Mechanistic Insights into Nitrite-Induced Cardioprotection Using an Integrated Metabonomic-Proteomic Approach

Online Data Supplement

Supplemental Methods

Animal Care and Nitrite Administration

Male Wistar rats (Harlan, Indianapolis, IN; 250-350 g) were allowed food (2018 rodent diet, Harlan Teklad) and water *ad libitum* and were kept on a 12 h/12 h light/dark cycle with at least 10 days of local vivarium acclimatization prior to experimental use. Rats were given a single intraperitoneal (i.p.) injection of sodium nitrite (SigmaAldrich, St. Louis, MO) dissolved in phosphate buffered saline (PBS, pH 7.4) at doses of 0, 0.1, 1.0 and 10 mg/kg body weight for nitrite bolus experiments. In order to avoid potential confounding effects of chronobiological changes of endogenous nitrite, nitrate and nitros(yl)ation levels in time-course experiments, nitrite injections were staggered such that organ harvest for each time point after nitrite administration was conducted at the same absolute time-of-day (thus, all animals were in the same chronobiological phase at the time of organ harvest).

Organ Perfusion and Homogenization

At 0, 2, 5, 10 and 30 min or 1, 3, 12, 24, 36, and 48 h post-application of nitrite, heparinized (0.07 units/g i.p.) animals were anesthetized with diethylether (2 min) and euthanized by cervical dislocation. Organs were perfused free of blood via retrograde perfusion with air-equilibrated PBS (50mM, pH 7.4) supplemented with *N*-ethylmaleimide (NEM)/ EDTA (10mM/2.5mM) for metabonomic measurements. The heart was excised, washed in perfusion buffer, blotted dry on filter paper, weighed, and homogenized immediately in ice-cold NEM/EDTA-containing perfusion buffer (5:1 v/w) For proteomic analysis of whole heart homogenate, animals were perfused with air-equilibrated PBS containing only EDTA (2.5 mM), and the heart was homogenized in PBS (50 mM, pH 7.4) buffer containing 2.5 mM EDTA, 1 mM DTT, 1% NP-40, phosphatase inhibitors (consisting of 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, and 50 mM β-glycerophosphate, final concentrations), and Complete™ protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). For either type of analysis, organs were homogenized on ice using a polytron homogenizer. Proteomic sample homogenates were incubated on ice for 30 min to facilitate solubilization, after which they were cleared of insolubles by centrifugation at 12,000 X *g* for 15 min at 4 °C, aliquoted, and stored at -80 °C. Prior to use, proteomic samples were thawed on ice and assayed for protein concentration using the BCA Assay™ (Pierce, Rockford, IL). An equal protein quantity from

each of the samples derived from each of the 3 animals at a particular dose were pooled, then dissolved and clarified in Destreak™ IEF buffer (GE Healthcare, Piscataway, NJ), as described below for mitochondrial sample preparation.

Isolated Perfused Heart Preparation

Hearts were isolated and perfused *ex vivo* in the Langendorff mode according to a previously described protocol.[19] Briefly, the thorax was rapidly opened, the heart excised, and arrested in ice-cold buffer. Retrograde perfusion was initiated via the aortic root at a constant coronary perfusion pressure of 70 mmHg at 37 °C. The coronary perfusate consisted of a modified Krebs-Henseleit buffer containing in mM: 118 NaCl, 5.3 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 0.5 EDTA, and 25 $NaHCO_3$. The perfusate was equilibrated with 95% O_2 and 5% CO_2 to yield a pH of 7.4 at 37 °C. A water-filled balloon custom-made from polyvinyl chloride film was inserted through the mitral valve into the left ventricle via an incision in the left atrium. The balloon was connected to a pressure transducer (MLT 844, ADInstruments, Colorado Springs, CO) for continuous recording of left ventricular pressure and HR. The balloon was inflated until an LV end-diastolic pressure (EDP) of 10 mm Hg was achieved, and the balloon volume was then held constant so that changes in EDP reflected changes in LV diastolic compliance. Isovolumic contractile data were collected on-line using a MacLab data acquisition system (ADInstruments). Hearts were stabilized for 15 min and subsequently subjected to 15 min of zero-flow ischemia, followed by 30 min of reperfusion. LVDevP (the difference between systolic pressure and EDP), the minimum and maximum values within a beat of the first derivative of left ventricular pressure (+dP/dt and -dP/dt), and RPP (product of LVDevP and HR) were calculated off-line.

Metabonomic and Redox Measurements

Nitroso and nitrosyl compounds were measured by group-specific denitrosation after injection of the biological sample into a triiodide-containing reaction mixture continuously purged with nitrogen. Evolved NO was quantified in the gas phase using an ozone-based chemiluminescence detector (CLD 77am sp; EcoPhysics, Ann Arbor, MI). Nitrite and nitrate concentrations were quantified simultaneously by ion chromatography (ENO20; Eicom, San Diego, CA). Ascorbate and dehydroascorbate and reduced and oxidized glutathione were measured essentially as described[18] (see Online Data Supplement). Protein carbonyls were quantified using a commercially available ELISA (Northwest LLC, Vancouver, WA).Ascorbate and dehydroascorbate were measured as described by Carr, et al., at a wavelength of 524 nm using a Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA), in which ascorbic acid was

oxidized to dehydroascorbic acid (DHA) by the addition of 2,6-dichlorophenolindophenol (DCIP) and incubated with the color producing reagent 2,4-dinitrophenylhydrazine (DNPH) Control samples were processed with water, rather than DCIP, prior to incubation with DHPH. Total ascorbate was determined as the raw absorbance difference between the DCIP-treated samples and control samples fitted to an ascorbic acid standard curve. A blank in which the color producing reagent, 2,4-dinitrophenylhydramine (DNPH) was added only after the reaction was terminated by the addition of sulfuric acid was used to account for tissue-specific background coloration of the assay solution. Ascorbic acid tissue concentration was found by determining the absorbance difference between the DCIP-treated sample and the blank and fitting it to the ascorbic acid standard curve.

Isolation Mitochondria and Post-mitochondrial Cytoplasm from the Heart

Hearts were perfused, isolated, washed, weighed and homogenized as described, except that homogenization was conducted in mitochondrial purification buffer (MPB, consisting of PBS, pH 7.4, supplemented with 50 mM sucrose, 200 mM mannitol, 5 mM potassium phosphate, 5 mM MOPS, 1 mM EGTA, 0.1% BSA, Complete™ protease inhibitors (Roche), and the above phosphatase inhibitor mix. Homogenates were incubated on ice for 30 min, after which homogenates from replicate animals within each treatment group were pooled, clarified by centrifugation at 1,500 X *g* for 10 min at 4 °C, then subjected to centrifugation at 15,000 X *g* for 20 min at 4 °C to separate crude mitochondria (pellet) from post-mitochondrial cytoplasm (supernatant). Pellets were washed twice by resuspension in 5 ml of fresh MPB and re-centrifuged at 15,000 X *g* for 20 min at 4 °C. Pellets were dissolved directly in Destreak™ IEF buffer (GE Healthcare), incubated at room temperature for 1 h to enhance solubilization, clarified by centrifugation at 21,000 X *g*, and assayed for protein concentration using the 2D-Quant Kit™ (GE Healthcare). Post-mitochondrial cytoplasmic supernatants were assayed for protein concentration using the BCA Assay™, equal amounts were precipitated using the 2D Clean-up Kit™ (BioRad) and dissolved in Destreak™ IEF buffer.

2D-PAGE Analysis

IEF for 2D-PAGE was conducted as follows: 17 cm ReadyStrips™ IPG strips (BioRad) of pI 3-10 or 4-7 were subjected to active rehydration overnight with normalized aliquots of protein in Destreak™ IEF buffer, supplemented with 0.02% pI 3-10 ampholytes (BioRad). Isoelectric focusing was performed in a Protean™ IEF cell (Bio-Rad) at 120 V for 1 h, 300 V for 30 min, a

linear increase up to 3500 V over 3 h, up to 5000 V over 10 min, and then at 8000 V steadily, until a total of 67,000 Vh had elapsed. Following IEF, IPG strips were subjected to reduction, alkylation, and equilibration for the second dimension, using Equilibration Buffers I & II (BioRad) for 30 min each, then SDS-PAGE, using 10% polyacrylamide ReadyGels™ (Bio-Rad). After staining, gels were imaged using a VersaDoc™ 3000 (BioRad, Hercules, CA). Protein transfer to PVDF membranes was accomplished using a Genie™ apparatus (Idea Scientific, Minneapolis, MN). Chemiluminescence was captured using a VersaDoc™ 3000 and analyzed using QuantityOne™ software.

In-Gel Digestion and MALDI-TOF Mass Spectrometry

In-gel digestion was conducted as previously described, by a modified procedure originally based on the work of Shevchenko, et al. [Shevchenko A, Wilm M, Vorm O, Mann M: Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem 1996, 68:850–858]. Briefly, the protein spots of interest were punch-excised using wide-orifice pipet tips, and were destained either with Commassie destain (100 mM ammonium bicarbonate (ABC), pH 9/50% acetonitrile(ACN)) or with silver destain (the potassium ferrocyanide-based reagents of the ProteoSilver™ Plus kit, SigmaAldrich, St. Louis, MO). The gel pieces were washed four times with alternating solutions of 100 mM ABC (pH 9), 100 mM ABC (pH 9)/ 50% ACN, and 100% ACN, swelled in digestion solution (approximately 10 ng Trypsin Gold (Promega, Madison, WI) in 50 mM ABC (pH 9)/ 10% ACN) and incubated overnight at 37 °C. Peptides were extracted twice with alternating solutions of 20 mM ABC, (pH 9), 1% trifluoroacetic acid (TFA)/ 50% ACN, and 100% ACN, and all extraction supernatants were pooled and spun to dryness in a SpeedVac™ (Thermo-Savant, Waltham, MA). For acquisition of mass spectra, instrument laser intensity was adjusted to be approximately 3% above the threshold value. Signals from 200 to 300 laser shots were summed for each mass spectrum. External calibration of the spectra was achieved using Bruker peptide standards, and internal calibration was achieved to within 50 ppm using the masses of known peptide ions.

MALDI-TOF MS Data Analysis and Peptide Mass Fingerprinting

Mascot™ peptide mass fingerprinting analysis was conducted against the SwissProt or NCBI non-redundant protein databases using the following parameters: a) species, rat or mouse b) trypsin digestion with up to 3 missed cleavages c) +/- 70 ppm error d) cysteine carbamidomethylation as a fixed modification, e) methionine oxidation as a variable

modification. Peak lists were submitted in batch to Mascot™ using the BUDSS software (written in-house).

Preparation of Peptides from Heart Homogenates for Phosphopeptide Enrichment and Mass Spectrometry

In order to avoid potential artifactual chemical modifications and losses associated with peptides extracted from stained gels, peptides were produced directly from heart homogenates in the following manner. Protein was precipitated from pooled vehicle-treated (control) and pooled nitrite-treated heart homogenates by adding 8 vol. ice-cold acetone / 1 vol. 100% trichloroacetic acid and incubating overnight at -20 °C. Precipitates were pelleted by centrifugation at 21,000 g for 10 min at 4 °C; pellets were then washed with 100% ice-cold acetone. Protein was solublized and subjected to reduction of disulfides by incubation in a solution of 50 mM Tris(hydroxymethyl)aminomethane (Tris, pH 8.8) containing 7M urea/ 2 M thiourea/ 10 mM dithiothreitol (DTT) for 4 h at room temperature (RT) with gentle rocking. Thiol alkylation was performed by the addition of 20 mM iodoacetamide and incubation at RT in the dark for 30 min followed by quenching with 50 mM DTT. Samples were diluted 2-fold with 50 mM Tris (pH 8.8)/ 20% ACN and subjected to digestion with Lys-C endoproteinase (Pierce) for 10 h at RT using a 1/250 (wt/wt) enzyme to substrate ratio. This was followed by a second phase of digestion, after a 2.5-fold dilution with 50 mM Tris (pH 8.8)/ 10% ACN, using trypsin (Promega) at a 1/50 (wt/wt) enzyme to substrate ratio and incubating at RT overnight. Digests were spun to dryness in a SpeedVac™.

Phosphopeptide Enrichment

Phosphopeptides were enriched from samples using a 2-stage process of calcium phosphate precipitation [Zhang X, Ye J, Jensen ON, Roepstorff P: Highly Efficient Phosphopeptide Enrichment by Calcium Phosphate Precipitation Combined with Subsequent IMAC Enrichment. Mol Cell Proteomics. 2007 6(11): 2032-42.], followed by titanium dioxide enrichment [Thingholm TE, Jørgensen TJ, Jensen ON, Larsen MR: Highly selective enrichment of phosphorylated peptides using titanium dioxide. Nat Protoc. 2006;1(4):1929-35.]. Dried samples were resuspended in water followed by the addition of ammonium hydroxide until the solutions achieved a pH of 10. Dibasic sodium phosphate was added to a final concentration of 20 mM, followed by calcium chloride ($CaCl_2$) to a final concentration of 80 mM. Insoluble precipitates were pelleted by centrifugation at 21,000 g for 10 min, washed with 80 mM $CaCl_2$, and dissolved in 1% TFA (or in 5% formic acid (FA)/ 3% ACN, for analysis directly by LC-MS). Peptides

were desalted by C18 solid phase extraction using Supelclean™ LC-18 devices (SigmaAldrich), using 1% TFA, 0.1% TFA, and 80% ACN/ 0.1% TFA/ 20 mg/ml dihydroxybenzoic acid (DHB) as the binding, washing, and elution solutions, respectively. Eluted peptides were then immediately subjected to titanium dioxide phosphopeptide enrichment using MonoTip™ pipette tips (GL Sciences, Torrance, CA), wherein peptides were bound to the tips in 80% ACN/ 0.1% TFA/ 20 mg/ml DHB solution, washed 3 times with 80% ACN/ 0.1% TFA/ 20 mg/ml DHB solution, washed 7 times with 80% ACN/ 0.1% TFA solution, and eluted with 75 mM ammonium hydroxide. Samples were prepared for LC-MS by the addition of 5% FA/ 3% ACN.

Nanoflow HPLC-coupled Tandem Mass Spectrometry

LC-MS was performed on the phosphopeptide-enriched samples using a nanoAcquity UPLC™ capillary high-performance liquid chromatography system (Waters Corp., Milford, MA) coupled to an LTQ-Orbitrap™ hybrid mass spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with a TriVersa NanoMate ion source (Advion, Ithaca, NY). Sample concentration and desalting were performed online using a nanoAcquity UPLC™ trapping column (180µm x 20mm, packed with 5 µm, 100 Å Symmetry C18 material, Waters Corp.) at a flow rate of 15 µL/ min for 1 min. Separation was accomplished on a nanoAcquity UPLC™ capillary column (100µm x 100mm, packed with 1.7µm, 130 Å BEH C18 material, Waters Corp.). A linear gradient of A and B buffers (buffer A: 3% ACN/ 0.1% FA; buffer B: 97% ACN/ 0.1% FA) from 7% to 45% buffer B over 124 min was used at a flow rate of 0.5 µL/ min to elute peptides into the mass spectrometer. Columns were washed and re-equilibrated between LC-MS experiments. Electrospray ionization was carried out at 1.7 kV using the NanoMate, with the LTQ heated capillary set to 150 °C. Mass spectra were acquired in the Orbitrap in the positive-ion mode over the m/z range of 300–2000 at a resolution of 60,000 (approximately 1 mass spectrum/ s). Mass accuracy after internal calibration was within 4 ppm. Simultaneously, tandem MS (MS/MS) were acquired using the LTQ for the five most abundant, multiply-charged species in the mass spectrum with signal intensities of >8000 NL, using multistage activation that targeted the ions of interest plus those resulting from the potential prompt neutral loss of one or multiple phosphoric acid moieties. MS/MS collision energies were set at 35%, using helium as the collision gas, and MS/MS were acquired over a range of m/z values dependent on the precursor ion. Dynamic exclusion was set such that MS/MS for each species was acquired a maximum of twice. All spectra were recorded in profile mode for further processing and analysis.

LC-MS Data Analysis

Xcalibur and Proteome Discoverer software (ThermoFisher Scientific) were used for MS and MS/MS data analysis, while peptide and protein assignments were conducted using Mascot™ to search against the rodent species subset of the SwissProt database (UniProt release 14.3) employing a error window of 6ppm on the precursor ions and 0.6 Da on the fragment ions. Fixed modifications were set to include carbamidomethylation of cysteine; variable modifications were set to include oxidation of methionine and phosphorylation of serine, threonine, and tyrosine residues. Mascot™ results were parsed, filtered, and analyzed using Scaffold (Proteome Software, Portland, OR). Potential phosphopeptides identified by Mascot™ were verified and refined by manual interpretation of the original spectra.

Supplemental Figures

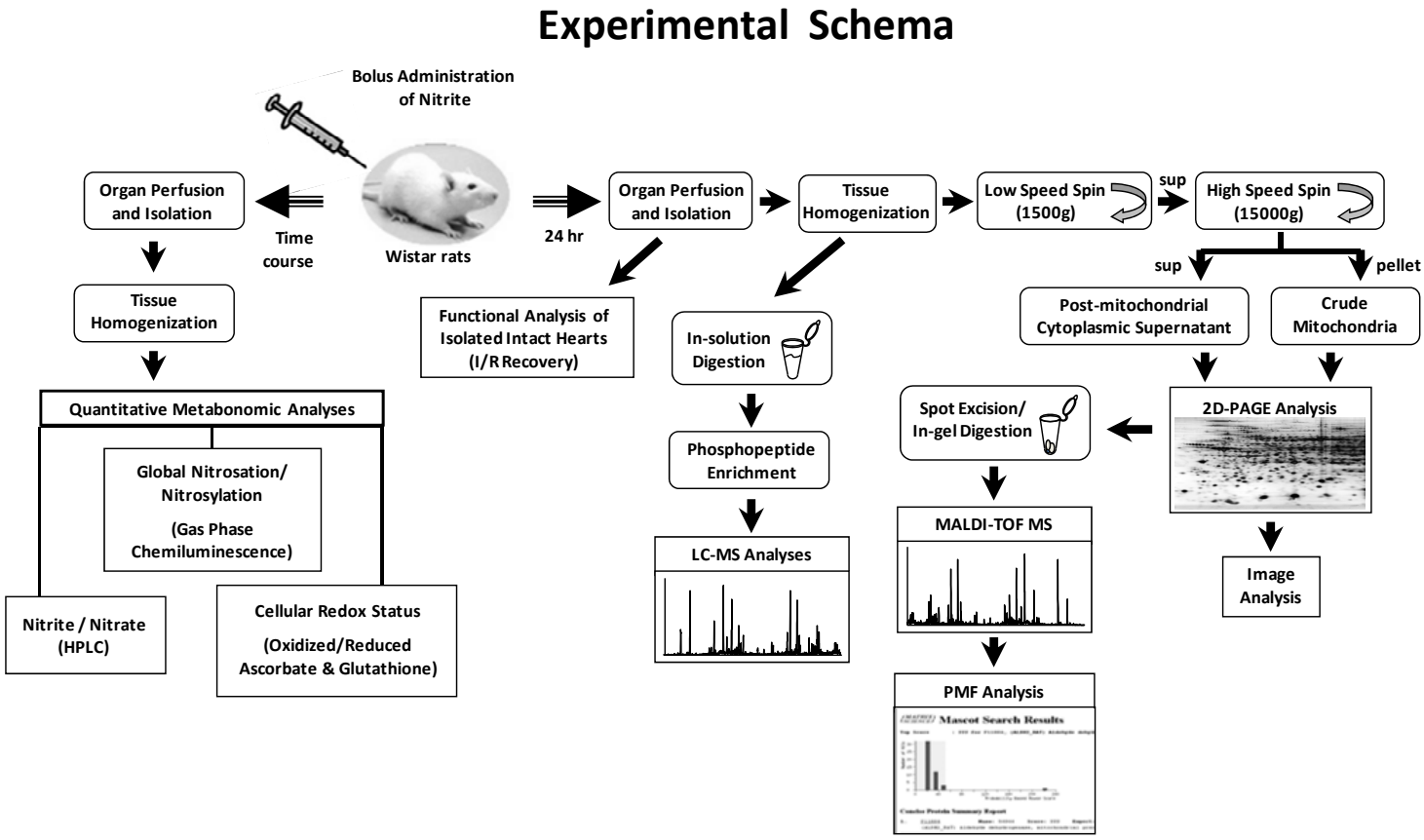

Figure S-1. Schema depicting the experimental design to study the effects of nitrite exposure on the heart using metabonomic, proteomic, and functional analyses. Age-matched male Wistar rats were administered a systemic bolus of nitrite through intraperitoneal injection to mimic an acute exposure event such as those caused by brief bouts of exercise, consumption of nitrate-rich vegetables, or other physiological process that leads to sharp increases in circulating nitrite levels. Hearts (as well as other organs) were isolated various times afterwards, homogenized, and either were subjected to quantitative metabonomic analysis, consisting of measurement of tissue nitrite and nitrate levels, nitrosation and nitrosylation, and cellular redox status, or were subjected to separation into mitochondrial and post-mitochondrial fractions by differential centrifugation, followed by 2D-PAGE, protein and protein modification-specific staining, in-gel digestion, MALDI-TOF MS and PMF analyses. Phosphopeptides enriched from in-solution digested tissue homogenate were analyzed by LC-MS. Alternatively, intact hearts were functionally assessed in response to ischemia/reperfusion *ex vivo* under Langendorff perfusion.

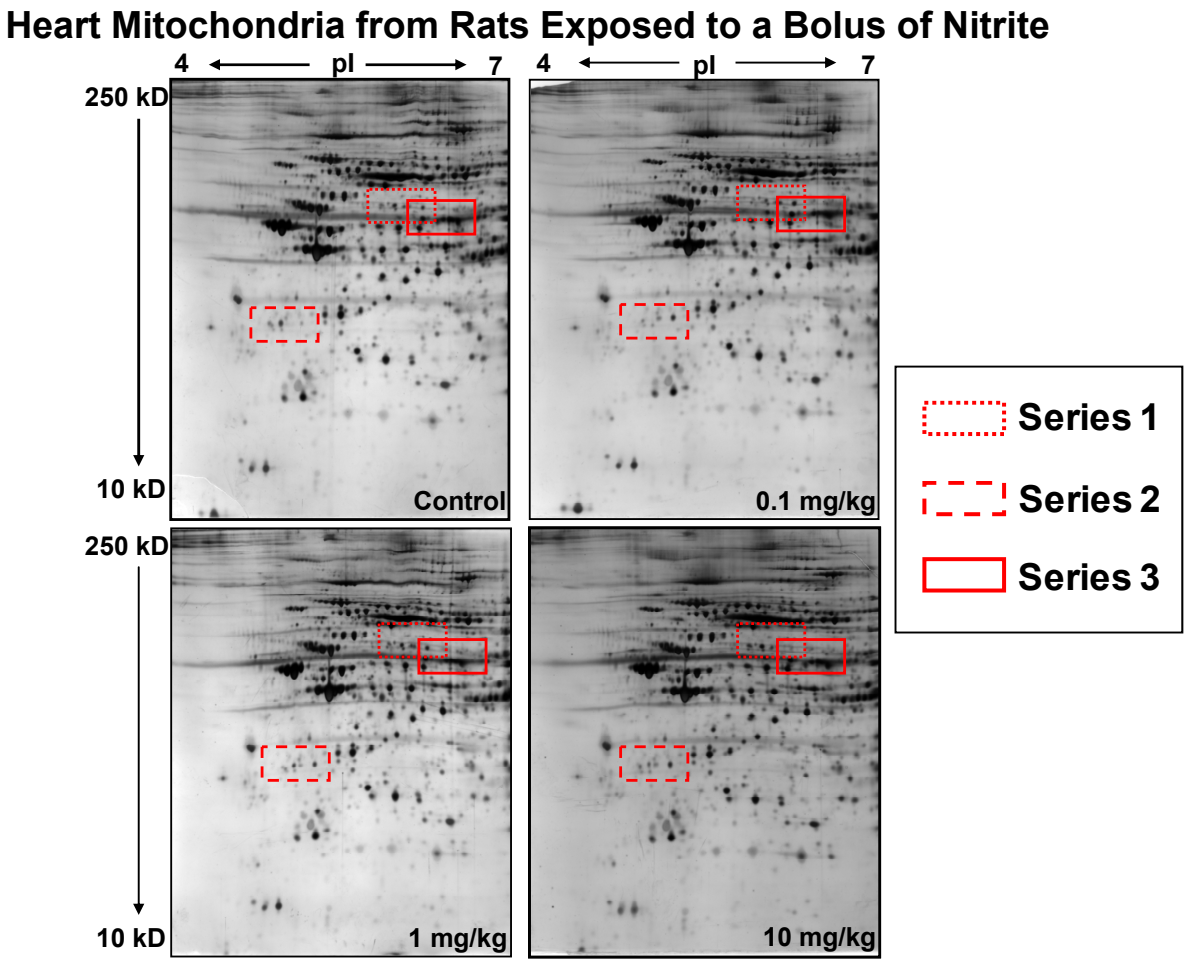

Figure S-2. Three regions of the cardiac mitochondrial 2D-PAGE proteome displaying pronounced changes in spot series. Isolated cardiac mitochondria from animals administered a bolus dose of 0.1, 1.0, 10 mg/kg nitrite, or saline (control) were subjected to 2D-PAGE analysis (as in Figure 3A), here using IEF over the pI range 4 to 7, as indicated, in order to expand regions displaying pronounced spot changes between levels of nitrite exposure. Regions boxed in red contain spot series showing pronounced changes (series 1, 2, 3, dotted, dashed, and solid lines, respectively) and are shown enlarged in Figure 3B.

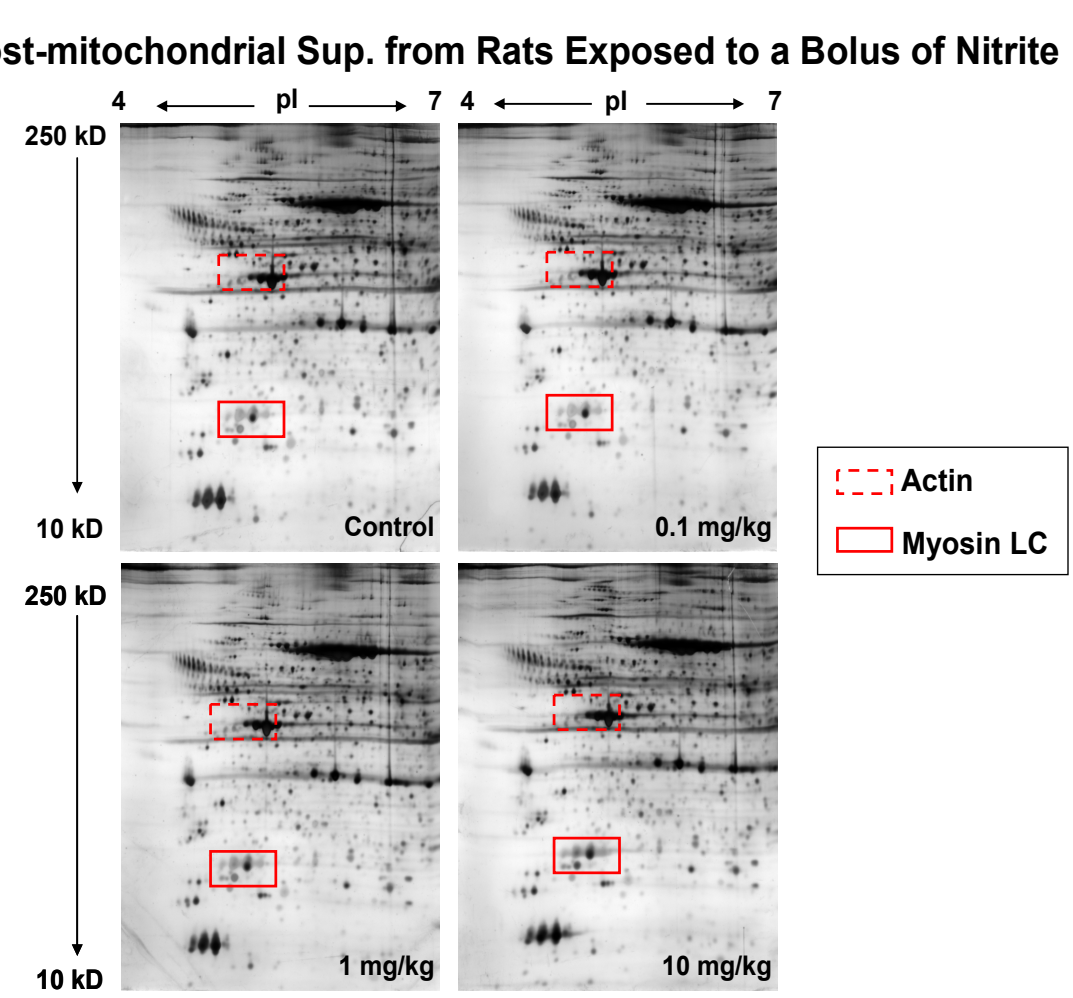

Figure S-3. Brief nitrite exposure induces long-term changes to structural/contractile proteins in the myocardium. Post-mitochondrial cytoplasmic supernatant purified by differential centrifugation from the cardiac tissue of animals administered a bolus dose of 0.1, 1.0, 10 mg/kg nitrite, or saline (control), as described, was analyzed by 2D-PAGE over the pI range 4 to 7 and molecular weight range of 250 kDa to 10 kDa. Regions boxed in red contain actin and myosin LC1 spot series that displayed pronounced changes in response to nitrite administration and are shown enlarged in Figure 4A and B.

Supplemental Tables

Table 1. Peptide Mass Fingerprint Analysis of Mitochondrial and Post-mitochondrial Cytoplasm 2D-gel Spots

SwissProt ID	Accession Number	Protein Name	Mascot Score	Expect Value
A1AT_RAT	P17475	Alpha-1-antiproteinase	99	2.40E-06
A1I3_RAT	P14046	Alpha-1-inhibitor 3	201	1.60E-16
ACON_RAT	Q9ER34	Aconitate hydratase, mitochondrial	218	3.10E-18
ACTC_MOUSE	P68033	Actin, alpha cardiac muscle 1	169	2.50E-13
AFAM_RAT	P36953	Afamin	222	1.20E-18
AL9A1_RAT	Q9JLJ3	4-trimethylaminobutyraldehyde dehydrogenase	102	1.20E-06
ALBU_RAT	P02770	Serum albumin	260	2.00E-22
ALDH2_RAT	P11884	Aldehyde dehydrogenase, mitochondrial	205	6.20E-17
ANXA6_RAT	P48037	Annexin A6	234	7.80E-20
APOA1_RAT	P04639	Apolipoprotein A-I	81	0.00018
ATPA_RAT	P15999	ATP synthase subunit alpha, mitochondrial	95	6.00E-06
ATPB_RAT	P10719	ATP synthase subunit beta, mitochondrial	182	1.20E-14
ATRX_MOUSE	Q61687	Transcriptional regulator ATRX	58	0.029
BCAT2_RAT	O35854	Branched-chain-amino-acid aminotransferase, mitochondrial	95	6.20E-06
CAH3_RAT	P14141	Carbonic anhydrase 3	112	1.20E-07
CALR_RAT	P18418	Calreticulin	156	4.90E-12
CATA_RAT	P04762	Catalase	197	3.90E-16
CH60_MOUSE	P63038	60 kDa heat shock protein, mitochondrial	290	2.00E-25
CI068_RAT	Q6AYJ3	Uncharacterized protein C9orf68 homolog	60	0.018

SwissProt ID	Accession Number	Protein Name	Mascot Score	Expect Value
CPI3_RAT	P05544	Serine protease inhibitor A3L	186	4.90E-15
CPI6_RAT	P09006	Serine protease inhibitor A3N	89	2.60E-05
CPSM_RAT	P07756	Carbamoyl-phosphate synthase [ammonia], mitochondrial	223	9.90E-19
CPT2_RAT	P18886	Carnitine O-palmitoyltransferase 2, mitochondrial	226	4.90E-19
CRYAB_RAT	P23928	Alpha-crystallin B chain	172	1.20E-13
CY1_MOUSE	Q9D0M3	Cytochrome c1, heme protein, mitochondrial	82	0.00012
DNJ5B_MOUSE	Q9CQ94	DnaJ homolog subfamily C member 5B	58	0.034
ECHA_RAT	Q64428	Trifunctional enzyme subunit alpha, mitochondrial	136	4.90E-10
ECHM_RAT	P14604	Enoyl-CoA hydratase, mitochondrial	103	9.90E-07
EPHA3_MOUSE	P29319	Ephrin type-A receptor 3	64	0.008
ETFB_RAT	Q68FU3	Electron transfer flavoprotein subunit beta	124	7.80E-09
EZRI_RAT	P31977	Ezrin	70	0.0021
FABPA_RAT	P70623	Fatty acid-binding protein, adipocyte	185	6.20E-15
FABPH_RAT	P07483	Fatty acid-binding protein, heart	113	9.90E-08
FTHFD_RAT	P28037	10-formyltetrahydrofolate dehydrogenase	172	1.20E-13
G3P_RAT	P04797	Glyceraldehyde-3-phosphate dehydrogenase	114	7.80E-08
GANAB_MOUSE	Q8BHN3	Neutral alpha-glucosidase AB	65	0.0068
GRP75_RAT	P48721	Stress-70 protein, mitochondrial	200	2.00E-16
GUAD_RAT	Q9WTT6	Guanine deaminase	128	3.10E-09
HBA_RAT	P01946	Hemoglobin subunit alpha-1/2	77	0.0004
HBB1_RAT	P02091	Hemoglobin subunit beta-1	134	7.80E-10
HBB2_RAT	P11517	Hemoglobin subunit beta-2	81	0.00016
HDAC2_MOUSE	P70288	Histone deacetylase 2	57	0.042
HEMO_RAT	P20059	Hemopexin	152	1.20E-11

SwissProt ID	Accession Number	Protein Name	Mascot Score	Expect Value
HS90B_RAT	P34058	Heat shock protein HSP 90-beta	147	3.90E-11
HSP7C_CRIGR	P19378	Heat shock cognate 71 kDa protein	73	0.001
HSPB1_RAT	P42930	Heat shock protein beta-1	63	0.01
IDH3B_RAT	Q68FX0	Isocitrate dehydrogenase [NAD] subunit beta, mitochondrial	72	0.0013
IQCB1_MOUSE	Q8BP00	IQ calmodulin-binding motif-containing protein 1	66	0.0053
IVD_RAT	P12007	Isovaleryl-CoA dehydrogenase, mitochondrial	81	0.00018
K1C14_RAT	Q6IFV1	Keratin, type I cytoskeletal 14	74	0.00077
KPYM_RAT	P11980	Pyruvate kinase isozymes M1/M2	82	0.00012
LDHB_RAT	P42123	L-lactate dehydrogenase B chain	225	6.20E-19
LONM_RAT	Q924S5	Lon protease homolog, mitochondrial	185	6.20E-15
MUG1_RAT	Q03626	Murinoglobulin-1	181	1.60E-14
MUG2_RAT	Q6IE52	Murinoglobulin-2	101	1.60E-06
MVP_RAT	Q62667	Major vault protein	189	2.50E-15
MYG_RAT	Q9QZ76	Myoglobin	86	5.30E-05
MYH4_RAT	Q29RW1	Myosin-4	64	0.0072
MYH8_MOUSE	P13542	Myosin-8	62	0.013
NALP6_MOUSE	Q91WS2	NACHT, LRR and PYD domains-containing protein 6	63	0.011
NDKA_RAT	Q05982	Nucleoside diphosphate kinase A	59	0.026
NDKB_RAT	P19804	Nucleoside diphosphate kinase B	121	1.60E-08
NDUAA_RAT	Q561S0	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial	208	3.10E-17
NDUS1_RAT	Q66HF1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	295	6.20E-26
NRAP_MOUSE	Q80XB4	Nebulin-related-anchoring protein	67	0.0041
ODO1_RAT	Q5XI78	2-oxoglutarate dehydrogenase E1 component, mitochondrial	290	2.00E-25

SwissProt ID	Accession Number	Protein Name	Mascot Score	Expect Value
ODO2_RAT	Q01205	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	63	0.0099
ODP2_RAT	P08461	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	118	3.10E-08
ODPB_RAT	P49432	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	102	1.20E-06
OVGP1_MOUSE	Q62010	Oviduct-specific glycoprotein	59	0.028
PCCA_RAT	P14882	Propionyl-CoA carboxylase alpha chain, mitochondrial	194	7.80E-16
PDIA1_RAT	P04785	Protein disulfide-isomerase	202	1.20E-16
PDIA3_RAT	P11598	Protein disulfide-isomerase A3	221	1.60E-18
PEBP1_RAT	P31044	Phosphatidylethanolamine-binding protein 1	81	0.00016
PGAM1_MOUSE	Q9DBJ1	Phosphoglycerate mutase 1	73	0.00094
PIMT_RAT	P22062	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	57	0.042
PKHA4_RAT	P60669	Pleckstrin homology domain-containing family A member 4	60	0.022
PRDX1_RAT	Q63716	Peroxiredoxin-1	146	4.90E-11
PRDX2_MOUSE	Q61171	Peroxiredoxin-2	58	0.033
PRDX6_RAT	O35244	Peroxiredoxin-6	68	0.0034
PSA1_RAT	P18420	Proteasome subunit alpha type-1	99	2.60E-06
PSA5_MOUSE	Q9Z2U1	Proteasome subunit alpha type-5	70	0.0019
PYC_RAT	P52873	Pyruvate carboxylase, mitochondrial	174	7.80E-14
RB11B_MOUSE	P46638	Ras-related protein Rab-11B	65	0.0058
REM1_MOUSE	O35929	GTP-binding protein REM 1	56	0.047
SCOT_MOUSE	Q9D0K2	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial	77	0.00038
SPA3K_RAT	P05545	Serine protease inhibitor A3K	154	7.80E-12

SwissProt ID	Accession Number	Protein Name	Mascot Score	Expect Value
SUOX_RAT	Q07116	Sulfite oxidase, mitochondrial	152	1.20E-11
TBB2C_MOUSE	P68372	Tubulin beta-2C chain	74	0.00075
TBX3_RAT	Q7TST9	T-box transcription factor TBX3	56	0.051
TCPE_RAT	Q68FQ0	T-complex protein 1 subunit epsilon	103	9.90E-07
TCPG_MOUSE	P80318	T-complex protein 1 subunit gamma	56	0.048
TPIS_RAT	P48500	Triosephosphate isomerase	170	2.00E-13
TPM1_MOUSE	P58771	Tropomyosin alpha-1 chain	166	4.90E-13
TPM2_MOUSE	P58774	Tropomyosin beta chain	88	2.90E-05
TPM3_MOUSE	P21107	Tropomyosin alpha-3 chain	70	0.0019
TRFE_RAT	P12346	Serotransferrin	219	2.50E-18
UBE1X_MOUSE	Q02053	Ubiquitin-like modifier-activating enzyme 1 X	81	0.00015
UCRI_RAT	P20788	Cytochrome b-c1 complex subunit Rieske, mitochondrial	103	9.90E-07
VINC_MOUSE	Q64727	Vinculin	248	3.10E-21
VPS41_MOUSE	Q5KU39	Vacuolar protein sorting-associated protein 41 homolog	66	0.0051
VTDB_RAT	P04276	Vitamin D-binding protein	151	1.60E-11

Table 1. Spots from mitochondrial and post-mitochondrial cytoplasm 2D-gels were subjected to trypsin digestion, MALDI-TOF MS and Mascot™ PMF analyses, as described, to build a roadmap of identified protein spots across the gels. The SwissProt rat proteomic database was used, except in circumstances where a database assignment could not be made, in which case the mouse proteomic database was used. Shown are the SwissProt ID and accession numbers, the gene names, the Mascot™ scores and expect values for each protein hit. The threshold for assignments was drawn at an expect value of 0.05, corresponding to a false discovery rate of 5%.

Table 2. Summary of Observed Nitrite-Dependent Proteomic Alterations

Name of Protein (or Family)	Observed Nitrite-Dependent Alteration	Figure and Spot of Reference	Apparent Expression				Method of Analysis
			Control	0.1 mg/kg	1 mg/kg	10 mg/kg	
Protein Disulfide Isomerase A3	Migration in pI	Fig. 3Bi: spot a	+++	+	++	++	2D-PAGE, gel staining, PMF analysis
		Fig. 3Bi: spot b	++++	+	++++	++	
		Fig. 3Bi: spot c	+	++	++	++	
		Fig. 3Bi: spot d	++	++++	++++	++++	
Ubiquinone Biosynthesis Protein CoQ9	Migration in pI	Fig. 3Bii: spot a	++	+	+++	+++	2D-PAGE, gel staining, PMF analysis
		Fig. 3Bii: spot b	+++	++	++++	++++	
		Fig. 3Bii: spot c	+	++	++	+	
		Fig. 3Bii: spot d	+++	++++	++++	+	
Aldehyde Dehydrogenase 2	Migration in pI	Fig. 3Biii: spot a	+	+++	++++	+++	2D-PAGE, gel staining, PMF analysis
		Fig. 3Biii: spot b	++	++	+++	++	
		Fig. 3Biii: spot c	++++	++++	++++	++++	
		Fig. 3Biii: spot d	++	++	+++	++	
		Fig. 3Biii: spot e	++++	++	+++	+++	
Myosin Light Chain Isoforms	Migration in pI	Fig. 4A: spot a	++	++	++	ˆ+	2D-PAGE, gel staining, PMF analysis
		Fig. 4A: spot b	++	++	++	+++	
		Fig. 4A: spot c	+++	+++	+++	++++	
		Fig. 4A: spot d	++	++	++	++	
		Fig. 4A: spot e	++	++	++	++	
		Fig. 4A: spot f	+++	++	+++	++++	
		Fig. 4A: spot g	+++	++	++	++	

Name of Protein (or Family)	Observed Nitrite-Dependent Alteration	Figure and Spot of Reference	Apparent Expression				Method of Analysis
			Control	0.1 mg/kg	1 mg/kg	10 mg/kg	
Cardiac α-actin	Migration in pI	Fig. 4B: spot a	+++	+	+++	+++	2D-PAGE, gel staining, PMF analysis
		Fig. 4B: spot b	+++	++	+++	+++	
		Fig. 4B: spot c	+++	++++	+++	+++	
		Fig. 4B: spot d	+++	++++	++++	+++	
		Fig. 4B: spot e	+++	+++	+++	+++	
Lactate Dehydrogenase B	Nitration	Fig. 4C: spot a	++	+++	++++	++++	2D-PAGE Western, gel staining, PMF analysis
Pyruvate Dehydrogenase Complex Component E2	Nitration	Fig. 4C: spot b	+++	++	+++	+++	2D-PAGE Western, gel staining, PMF analysis
Cardiac α-actin	Nitration	Fig. 4C: spot c	+++	++	+++	+++	2D-PAGE Western, gel staining, PMF analysis
Unidentified	Nitration	Fig. 4C: spot d	+++	+	++	+++	2D-PAGE Western, gel staining, PMF analysis
Unidentified	Nitration	Fig. 4C: spot e	+	+	++	+++	2D-PAGE Western, gel staining, PMF analysis

Name of Protein (or Family)	Observed Nitrite-Dependent Alteration	Figure and Spot of Reference	Apparent Expression		Method of Analysis
			Control	Pooled Nitrite Treated	
Filamin-C	Phosphorylation	Fig. 5A	+	+++	Phospho-peptide enrichment, LC-MS
cAMP-dependent Protein Kinase	Phosphorylation	Fig. 5B	+	+++	Phospho-peptide enrichment, LC-MS
Serine/threonine Protein Phosphatase 2A	Phosphorylation	Fig. 5C	+	+++	Phospho-peptide enrichment, LC-MS

Table 2. Nitrite-treatment dependent proteomic alterations observed in this study. Given are names of proteins or protein families (when precise protein isoform was not distinguished), observed alterations, references to figures and gel spots upon which these observations were made, apparent relative changes in expression levels of each of these species, and the methods of analysis that were used in each case. Relative expression levels: +, low to undetectable; ++, medium-low; +++, medium-high; ++++, high.