SUPPLEMENTAL MATERIAL

Detailed Materials and Methods

Animals

Male C57BL/6 mice (n = 25) were obtained from Jackson Laboratories (Bar Harbor, ME). All animals utilized in this study were between the ages of 12-15 weeks. Prior to myocardial excision, mice were anesthetized with pentobarbital sodium (50-100 mg/kg) via intraperitoneal injection. This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was approved by the Institutional Laboratory Animal Care and Use Committee.

Solutions and drugs

Krebs-Henseleit buffer (KHB) consisted of (in mmol/L): NaCl (120), KCl (4.7), NaH_2PO_4 (1.2), $NaHCO_3$ (25), $MgSO_4$ (1.2), Glucose (10), and $CaCl_2$ (1.75); pH 7.4. KHB was bubbled with 95% O_2/5% CO_2. Ascorbate (Sigma, St. Louis, MO) was used as an *S*-nitrosylation (SNO)-specific reducing agent. Dithiothreitol (DTT; Pierce, Rockford, IL) was used as a general reducing agent. All solutions were made fresh on the day of experimentation.

Ischemia-Reperfusion treatment protocol

Hearts were Langendorff-perfused in the dark as previously described;[1, 2] treatment protocols are shown in Fig. 1a. Hearts were randomly subjected to a perfusion protocol (60 minute perfusion period), an IPC protocol (20 minute equilibration period, 4 cycles of 5 minutes ischemia and 5 minutes reperfusion), an IR protocol (60 minute equilibration period, 20 minute ischemic period, 5 minute reperfusion period), an IPC-IR protocol (20 minute equilibration period, 4 cycles of 5 minutes ischemia and 5 minutes reperfusion, 20 minute ischemic period, 5 minute reperfusion period), or an IPC-R protocol (20 minute equilibration period, 4 cycles of 5 minutes ischemia and 5 minutes reperfusion, 25 minute reperfusion period). Hearts were snap frozen in liquid nitrogen immediately following the treatment protocol.

Crude homogenate preparation

Crude heart homogenates were prepared as described previously.[1, 2] All subsequent procedures were performed in the dark. Hearts were powdered on liquid nitrogen with a mortar and pestle, and resuspended in 1.5 mL of homogenization buffer containing (in mmol/L): sucrose (300), HEPES-NaOH 7.7 (250), EDTA (1), and Neocuproine (0.1). An EDTA-free protease inhibitor tablet (Roche Diagnostics Corporation, Indianapolis, IN) was introduced just before use. Samples were then homogenized via Dounce glass homogenization on ice and centrifuged at 1,000 g for 2 minutes. The supernatant was recovered as total crude homogenate. Protein concentration was determined using the Bradford protein assay.

Oxidation site identification with resin-assisted capture

For oxidation site identification (Ox-RAC), samples (1 mg) were diluted in HEN buffer containing (in mmol/L): **H**EPES-NaOH 7.7 (250), **E**DTA (1), and **N**eocuproine (0.1) with 2.5% SDS and an EDTA-free protease inhibitor tablet (Roche Diagnostics Corporation). All buffers were de-gassed prior to use in order to prevent oxidation of the resin. Homogenates were then incubated with 20 mmol/L ascorbate for 45 minutes at room temperature in order to remove SNO. The inclusion of this step serves to distinguish SNO from other oxidative modifications; this step can be eliminated for the combined examination of SNO and other oxidative modifications. Samples were then incubated with 50 mmol/L *N*-ethylmaleimide (NEM; Sigma) for 20 minutes at 50°C in order to block non-modified (i.e., free) and ascorbate-reduced thiol groups from modification; ascorbate and NEM were removed via acetone precipitation. Samples

were then resuspended in HENS and oxidized thiols were then reduced with 10 mmol/L DTT for 10 minutes at room temperature; DTT was removed via acetone precipitation. Samples were then resuspended in HEN with 1% SDS (HENS). Thiopropyl sepharose (GE Healthcare, Piscataway, NJ) was rehydrated for 25 minutes in DEPC H_2O. Following rehydration, 25 μL of the resin slurry was added to a Handee Mini Spin Column (Pierce) and washed with 5 x 0.5 mL DEPC H_2O, followed by 10 x 0.5 mL HEN buffer. Blocked samples were then added to the thiopropyl sepharose-containing spin column and rotated for 4 hours in the dark at room temperature. Proteins bind to the resin by forming disulfide linkages between reduced thiol groups of the protein and the thiol groups of the resin. Resin-bound proteins were then washed with 8 x 0.5 mL HENS buffer, followed by 4 x 0.5 mL HENS buffer diluted 1:10. Samples were then subjected to trypsin digestion (sequencing grade modified; Promega, Madison, WI) overnight at 37°C with rotation in buffer containing (in mmol/L): NH_4HCO_3 (50) and EDTA (1). Resin-bound peptides were then washed with 5 x 0.5 mL HENS buffer diluted 1:10, 5 x 0.5 mL 2 mol/L NaCl, 5 x 0.5 mL 80% acetonitrile/0.1% trifluoroacetic acid, and 5 x 0.5 mL HEN buffer diluted 1:10. Peptides were eluted for 30 minutes at room temperature in elution buffer containing (in mmol/L): DTT (20), NH_4CO_3 (10), and 50% methanol. The resin was then washed with an additional volume of elution buffer, followed by 2 volumes of DEPC water. All fractions were combined and concentrated via speedvac. Samples were then resuspended in 0.1% formic acid, and cleaned with a C_{18} column (ZipTip; Millipore, Billerica, MA). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was then performed using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA) as described below. The MASCOT database search engine was used for protein identification as described below.

S-nitrosylation site identification with resin-assisted capture

A modified version of the SNO-RAC protocol was developed in order to examine protein SNO.[3] Samples (1 mg) were diluted in HEN buffer with 2.5% SDS and an EDTA-free protease inhibitor tablet (Roche Diagnostics Corporation). All buffers were de-gassed prior to use in order to prevent oxidation of the resin. Homogenates were then incubated with 50 mmol/L NEM for 20 minutes at 50°C in order to block non-modified (i.e., free) thiol groups from modification; NEM was removed via acetone precipitation. Samples were then resuspended in HENS. Thiopropyl sepharose resin (GE Healthcare, Piscataway, NJ) was rehydrated for 25 minutes in DEPC H_2O. Following rehydration, 25 μL of the resin slurry was added to a Handee Mini Spin Column (Pierce) and washed with 5 x 0.5 mL DEPC H_2O, followed by 10 x 0.5 mL HEN buffer. Blocked samples were then added to the thiopropyl sepharose-containing spin column, along with 20 mmol/L ascorbate to reduce SNO, and rotated for four hours in the dark at room temperature. Proteins bind to the resin by forming disulfide linkages between reduced thiol groups of the protein and the thiol groups of the resin. Resin-bound proteins were then washed with 8 x 0.5 mL HENS buffer, followed by 4 x 0.5 mL HENS buffer diluted 1:10. Samples were then subjected to trypsin digestion (sequencing grade modified; Promega) overnight at 37°C with rotation in buffer containing (in mmol/L): NH_4HCO_3 (50) and EDTA (1). Resin-bound peptides were then washed with 5 x 0.5 mL HENS buffer diluted 1:10, 5 x 0.5 mL 2 mol/L NaCl, 5 x 0.5 mL 80% acetonitrile (v/v)/0.1% trifluoroacetic acid (v/v), and 5 x 0.5 mL HEN buffer diluted 1:10. Peptides were eluted for 30 minutes at room temperature in elution buffer containing (in mmol/L): DTT (20), NH_4HCO_3 (10), and 50% methanol (v/v). The resin was then washed with an additional volume of elution buffer, followed by 2 volumes of DEPC water. All fractions were combined and concentrated via speedvac. Samples were then resuspended in 0.1% formic acid, and cleaned with a C_{18} column (ZipTip; Millipore). LC-MS/MS was then performed using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) as described below. The MASCOT database search engine was used for protein identification as described below.

Liquid chromatography-tandem mass spectrometry analysis on LTQ Orbitrap XL

LC-MS/MS was performed using an Eksigent nano-LC 1D plus system (Dublin, CA) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) using CID fragmentation. Peptides were first loaded onto a Zorbax 300SB-C_{18} trap column (Agilent, Palo Alto, CA) at a flow rate of 5 μL/minute for 10 minutes, and then separated on a reversed-phase PicoFrit analytical column (New Objective, Woburn, MA) using a 40-minute linear gradient of 2-40% acetonitrile in 0.1% formic acid at a flow rate of 300 nL/minute. LTQ Orbitrap XL settings were as follows: spray voltage 1.5 kV, and full MS mass range: m/z 200 to 2000. The LTQ Orbitrap XL was operated in a data-dependent mode (i.e., one MS1 high resolution [30,000] scan for precursor ions followed by six data-dependent MS2 scans for precursor ions above a threshold ion count of 2000 with collision energy of 35%).

MASCOT Database Search

The raw file generated from the LTQ Orbitrap XL was analyzed using Proteome Discoverer v1.1 software (Thermo Fisher Scientific) with the NIH six-processor MASCOT cluster search engine (http://biospec.nih.gov, version 2.3). The following search criteria were used: database, Swiss-Prot (Swiss Institute of Bioinformatics); taxonomy, Mus musculus (mouse); enzyme, trypsin; miscleavages, 3; variable modifications, oxidation (M), *N*-methylmaleimide (C), deamidation (NQ); MS peptide tolerance 25 ppm; MS/MS tolerance as 0.8 Da. All peptides were assigned an ion score. The ion score is a measure of how well the MS/MS spectra matches the stated peptide; higher scores represent more confident matches. Ion scores were generated as $-10*LOG_{10}(P)$, where P represents the probability that the match is random. For a more detailed explanation of the ion score, please see the following reference.[4] Peptides with ion scores below 30 were not accepted. Peptides were filtered at a false discovery rate (FDR) of 5%, as determined by a targeted decoy database search with a significance threshold of 0.03. Identifications from non-specifically bound peptides (i.e., non-cysteine containing peptides) accounted for approximately 6% of all peptide identifications for both the SNO-RAC and Ox-RAC protocols; non-cysteine containing peptides were filtered from the data set.

Label-free peptide quantification and analysis

Relative quantification of SNO and oxidation was performed using QUOIL (**QU**antification with**O**ut **I**sotope **L**abeling), an in-house software program designed as a label-free approach to peptide quantification by LC-MS/MS.[5] This label-free approach relies on the direct comparison of peptide area-under-the-curve peaks from each LC-MS/MS run. More specifically, a peptide's chromatogram peak in each LC-MS/MS run was reconstructed based on its precursor m/z value. Quantitative ratios were then obtained by normalizing the peptide peak areas against a chosen reference (i.e., perfusion control for common peptides). The resulting ratios reflect the relative quantity of a peptide (and hence the corresponding SNO or oxidation level) in different samples, but the absolute amounts of the protein SNO and oxidation cannot be determined, since unmodified protein does not bind to the column and was not measured.

Glyceraldehyde-3-phosphate dehydrogenase activity assay

GAPDH activity was measured as described previously.[6] The assay buffer consisted of (in mmol/L): sodium pyrophosphate (10), sodium arsenate (25), NAD^+ (0.25); pH 8.5. Whole heart homogenate (10 μg) was diluted in 100 μL of assay buffer and the activity of GAPDH was monitored by following the reduction of NAD^+ to NADH at 340 nm using a FLUOstar Omega plate reader (BMG Labtech, Offenburg, Germany). The assay was initiated upon the addition of 0.5 mmol/L glyceraldehyde-3-phosphate (Sigma).

Statistics

Statistical significance ($p<0.05$) was determined between groups using an ANOVA for multiple groups or a Student's *t*-test for two groups.

Online Table I. **Selected peptides showing cysteine oxidation as identified via Ox-RAC proteomic analysis.**

Protein Name	Protein ID	Peptide Sequence	Ox Cys	Perfusion		IPC		IR		IPC-IR	
				Ratio	Ion Score	Ratio	Ion Score	Ratio	Ion Score	Ratio	Ion Score
Citrate synthase (M)	Q9CZU6	GYSIPECQK	101								
Cytochrome b-c1 complex subunit 1 (M)	Q9CZ13	VYEEDAVPGLTPCR	268	1	81	1.25	101	0.93	84	1.24	94
		LCTSATESEVTR	380	1	95	0.23	93	0.50	93	0.50	95
Fructose-bisphosphate aldolase A	P05064	ALANSLACQGK	339	1	83	1.13	86	$1.85^{\dagger,*}$	80	0.32	67
Glyceraldehyde-3-phosphate dehydrogenase	P16858	IVSNASCTTNCLAPLAK	150,154	1	124	1.38	138	1.65^{*}	122	$0.75^{\dagger,\ddagger}$	122
		VPTPNVSVVDLTCR	245	1	100	3.25^{*}	100	3.79^{*}	101	$1.83^{\dagger,\ddagger}$	102
Isocitrate dehydrogenase [NADP] (M)	P54071	SSGGFVWACK	308	1	77	1.34	76	1.28	83	1.01	63
		VCVQTVESGAMTK	402	1	110	17.88^{*}	112	$41.33^{*,\dagger}$	106	$7.93^{*,\dagger}$	107
L-lactate dehydrogenase A chain	P06151	IVSSKDYCVTANSK	84	1	93	2.05	85	2.79^{*}	89	$0.33^{*,\dagger}$	87
		VIGSGCNLDSAR	163	1	103	5.46^{*}	95	$0.89^{\dagger}$	99	$0.80^{\dagger}$	106
Malate dehydrogenase	P14152	VIVVGNPANTNCLTASK	137	1	89	1.97^{*}	89	1.62	88	$1.05^{\dagger}$	81
Malate dehydrogenase (M)	P08249	EGVVECSFVQSK	275	1	84	2.22^{*}	72	3.33^{*}	76	2.35^{*}	75
Protein NipSnap homolog 2	O55126	ICQEVLPK	89								
Succinyl-CoA ligase α (M)	Q9WUM5	IICQGFTGK	60				47		55		48
Triosephosphate isomerase	P17751	IAVAAQNCYK	67	1	40	1.61	48	1.81^{*}	46	$0.68^{*,\dagger,\ddagger}$	42
		IIYGGSVTGATCK	218	1	103	2.88^{*}	103	2.33^{*}	98	$1.04^{\dagger,\ddagger}$	90
Aconitate hydratase (M)	Q99KI0	VGLIGSCTNSSYEDMGR	385	1	132	2.88	128	2.03^{*}	137	$0.95^{\ddagger}$	128
Aspartate aminotransferase	P05201	INMCGLTTK	391								
Aspartate aminotransferase (M)	P05202	VGAFTVVCK	295	1	95	1.29^{*}	99	1.70^{*}	97	$1.30^{*,\ddagger}$	115
β-enolase	P21550	VNQIGSVTESIQACK	357	1	128	1.35^{*}	115	1.71^{*}	123	$0.69^{*,\dagger,\ddagger}$	123
Cysteine and glycine-rich protein 3	P50462	TVYHAEEIQCNGR	25	1	72	2.49	84	1.61^{*}	73	$0.61^{*,\ddagger}$	87
Cysteine-rich protein 2	Q9DCT8	ASSVTTFTGEPNMCPR	126	1	89	2.27^{*}	90	1.74^{*}	88	$1.04^{\dagger,\ddagger}$	98
Cytochrome c oxidase subunit 5b (M)	P19536	cPNCGTHYK	115						33		
Electron transfer flavoprotein subunit α (M)	Q99LC5	TIYAGNALCTVK	155	1	63	2.38	108	3.87	67	0.96	65
Electron transfer flavoprotein subunit β (M)	Q9DCW4	EIIAVSCGPSQCQETIR	66,71	1	124	1.01	107	1.42^{*}	127	$0.44^{\ddagger}$	128
Enoyl-CoA hydratase (M)	Q8BH95	LVEEAIQCAEK	225	1	88	6.06^{*}	92	7.28^{*}	86	$2.24^{\dagger,\ddagger}$	78
Isocitrate dehydrogenase [NAD] subunit α (M)	Q9D6R2	IEAACFATIK	331				56		57		
Long-chain-fatty-acid-CoA ligase 1	P41216	GIQVSNNGPCLGSR	109								
Metallothionein-1	P02802	SCCSCCPVGCSK	33,34,36 37,41								
		GAADKCTCCA	57,59,60								
NADH dehydrogenase [ubiquinone] 1 α subcomplex subunit 10 (M)	Q99LC3	VITVDGNICSGK	67	1	61	1.31	67	1.45^{*}	70	$1.30^{\ddagger}$	68
Peroxiredoxin-6	O08709	DFTPVCTTELGR	47	1	67	8.47^{*}	71	9.17^{*}	65	$5.11^{*,\dagger,\ddagger}$	65
Propionyl-CoA carboxylase α chain	Q91ZA3	MADEAVCVGPAPTSK	107								
Succinate dehydrogenase [ubiquinone] flavoprotein (M)	**Q8K2B3**	VGSVLQEGCEK	536								
Succinyl-CoA ligase subunit β (M)	Q9Z2I9	ILACDDLDEAAK	430	1	75	4.50^{*}	69	5.38^{*}	77	$1.71^{*,\dagger,\ddagger}$	75
Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	O55143	SMSVYCTPNKPSR	498	1	57	2.98	65	1.36	55	1.31	59
Tubulin β-4 chain	**P04350**	NMMAACDPR	303	1	62	3.28^{*}	59	1.60^{*}	64	$0.36^{*,\dagger,\ddagger}$	62
		TAVCDIPPR	354	1	53			2.05^{*}	52	$1.35^{\ddagger}$	52
Very long-chain specific acyl-CoA dehydrogenase (M)	P50544	SSAIPSPCGK	238	1	35	4.00^{*}	42	$1.17^{\dagger}$	39	$0.39^{*,\dagger}$	40
Voltage-dependent anion channel protein 2 (M)	Q60930	SCSGVEFSTSGSSNTDTGK	48	1	122	3.24^{*}	127	$2.11^{*,\dagger}$	131	$1.50^{\dagger,\ddagger}$	137

Proteins selected for inclusion in Online Table I are the same proteins from Table 1. Each protein/peptide was identified from at least three of five SNO-RAC/LC-MS/MS proteomic analyses (peptides were filtered at a false discovery rate of 5%; peptides with ion scores below 30 were not accepted). (M): mitochondrial isoform; (C): SNO cysteine residue; (c): NEM-blocked cysteine residue; Peptides not detected under the specified condition contain a blank space in the ion score column. Peptide quantitative ratio was determined via label-free peptide analysis. $^{*}p<0.05$ vs. Perfusion; $^{\dagger}p<0.05$ vs. IPC; $^{\ddagger}p<0.05$ vs. IR.

Additional Online Table Legends

Online Table II. **Oxidized protein/peptide identifications from perfusion hearts as identified via Ox-RAC proteomic analysis.** LC-MS/MS derived peptide sequences and MASCOT MS2 search identifications (false discovery rate of 5%). Peptide identifications with ions scores below 30 were not accepted; non-cysteine containing peptides were filtered from the data set. To view peptide sequences, click on the '+' symbol found on the left side of the spreadsheet. Each of 5 biological replicates was run in order to increase protein/peptide identifications; replicates are identified in column headings as A2 (Perfusion Heart 1), B2 (Perfusion Heart 2), C2 (Perfusion Heart 3), D2 (Perfusion Heart 4), and E2 (Perfusion Heart 5). Please note that Online Table II includes all Ox-RAC protein/peptide identifications from perfusion hearts, including those observed in fewer than three of five Ox-RAC proteomic analyses.

Online Table III. **Oxidized protein/peptide identifications from IR hearts as identified via Ox-RAC proteomic analysis.** LC-MS/MS derived peptide sequences and MASCOT MS2 search identifications (false discovery rate of 5%). Peptide identifications with ions scores below 30 were not accepted; non-cysteine containing peptides were filtered from the data set. To view peptide sequences, click on the '+' symbol found on the left side of the spreadsheet. Each of 5 biological replicates was run in order to increase protein/peptide identifications; replicates are identified in column headings as A2 (IR Heart 1), B2 (IR Heart 2), C2 (IR Heart 3), D2 (IR Heart 4), and E2 (IR Heart 5). Please note that Online Table III includes all Ox-RAC protein/peptide identifications from IR hearts, including those observed in fewer than three of five Ox-RAC proteomic analyses.

Online Table IV. **Oxidized protein/peptide identifications from IPC hearts as identified via Ox-RAC proteomic analysis.** LC-MS/MS derived peptide sequences and MASCOT MS2 search identifications (false discovery rate of 5%). Peptide identifications with ions scores below 30 were not accepted; non-cysteine containing peptides were filtered from the data set. To view peptide sequences, click on the '+' symbol found on the left side of the spreadsheet. Each of 5 biological replicates was run in order to increase protein/peptide identifications; replicates are identified in column headings as A2 (IPC Heart 1), B2 (IPC Heart 2), C2 (IPC Heart 3), D2 (IPC Heart 4), and E2 (IPC Heart 5). Please note that Online Table IV includes all Ox-RAC protein/peptide identifications from IPC hearts, including those observed in fewer than three of five Ox-RAC proteomic analyses.

Online Table V. **Oxidized protein/peptide identifications from IPC-IR hearts as identified via Ox-RAC proteomic analysis.** LC-MS/MS derived peptide sequences and MASCOT MS2 search identifications (false discovery rate of 5%). Peptide identifications with ions scores below 30 were not accepted; non-cysteine containing peptides were filtered from the data set. To view peptide sequences, click on the '+' symbol found on the left side of the spreadsheet. Each of 5 biological replicates was run in order to increase protein/peptide identifications; replicates are identified in column headings as A2 (IPC-IR Heart 1), B2 (IPC-IR Heart 2), C2 (IPC-IR Heart 3), D2 (IPC-IR Heart 4), and E2 (IPC-IR Heart 5). Please note that Online Table V includes all Ox-RAC protein/peptide identifications from IPC-IR hearts, including those observed in fewer than three of five Ox-RAC proteomic analyses.

Supplemental References

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