figure S4). Note, vastly low level of complex I sulfonation can be detected in the residues of C₇₂₇/C₅₆₄ at the 75 kDa subunit and C₁₈₃ at the 42 kDa subunit. EPR spin-trapping analysis indicated NADH-linked $\bullet\text{O}_2^-$ generation by post-ischemic mitochondria was marginally increased by 17.8% and 32.9% under state 2 and state 3 conditions (Figure S4). Thereby, reperfusion-induced cysteine sulfonation of complex I was not mainly controlled by the kinetics of ROS burst, but predominantly by a thermodynamic procedure that depends on time and pO₂ in myocardium.

NADH-linked $\bullet\text{O}_2^-$ (Figure 2) and downstream H_2O_2 generation [4] mediated by isolated mitochondria have been reported to be enhanced in the post-ischemic rat heart, thus supporting the conclusion that mitochondrial dysfunction and related complex I injury occurring in the post-ischemic heart are caused by oxidative stress. A redox dysfunction was also detected in the post-ischemic myocardium and mitochondria (Figure 2) [4]. Post-ischemic injury of complex I has been also reported in a mouse model of in vivo myocardial ischemia and reperfusion [7]. Thus I/R was expected to induce irreversible oxidative post-translational modifications, which is associated with oxidative injury to complex I in the post-ischemic heart.

Previous studies also established that I/R increases reversible S-glutathionylation of complex I resulting from oxidative stress [11]. Current results support the conclusion that I/R-enhanced S-glutathionylation is not associated with oxidative injury to complex I in the post-ischemic heart because IR-induced complex I injury was not reversed by DTT treatment. Complex I conclusively contributed to the excess level of $\bullet\text{O}_2^-$ and downstream H_2O_2 generation mediated by post-ischemic mitochondria [4]. As the pathological conditions of I/R induced oxidative injury to complex I in mitochondria, the impaired complex I could further mediate an enhanced electron leakage for $\bullet\text{O}_2^-$ production by post-ischemic mitochondria. Studies with the isolated enzyme have indicated that the $\bullet\text{O}_2^-$ production from complex I induces self-inactivation along with protein thiol radical formation and protein sulfonation [21, 22]. Thus under the disease conditions of I/R, significant elevation in vivo of $\bullet\text{O}_2^-$ and its derived oxidants by complex I might induce oxidative impairment and self-inactivation with a similar mechanism of irreversible sulfonation.

4.2a. Sulfonation of the cysteines of clusters N3 and N1a in the post-ischemic heart –

Under conditions of I/R, enhanced sulfonation of C_{425} in the 51 kDa subunit and of $\text{C}_{134}/\text{C}_{139}$ in the 24 kDa subunit may represent one of the major mechanisms of irreversible oxidative injury in the flavoprotein (Fp) subcomplex hosting the NADH dehydrogenase activity of complex I. Previous studies have demonstrated that impairment of the NADH ferricyanide reductase activity associated with the Fp subcomplex was marked in the post-ischemic rat heart [9] and the disease model of cardiac arrest [44].

C_{425} is one of the ligands for the 4Fe-4S N3 center of complex I, which was verified by X-ray crystal structure [10, 45]. The cluster N3 is within 14 Å of FMN in the 51 kDa, and can effectively accept electron transfer from FMNH_2 [10]. Sulfonation of C_{425} thus diminished the efficiency of electron transfer from FMNH_2 to cluster N3. We have previously demonstrated that destruction of C_{425} by in vitro S-glutathionylation results in a decrease in the electron transfer activities of the Fp subcomplex and associated complex I [20]. In the current study, an enhanced C_{425} sulfonation of the Fp was detected in the post-ischemic heart, this structural thiol involved in the binding of N3 was destroyed by sulfonation, and subsequently impaired the main electron transfer pathway for complex I activity and mitochondrial function in the post-ischemic myocardium.

The binuclear 2Fe-2S cluster N1a of 24 kDa has a more hydrophobic environment [10] and a very low midpoint potential ($E_{m,7.0} = -370$ mV) and functions as temporary storage for electrons [46]. Cluster N1a is coordinated by C_{134} , C_{139} , C_{175} , and C_{179} of the 24 kDa

subunit. The N1a cluster is within a 13 Å distance from the FMN of 51 kDa, and thus can accept electrons from reduced FMN, but cannot pass electrons directly to the nearest cluster N3 due to a longer edge-to-edge distance of 20.7 Å [10, 47]. As the cluster N1a is not located in the main electron transport pathway of enzyme turnover, it has been proposed that N1a may play an antioxidant role to prevent excessive accumulation of flavin semiquinone intermediate (FMNH[•]), thus reducing the [•]O₂⁻ generation by complex I via autooxidation of FMNH[•] ($E_{m,7.0} = -390$ mV is for FMNH[•] → FMN) [45]. Previous study using isolated Fp subcomplex has indicated that [•]O₂⁻ generation by FMN moiety could be enhanced by blocking the cysteinyl ligands of N3 and N1a by the thiol reagent *p*-chloromercuribenzoate [22]. Thereby, an enhancement of C₁₃₄ and C₁₃₉ sulfonation from the 24 kDa subunit by I/R destroyed the cluster N1a serving as temporary storage of electrons and as an antioxidant, which likely partially contribute to increasing overall [•]O₂⁻ generation by the complex I in the post-ischemic heart (Figure 2A–2B).

4.2b. Sulfonation of the cysteines of clusters N1b, N4, and N2 in the post-ischemic heart

Our previous study indicated that C₂₂₆ oxidation in vitro (C₂₂₆ is one of the ligands involved in tetranuclear cluster N4 of the 75 kDa subunit) accounted for impairing the electron transfer activity of complex I due to destruction of N4 cluster, while self-inactivation of the isolated enzyme was induced when it was attacked by [•]O₂⁻ generation [21]. Here increased sulfonation of C₂₂₆ and C₉₂ (C₉₂ binds the metal of the binuclear N1b center of the 75 kDa subunit) was detected in the post-ischemic rat heart (Figure 3A), accounting for part of the post-ischemic injury because clusters N1b and N4 are in the main electron transfer pathway.

The tetranuclear cluster N2 is hosted by the PSST subunit, and functions as an electron donor of ubiquinone. The PSST subunit is one of the subunits involved in ubiquinone binding [47]. The cluster N2, which resides well above the ND1 membrane domain plane, is close to the redox-active headgroup of ubiquinone (Figure 5). Together with N3 sulfonation, increased cysteine sulfonation of N1b, N4, and N2 may exert a synergistic effect in the post-ischemic heart on impairment of electron transfer and stimulation of electron leakage for [•]O₂⁻ generation from FMNH₂ or FMNH[•]. This conclusion is supported by the previous in vitro study indicating that [•]O₂⁻ generation by FMN moiety could be enhanced by blocking iron-sulfur clusters of Fp subcomplex or intact isolated complex I by the thiol reagent *p*-chloromercuribenzoate [22].

4.3. Cysteine sulfonation of the 51 kDa subunit in the post-ischemic heart –

Enhanced sulfonation of C₂₀₆ was detected in the mitochondrial complex I of the post-ischemic heart. C₂₀₆ is conserved among the proteins from *E. coli* (C₁₈₀ of NUO F), *bovine heart* (C₂₀₆ of 51 kDa), *Thermus thermophilus* (C₁₈₂ of NQO1), *Yarrowia lipolytica* (C₉₉ of NUO51), and *Neurospora crassa* (C₂₁₈ of 51 kDa) [48, 49]. C₂₀₆ is the only conserved cysteine of the complex I 51 kDa subunit apart from the four remaining conserved cysteine residues involved in binding to the N3 cluster. C₂₀₆ is very near the FMN-binding site (~ 6 Å based on X-ray structure), where the major catalysis of electron transfer and [•]O₂⁻ production occurs [10]. The electron transfer coupled with [•]O₂⁻ generation as induced by NADH is tightly controlled by the FMN cofactor and the FMN-binding site at the 51 kDa subunit [22].

Therefore, increased sulfonation at C₂₀₆ can induce a small conformational change near the FMN/NADH binding site that marginally decreases the efficiency of electron transfer from FMN to cluster N3 and subsequently increases the electron leakage for $\bullet\text{O}_2^-$ production by post-ischemic mitochondria.

Previous studies using the isolated enzyme confirmed C₂₀₆ as a target susceptible to oxidative attack by $\bullet\text{O}_2^-$, forming a protein thiyl radical, and involved in GSSG-mediated S-glutathionylation [20, 22]. Therefore, it is logical that C₂₀₆ of the 51 kDa subunit would be susceptible to sulfonation under the conditions of oxidative stress induced by I/R.

Residues C₁₂₅, C₁₈₇, C₂₃₈, C₂₅₅, and C₂₈₆ are not conserved between mammalian and bacterial proteins. In the previous study using the isolated enzyme, they were not found to be involved in protein thiyl radical formation. However, C₁₈₇ is involved in GSSG-mediated S-glutathionylation [20]. Likely, both C₁₈₇ and the other four cysteinyl residues are part of the regulatory thiols hosted in the complex I 51 kDa subunit, but they are not necessarily essential for enzymatic activity. Examination of the 3-D atomic structure of ovine respiratory complex I (pdb: 5LNK) suggests that these cysteine residues are surface-exposed, and thus susceptible to the redox modification of sulfonation induced by I/R [32].

4.4. Cysteine sulfonation of the 75 kDa subunit in the post-ischemic heart –

The residues of C₃₆₇, C₅₅₄, and C₅₆₄ are substantially distal, away from the redox-active components of clusters N1b, N4, and N5 as indicated by the atomic structure of ovine complex I (Figure 4). The residue of C₇₂₇ is positioned at the unstructured C-terminus (Figure 4). They all are surface-exposed, and expected to be susceptible to redox modifications by I/R.

4.4a. C₃₆₇ of 75 kDa —The C₃₆₇ S-glutathionylation of isolated bovine complex I induced by in vitro GSH-mixed disulfide exchange has been reported previously [20, 21], which supports C₃₆₇ as being one of the surface-exposed reactive thiols in complex I. In vitro evidence further suggests that GSSG-mediated S-glutathionylation of C₃₆₇ modestly enhances isolated complex I activity [20]. It is unlikely that C₃₆₇ sulfonation/sulfination accounts for the oxidative injury to complex I activity in the post-ischemic heart. However, detection of C₃₆₇ sulfonation/sulfination in the post-ischemic heart reinforces its role as a redox regulator in response to oxidative attack induced by I/R.

4.4b. C₅₅₄ and C₇₂₇ of 75 kDa —In vitro S-glutathionylation of C₅₅₄ and C₇₂₇ in the mitochondria of the rat heart can be mediated by diamide-induced oxidative injury of complex I as reported by Hurd *et al.*, suggesting an important role for C₅₅₄ and C₇₂₇ in redox regulation associated with antioxidant defense [23]. However, deglutathionylation of C₅₅₄ and C₇₂₇ via DTT treatment of mitochondria failed to reverse diamide-impaired complex I activity.

In vitro studies using the isolated enzyme with immuno-spin trapping with DMPO (5,5-dimethyl-1-pyrroline N-oxide) have supported the conclusion that protein thiyl radical formation at C₅₅₄ and C₇₂₇ mediates sulfonation of complex I [21]. The spin trap DMPO also mediates an incomplete cysteine oxidation at C₅₅₄ and C₇₂₇ under conditions of $\bullet\text{O}_2^-$

overproduction, resulting in cysteine sulfonation at C₅₅₄ and C₇₂₇ [21]. Furthermore, sulfonation of the C₅₅₄ containing peptide, ⁵⁴⁴LLFLLGADGGC(SO₂H)⁵⁵⁴ITR⁵⁵⁷ (*m/z* 1479.7746²⁺), was detected in the post-ischemic mitochondria, but not in sham control or ischemic mitochondria. C₅₅₄/C₇₂₇ sulfonation induced by I/R thus likely followed the mechanism involving both the protein thiyl radical and the protein sulfinyl radical (Eq. 1). The formation of protein thiyl radicals at C₅₅₄ and C₇₂₇ accelerates the corresponding cysteine oxidation to form the protein cysteinyl sulfinyl radical; and protein sulfonation is a byproduct derived from further reactions with oxidants (*e.g.*, H₂O₂) overproduced by I/R.

4.4c. C₅₆₄ of 75 kDa —The cysteine oxidation of the C₅₆₄ residue from the 75 kDa subunit has not been detected by in vitro studies. However, a drastic enhancement (Figure 3C) of C₅₆₄ sulfonation was marked in the mitochondria of the post-ischemic heart in the current study, thus representing a new finding. On the basis of the 3-D model [21, 32] (Figure 4), the residue C₅₆₄ is located next to C₅₅₄ on the surface of the 75 kDa subunit without hindrance from other associated subunits. A positively charged amino acid (K₅₆₂) neighbor of C₅₆₄ (with a distance of 5.7 Å between K₅₆₂ and C₅₆₄) can increase the thiolate formed at C₅₆₄. The above C₅₆₄-S⁻ intermediate may render C₅₆₄ susceptible to persistent oxidative attack leading to subsequent protein sulfonation during I/R.

4.5a. PSST and 49 kDa —The PSST (Ndufs7) and 49 kDa subunits (Ndufs2) are two of 14 highly conserved core subunits from complex I. As indicated by the X-ray structure of the intact respiratory complex I of *T. thermophilus* and a 3.9 Å resolution atomic structure derived from a cryo-electron microscopy map of the ovine heart complex I [32, 50], the 49 kDa and PSST subunits form a cleft as part of the ubiquinone-binding channel, and function as the ubiquinone reduction site [32, 47, 50, 51] (Figure 5, black dotted enclosed area). Tyr₁₄₁ (Y₁₄₁ or Y₁₀₅ in mature 49 kDa) and His₉₂ (H₉₂ or H₅₆ in mature 49 kDa) of the 49 kDa subunit form hydrogen bonds to the redox-active headgroup of the bound ubiquinone [51], and the PSST subunit coordinates cluster N2 as the immediate electron donor for ubiquinone. The C₁₄₆ residue is buried inside the 49 kDa subunit, and lies close to Y₁₄₁ and H₉₂ (Figure 5). It is likely that enhanced sulfonation of C₁₄₆ at the 49 kDa subunit may directly impact the conformation of the ubiquinone-binding domain and ubiquinone reduction, increasing •O₂⁻ generation via semiquinone autooxidation. The C₃₄₇ and C₃₂₆ residues are positioned at the same α-helical backbone of the 49 kDa (Figure 5). They are surface-exposed and susceptible to oxidative attack by I/R. Protein sulfonation of C₁₄₆, C₃₂₆, and C₃₄₇ can induce conformational change of the 49 kDa subunit, which may also indirectly provoke C₁₅₈ and C₁₈₈ oxidation at the N2 cluster of the PSST subunit, impairing electron transport efficiency and synergistically enhancing •O₂⁻ generation during I/R.

The C₁₇₅ residue serves as a regulatory thiol unique in the PSST subunit, and is positioned at a surface-exposed random-coil conformation connecting to the N2 cluster metal binding sites of C₁₅₈ and C₁₈₈ (Figure 5). IR-induced sulfonation of C₁₇₅ may also directly or

indirectly promote oxidation of C₁₅₈ and C₁₈₈, and subsequently impair electron transport and ubiquinone reduction.

4.5b. 42 kDa —The 42 kDa subunit (Ndufa10), one of 31 supernumerary subunits, is a large globular protein, residing on the matrix face of the membrane and the peripheral arm without contacting any hydrophilic core subunits [51, 52]. The structural extensions of the 42 kDa subunit dock it to the matrix face of ND2 [51] (Figure 6). Its presence in complex I is *metazoan-specific*, found only in selected branches of higher eukaryotes [52, 53]. The 42 kDa subunit has been reported to be loosely bound to complex I with the functional implication of stabilizing complex I and fine tuning movement during enzyme turnover [32, 53, 54]. All four available cysteine residues of C₆₇, C₁₁₂, C₁₈₃, and C₂₅₃ are localized in the nucleoside kinase fold of the 42 kDa subunit (Figure 6). They are surface-exposed to the matrix compartment, thus highly susceptible to oxidative attack by IR, forming cysteine sulfonic acid. Both C₁₈₃ and C₁₁₂ are positioned near the helical extensions docked to ND2 (Figure 6). The current studies indicate that I/R drastically enhanced sulfonation of 42 kDa at all four available cysteine residues (Table 4 and Figure 3D), supporting the low immobilization of the 42 kDa subunit in mammalian complex I. Substantial enhancement of C₁₈₃ and C₁₁₂ sulfonation by I/R may weaken docking of the 42 kDa subunit to the ND2 subunit. Together with the increase of C₆₇ and C₂₅₃ sulfonation, may synergistically influence the structural integrity of complex I during I/R.

4.5c. 15 kDa —The 15 kDa subunit (Ndufs5) is a supernumerary subunit confined to the face of the intermembrane space (IMS face, see Figure 7c) with the subunits PGIV (Ndufa8), PDSW (Ndubf10), and B18 (Ndubf7) [51]. Together with PGIV and B18, the 15 kDa subunit contains twin CX₉C motifs that form two intramolecular disulfide bonds within CHCH domains (pairs of helices linked by two intramolecular disulfide bonds), which is functionally important for enzymatic stability in the oxidizing environment of the IMS [32, 51]. The 15 kDa subunit hosts two disulfide bonds, C₃₃-C₆₆ and C₄₃-C₅₆. The physiological conditions of ischemia are known to induce a highly reductive milieu in the myocardium [55], which likely facilitates reduction of the C₄₃-C₅₆ disulfide bond. Overproduction of ROS during reperfusion thus subsequently enhances oxidative modification of the 15 kDa subunit at the C₄₃ thiol with sulfonation (Table 4 and Figure 3D).

4.5d. 13 kDa —The 13 kDa subunit (Ndufs6) is a Zn²⁺-binding protein, which has been reported to be functionally important for complex I assembly [56]. Structural information indicates that the 13 kDa subunit is surface-exposed to the matrix, and interacts with the 75 kDa, 49 kDa, and TYKY subunits [51, 52]. Together with the other six supernumerary subunits, it participates in stabilizing the domain of NADH dehydrogenase and interfaces between the hydrophilic and membrane domains. Mammalian Ndufs6 is conserved in the complex I of yeast (NUMM subunit in *Y. lipolytica*). It has been reported that deletion of the NUMM subunit in yeast blocks a late step of complex I assembly and biogenesis involved in insertion or stabilization of Fe/S cluster N4 [57]. In the rat, the 13 kDa subunit contains a Zn-binding domain with a binding motif of CX₈HX₁₅CX₂C [32, 57], where the Zn²⁺ ion is coordinated to the C₇₉ residue [32]. The current studies revealed that I/R dramatically

enhances C₇₉ sulfonation (Figure 3D) of the 13 kDa subunit, which can impair the Zn²⁺-binding site and presumably affects the structural integrity of complex I.

4.6. Functional impact of complex I sulfonation on the mitochondrial dysfunction in the post-ischemic rat heart –

Table 5 summarizes the rating of functional impact resulted from I/R-enhanced cysteine oxidation of complex I based on quantitative analysis (Figure 3). I/R significantly enhanced site-specific sulfonation of the 42 kDa, 75 kDa, 49 kDa, and 13 kDa subunits, being responsible for the primary effects on complex I injury and associated mitochondrial dysfunction. The mutual primary effects, including affecting structural integrity (C₁₈₃/C₂₅₃ of 42 kDa and C₇₉ of 13 kDa), impairing redox regulation (C₅₆₄/C₇₂₇ of 75 kDa), weakening ubiquinone-binding (C₁₄₆ of 49 kDa) and electron transport activity (C₉₂ and C₂₂₆ of 75 kDa and C₁₄₆ of 49 kDa), can synergistically contribute to the reperfusion injury of complex I. A modestly increased sulfonation of 24 kDa and PSST subunits provides the secondary effect on the reperfusion injury of complex I via impairing antioxidant function of N1a cluster (C₁₃₄/C₁₃₉ of 24 kDa), increasing ROS production, and decreasing ubiquinone reduction and electron transport function (C₁₇₅ and C₁₅₈/C₁₈₈ of PSST). Lower level of cysteine sulfonation of 51 kDa and 15 kDa subunits adds a tertiary impact on the reperfusion injury of complex I by affecting redox regulation, electron transport activity (C₄₂₅ of 51 kDa), and stability (C₄₃ of 15 kDa).

5. SUMMARY and CONCLUSIONS

Mitochondrial thiols are composed of protein thiols and the GSH pool. The complex I of the mitochondria is rich in protein thiols. Therefore, the protein thiols of complex I appear to play a critical role in controlling mitochondrial dysfunction in the post-ischemic rat heart. Complex I is the major component of the ETC; it hosts two important types of protein thiols: the structural thiols involved in the metal binding of iron sulfur clusters as the redox-active components, and the regulatory thiols that are thought to have biological functions of antioxidant and redox signaling. For example, the physiological role of the complex I-derived regulatory thiols has been related to inhibited respiration by nitrosative stress [16].

Our data obtained from an in vivo model provides deeper insights into the major disease mechanism of ROS-mediated redox modifications by I/R injury. Reperfusion-induced hyperoxygenation and overproduction of $\cdot\text{O}_2^-$ following ischemic conditions is obviously the major causative factor of the above disease marker. This finding also provides a detailed map of cysteine residues that are vulnerable to damage during I/R. As illustrated in Figure 7, the current studies reveal that the cysteinyl residues involved in I/R-enhanced oxidative sulfonation in complex I are across redox-active components (N1a, N1b, N2, N3, N4), core subunits [Ndufv1 (51 kDa), Ndus1 (75 kDa), Ndufs2 (49 kDa), Ndufs7 (PSST)], and supernumerary subunits [Ndufa10 (42 kDa), Ndufs5 (15 kDa), Ndufs6 (13 kDa)]. Site-specific sulfonation of the cysteinyl ligands for N1b, N2, N3, and N4 was unequivocally associated with impaired ETC in the post-ischemic heart. Increased C₁₃₄/C₁₃₉ oxidation of cluster N1a in the 24 kDa subunit contributed to ROS overproduction via FMNH $\cdot$ autooxidation in the post-ischemic heart. Increased site-specific sulfonation of non-metal-

binding and regulatory thiols in the 51 kDa (Figure 3B) and 75 kDa subunits (Figure 3C) likely promoted the redox status of the post-ischemic myocardium to a more oxidized physiological setting. Together with C₁₅₈/C₁₈₈ oxidation of cluster N2 and C₁₇₅ oxidation of unique regulatory thiols in the PSST subunit, enhanced site-specific sulfonation of the 49 kDa subunit might facilitate incomplete ubiquinone reduction, contributing to I/R-mediated ROS production by ubisemiquinone. Increased site-specific cysteine oxidation of the 42 kDa, 15 kDa, and 13 kDa subunits may negatively impact the structural integrity of mammalian complex I, synergistically promoting I/R-mediated complex I injury and consequent mitochondrial dysfunction in the post-ischemic heart. Recognition of this molecular event is valuable in understanding the fundamental basis of the disease pathogenesis of reperfusion injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ETC	mitochondrial electron transport chain
I/R	myocardial ischemia and reperfusion
ROS	reactive oxygen species
O₂⁻	superoxide anion radical
OCR	oxygen consumption rate
Q₂	ubiquinone-2
PAGE	polyacrylamide gel electrophoresis
EPR	electron paramagnetic resonance
MS	mass spectrometry
MS/MS	tandem mass spectrometry
IMS	intermembrane space
SOD	superoxide dismutase
Fp	the flavoprotein subcomplex of complex I

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Figure 1.

Mitochondria were prepared from the tissue of sham control (SC) rat hearts (sham mitochondria) and the risk region of ischemic rat hearts (ischemic mitochondria, I) and post-ischemic rat hearts (I/R mitochondria, I/R), and then subjected to measurement of the oxygen consumption rate (OCR) by oxygen polarography at 30 °C as described in a previous publication [4]. **A**, state 3, oligomycin-induced state 4, and FCCP-mediated OCRs from the isolated mitochondria of sham control, ischemic, and post-ischemic hearts. **B**, statistical analysis of state 3 OCRs based on oxygen polarographic measurement in **A** (data are reported as group averages $\pm$ SE, $n = 7-10$, *** $p < 0.001$). **C**, measurement of the enzymatic activity of complex I in mitochondria by rotenone-sensitive Q₂-mediated NADH oxidation with or without dithiothreitol (DTT) treatment [9]. (data are reported as group averages $\pm$ SE, $n = 6$; ** $p < 0.01$, assessed by Student's *t*-test for sham mitochondria vs I/R mitochondria and ischemic mitochondria vs I/R mitochondria).

Figure 2.

The $\bullet\text{O}_2^-$ generation (*A-B*) and redox activity (*C-D*) mediated by isolated mitochondria in the presence of glutamate and malate (in mM, potassium glutamate 140 and malate 5, NADH-linked) was measured by EPR spin trapping with DMPO and direct EPR according to *experimental conditions and instrumental settings* described in the previous publications [4, 24, 41]. *A*, The EPR spectra of SOD1-sensitive DMPO/ $\bullet\text{OH}$ adduct mediated by isolated mitochondria of sham control and post-ischemic rat hearts under the conditions of state 2 (*spectra b and d*) and state 3 (*spectra c and e*), and the effect of SOD1 on the detected DMPO/ $\bullet\text{OH}$ (*spectrum f*). *B*, Comparison between two groups of sham control and post-ischemic mitochondria in the mediation of NADH-linked $\bullet\text{O}_2^-$ production. Data were collected based on spin quantitation of DMPO/ $\bullet\text{OH}$. Data were analyzed by student's *t* test ($n = 4$, $*p < 0.05$ and $**p < 0.01$). *C*, Mitochondria (0.4 mg/ml) in respiration buffer (in mM, NaCl 10, MgCl₂ 1, EGTA 1, Trizma 1. Phosphate 2.5, and cytochrome *c* 0.01; adjusted to pH 7.4) was incubated CM-H (1 mM) at room temperature. Oxidation of CM-H was immediately initiated by glutamate plus malate (glu. plus mal) and then subjected to EPR measurement for converting the hydroxylamine to a stable nitroxide with three-line spectrum (*inset*). *D*, the same as C, except the redox activity of converting CM-H to a stable nitroxide is presented in a bar graph ($n = 4$).

Figure 3.

The label-free quantitation (LFQ) of sulfonated or sulfinated peptides from complex I. LFQ is presented as the percentage of the signal intensity of sulfonated/sulfinated peptide in total peptides (sulfonated peptides plus carbamidomethylated peptides). The icon of each sulfonated or sulfinated peptide is denoted in Tables 1–4. Data are reported as mean $\pm$ SE ($n = 3$, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Comparisons between two groups (I/R vs sham control) were assessed by Student's t -test to analyze the significance of differences. §Results of control or ischemic mitochondria with no detectable protein sulfonation or sulfinylation in the denoted subunit of complex I were not included in the statistical analysis.

Figure 4.

The core subunit of 75 kDa (blue) with the FMN, Fe/S clusters (N1b, N3, N4, N5), and Cys₃₆₇, Cys₅₅₄, Cys₅₆₅, Cys₇₂₇ (magenta) that were detected with enhanced sulfonation mediated by I/R.

Figure 5.

The core subunits of 49 kDa (blue), PSST (green), and ND1 (orange) subunits with the N2 tetranuclear cluster. The interface of the hydrophilic 49 kDa and PSST subunits and the membrane ND1 subunit form a ubiquinone-binding pocket (marked with a black dotted enclosed area). The conserved Tyr₁₄₁ (Y₁₄₁ or Y₁₀₅ in mature 49 kDa) and His₉₂ (H₉₂ or H₅₆ in mature 49 kDa) of the 49 kDa subunit have been proposed as a ubiquinone-binding site, and they interact with the bound ubiquinone via hydrogen bonds at the top of the pocket. Cys₁₄₆, Cys₃₂₆, and Cys₃₄₇ (magenta) of the 49 kDa subunit, Cys₁₅₈, Cys₁₈₈ (magenta, metal binding sites of N2), and Cys₁₇₅ of the PSST subunit were identified as enhancing sulfonation mediated by I/R. Icons at the right indicate the location of the 49 kDa, PSST, and ND1 subunits in complex I viewed from the peripheral arm (top) and rotated 90° (bottom).

Figure 6.

The supernumerary subunit of 42 kDa with the core α/β nucleoside-kinase fold (dark green and green) and three extensions (gray) docked to the core subunit of ND2 (orange). All four available cysteines, Cys₆₇, Cys₁₁₂, Cys₁₈₃, and Cys₂₅₃ (magenta) of the 42 kDa subunit exhibited considerably increased sulfonation mediated by I/R. The icon at the lower right indicates the viewpoint and location of the 42 kDa subunit in complex I, suggesting that the 42 kDa is loosely bound to mammalian complex I.

Figure 7.

a, Overview of the subunits with significantly enhanced cysteine oxidation as mediated by I/R. Structure depicted with core subunits of 51 kDa in dark green, 24 kDa in light pink, 75 kDa in dark blue, 49 kDa in blue, PSST in green, and the supernumerary subunits of 42 kDa in dark turquoise, and 13 kDa in brick red. Specific cysteine residues involved in I/R-mediated enhanced sulfonation (C-SO₃H) were colored by magenta within the structure of the polypeptide or with red balls as the metal-binding sites of Fe/S clusters. Noteworthy cysteine sulfonation enhanced by I/R with a detrimental effect on the function of complex I in mitochondria was remarked. **b**, Fe/S clusters are shown as spheres with the metal-binding cysteine residues in red balls. **c**, Icon at the lower right indicates viewpoint and location of subunits in **a**, redox active components in **b**, and the 15 kDa subunit (in magenta) confined to the IMS face.

Summary of the peptide sequence and corresponding sulfonic/sulfinic cysteines involved in the ligands of iron sulfur clusters of mitochondrial complex I in the post-ischemic rat heart

Table 1:

Peptide Name	Amino Acid Sequence	Theoretical M+H	Observed M+H	Oxidized cysteine	Fe-S clusters of complex I
N3_C425 ^a	⁴¹⁸ Q(de)IEGHTIC(SO ₃ H)ALGDGAAPVQGLIR ⁴⁴⁰	818.7361 ³⁺	818.7462 ³⁺	C ₄₂₅ Trioxidation	N3 (51 kDa)
	⁴¹⁸ QIEGHTIC(SO ₃ H)ALGDGAAPVQGLIR ⁴⁴⁰	818.4027 ³⁺	818.4101 ³⁺		
N1a_C134 ^c	¹²⁹ YHIQVC(SO ₂ H)TTTPC(cam)M(ox)LR ¹⁴²	885.8950 ²⁺	885.9027 ²⁺	C ₁₃₄ Dioxidation	N1a (24 kDa)
N1a_C134	¹²⁹ YHIQVC(SO ₃ H)TTTPC(cam)M(ox)LR ¹⁴²	893.9003 ²⁺	893.8997 ²⁺	C ₁₃₄ Trioxidation	N1a (24 kDa)
N1a_C139	¹²⁹ YHIQ(de)VC(cam)TTTPC(SO ₃ H)M(ox)LR ¹⁴²	894.3961 ²⁺	894.3970 ²⁺	C ₁₃₉ Trioxidation	N1a (24 kDa)
N1b_C92	⁸⁸ VVAAC(SO ₃ H)AM(ox)PVM(ox)K ⁹⁸	600.2735 ²⁺	600.2777 ²⁺	C ₉₂ Trioxidation	N1b (75 kDa)
N4_C226a	²¹³ M(ox)FM(ox)SELSGN(de)IIDIC(SO ₃ H)PVGALTSKPYAFTAR PWETR ²⁴⁶	1295.2796 ³⁺	1295.2929 ³⁺	C ₂₂₆ Trioxidation	N4 (75 kDa)
N4_C226b	²¹³ M(ox)FM(ox)SELSGN(de)IIDIC(SO ₃ H)PVGALTSK ²³⁴	1204.0539 ²⁺	1204.0669 ²⁺	C ₂₂₆ Trioxidation	N4 (75 kDa)
N2_C158	¹⁵¹ YVVSM(ox)GSC(SO ₃ H)ANGGGYYHYSYVVR ¹⁷³	1292.0458 ²⁺	1292.0502 ²⁺	C ₁₅₈ Trioxidation	N2 (PSST)
N2_C188	¹⁷⁸ VPVDIYVPGC(SO ₃ H)APPTAEALLYGILQLQR ²⁰⁴	996.1962 ³⁺	996.2049 ³⁺	C ₁₈₈ Trioxidation	N2 (PSST)

^aOne of two sulfonated peptides from N3_C425, ⁴¹⁸Q(de)IEGHTIC(SO₃H)⁴²⁵ALGDGAAPVQGLIR⁴⁴⁰ (m/z 818.7462³⁺) of the 51 kDa subunit, was a result of glutamine deamidation, rehydration, and isomerization during in-gel proteolytic digestion of complex I with trypsin plus LysC [58, 59]. Glutamine (Q) of ⁴¹⁸QIEGHTIC(SO₃H)⁴²⁵ALGDGAAPVQGLIR⁴⁴⁰ was converted to the six-membered ring glutarimide intermediate via deamidation, which was then rehydrated to glutamic acid or pyroglutamic acid [Q(de)] of ⁴¹⁸Q(de)IEGHTIC(SO₃H)⁴²⁵ALGDGAAPVQGLIR⁴⁴⁰. The resulting mass adduct was thus a +1 Da shift. The detection of ⁴¹⁸QIEGHTIC(SO₃H)⁴²⁵ALGDGAAPVQGLIR⁴⁴⁰ with m/z 818.4101³⁺ confirmed the above mechanism. A similar mechanism was also observed in N4_226.

^bThe amino acid sequences of S-sulfonated or S-sulfinated peptides in Tables 1-4 were numbered based on the amino acid sequences of the precursors containing the transient signaling peptides.

Table 2:

Summary of the peptide sequence and corresponding sulfonic/sulfenic cysteines from the FMN-binding 51 kDa protein of mitochondrial complex I in the post-ischemic rat heart

Peptide Name	Amino Acid Sequence	Theoretical M+H	Observed M+H	Oxidized Cysteine	FMN-binding subunit of complex I
51kDa_C125	¹¹² YLVVNADEGEPTC(SO ₃ H)KDR ¹²⁸	957.4311 ²⁺	957.4329 ²⁺	C ₁₂₅ Trioxidation	Ndufv1 51 kDa
51kDa_C187	¹⁸⁵ NAC(SO ₂ H)DSYDFDFVVR ¹⁹⁹	898.8716 ²⁺	898.8759 ²⁺	C ₁₈₇ Dioxidation	Ndufv1 51 kDa
51kDa_C187	¹⁸⁵ NAC(SO ₃ H)DSYDFDFVVR ¹⁹⁹	906.8659 ²⁺	906.8730 ²⁺	C ₁₈₇ Trioxidation	Ndufv1 51 kDa
51kDa_C206	²⁰⁰ GAGAYTC(SO ₃ H)GEETALIESIEGK ²¹⁹	1029.9768 ²⁺	1029.9814 ²⁺	C ₂₀₆ Trioxidation	Ndufv1 51 kDa
51kDa_C238	²²⁵ LKPPFPADVG(VFGC(SO ₃ H)PTTVANVETVAVS PTIC(cam)R ²⁵⁶	1130.9024 ³⁺	1130.9068 ³⁺	C ₂₃₈ Trioxidation	Ndufv1 51 kDa
51kDa_C255	²²⁵ LKPPFPADVG(VFGC(cam)PTTVANVETVAVS PTIC(SO ₃ H)R ²⁵⁶	1130.9024 ³⁺	1130.9079 ³⁺	C ₂₅₅ Trioxidation	Ndufv1 51 kDa
51kDa_C286	²⁷⁵ LFNISGHVN(de)HPC(SO ₃ H)TVBEEM(ox)SVPLK ²⁹⁷	882.4075 ³⁺	882.4191 ³⁺	C ₂₈₆ Trioxidation	Ndufv1 51 kDa

Summary of the peptide sequence and corresponding sulfonic/sulfenic cysteines from the 75 kDa polypeptide of mitochondrial complex I in the post-ischemic rat heart

Table 3:

Peptide Name	Amino Acid Sequence	Theoretical M+H	Observed M+H	Oxidized Cysteine	75 kDa subunit of complex I
75kDa_C367a	³⁵⁶ DLNKNKVDSDTLC(SO ₃ H)TEEIFPN(de)EGAGTDLR ³⁸²	1005.4594 ³⁺	1005.4642 ³⁺	C ₃₆₇ Trioxidation	Ndus1 75 kDa
75kDa_C367b	³⁵⁶ DLNKNKVDSDTLC(SO ₃ H)TEEIFPNNEGAGTDLR ³⁸²	1005.11314 ³⁺	1005.1365 ³⁺	C ₃₆₇ Trioxidation	
75kDa_C367c	³⁶¹ VDSDTLC(SO ₃ H)TEEIFPNNEGAGTDLR ³⁸²	1215.5307 ²⁺	1215.5327 ²⁺	C ₃₆₇ Trioxidation	
75kDa_C367'	³⁶¹ VDSDTLC(SO ₃ H)TEEIFPNNEGAGTDLR ³⁸²	1207.5332 ²⁺	1207.5371 ²⁺	C ₃₆₇ Dioxidation	Ndus1 75 kDa
75kDa_C554	⁵⁴⁴ LLFLGADGGC(SO ₃ H)YTR ⁵⁵⁷	748.8889 ²⁺	748.8933 ²⁺	C ₅₅₄ Trioxidation	Ndus1 75 kDa
75kDa_C564	⁵⁶³ DC(SO ₃ H)FIVYQGHGVDVGAPIADVILPGAAYTEK ⁵⁹²	1068.8435 ³⁺	1068.8497 ³⁺	C ₅₆₄ Trioxidation	Ndus1 75 kDa
75kDa_C727	⁷¹³ AVTEGAQAVEEPSIC(SO ₃ H) ⁷²⁷	776.3422 ²⁺	776.3456 ²⁺	C ₇₂₇ Trioxidation	Ndus1 75 kDa

Table 4:

Summary of the peptide sequence and corresponding sulfonic/sulfinic cysteines from the 1 α subcomplex (PSST, 49 kDa, 42kDa, 15 kDa, and 13 kDa subunits) of mitochondrial complex I in the post-ischemic rat heart

Peptide Name	Amino Acid Sequence	Theoretical M+H	Observed M+H	Oxidized Cysteine	Subcomplex 1 α of complex I
PSST_C175	¹⁷⁰ SVVVRGC(SO ₃ H)DRIVPVDIY ¹⁸⁴	869.9357 ²⁺	869.9433 ²⁺	C ₁₇₅ Trioxidation	Ndufs7 PSST
49kDa_C146	¹³⁹ LDYVSM(ox)M(ox)C(SO ₃ H)NEQAYS ¹⁵⁷ SLAVEK	1137.4801 ²⁺	1137.4858 ²⁺	C ₁₄₆ Trioxidation	Ndufs2 49 kDa
49kDa_C326	³¹⁷ DVPIGSRGDC(SO ₃ H)YDRY ³³⁰	832.3470 ²⁺	832.3545 ²⁺	C ₃₂₆ Trioxidation	Ndufs2 49 kDa
49kDa_C347	³⁴³ IEQC(SO ₃ H)LNK ³⁵⁰	504.7515 ²⁺	504.7558 ²⁺	C ₃₄₇ Trioxidation	Ndufs2 49 kDa
42kDa_C67	⁵⁹ VITVDGNIC(SO ₃ H)SGKNK ⁷²	748.3748 ²⁺	748.3747 ²⁺	C ₆₇ Trioxidation	Ndufa10 42 kDa
42kDa_C112	⁸⁵ HYPEAGIQYSSSTTGDGRPLDIEFGSC(SO ₃ H)SLEK ¹¹⁶	1160.5181 ³⁺	1160.5258 ³⁺	C ₁₁₂ Trioxidation	Ndufa10 42 kDa
42kDa_C183	¹⁸¹ KQC(SO ₃ H)VDHYN ¹⁹¹ EIK	712.8238 ²⁺	712.8274 ²⁺	C ₁₈₃ Trioxidation	Ndufa10 42 kDa
42kDa_C183'	¹⁸¹ KQC(SO ₃ H)VDHYN ¹⁹¹ EIK	522.2568 ³⁺	522.2561 ³⁺	C ₁₈₃ Dioxidation	Ndufa10 42 kDa
42kDa_C253	²⁴⁹ M(ox)SEIC(SO ₃ H)EVLVYSSWEAEDSTK ²⁶⁸	1185.9963 ²⁺	1186.0051 ²⁺	C ₂₅₃ Trioxidation	Ndufa10 42 kDa
15kDa_C43	³⁹ EWIEC(SO ₃ H)AHGIGATR ⁵¹	745.8347 ²⁺	745.8391 ²⁺	C ₄₃ Trioxidation	Ndufs5 15 kDa
13kDa_C79	⁷⁶ IIAC(SO ₃ H)DGGGGALGHPK ⁹⁰	707.3396 ²⁺	707.3430 ²⁺	C ₇₉ Trioxidation	Ndufs6 13 kDa

Table 5:

Summary of functional effects of site-specific protein sulfonation/sulfination on the mitochondrial complex I in the post-ischemic rat heart

Rating of effect	Subunit and cysteine residues of I/R-mediated protein sulfonation/sulfination	%, enhancement of cysteine oxidation	Functional impact resulted from specific cysteine sulfonation/sulfination	References
Primary	42 kDa (C ₁₁₂ /C ₁₈₃ /C ₂₅₃)	10%–25%	Affecting the structural integrity of complex I via weakening docking to ND2	32, 51
Primary	75 kDa (C ₅₆₄ /C ₇₂₇)	10%–20%	Affecting redox regulation and antioxidant defense	14, 23
	75 kDa (C ₅₅₄)	4%–9%		
	75 kDa N4 (C ₂₂₆)	6%–8%	Impairing main electron transfer pathway	10, 45, 21
	75 kDa N1b (C ₉₂)	3%–4%		
Primary	49 kDa (C ₁₄₆)	15%–20%	Affecting ubiquinone-binding and main electron transfer pathway	32,50,51
	49 kDa (C ₃₂₆ /C ₃₄₇)	2%–5%		
Primary	13 kDa (C ₇₉)	10%–20%	Affecting the structural integrity of complex I structure via impairing Zn ²⁺ -binding	32, 57
Secondary	24 kDa N1a (C ₁₃₄)	10%–15%	Impairing antioxidant function of N1a, increasing accumulation of FMNH [•] for •O ₂ ⁻ generation	10, 45, 46
	24 kDa N1a (C ₁₃₉)	3%–5%		
Secondary	PSST (C ₁₇₅)	4%–7%	With 49 kDa sulfonation to affect ubiquinone-binding, increasing semiquinone for •O ₂ ⁻ generation	32,51
	PSST N2 (C ₁₈₈)	2%–4%		
	PSST N2 (C ₁₅₈)	1%–3%		
Tertiary	51 kDa (C ₂₈₆ /C ₁₂₅ /C ₁₈₇ /C ₂₀₆ /C ₂₃₈)	3%–8%	Affecting redox regulation	10, 14, 20, 22
	51 kDa N3 (C ₄₂₅)	2%–3%	Impairing main electron transfer pathway	10, 20, 45
Tertiary	15 kDa (C ₄₃)	2%–4%	Breaking intramolecular disulfide bond, and impacting structural integrity	32, 51