SUPPLEMENTAL INFORMATION - Mitochondrial nitro-alkene formation and mild uncoupling in ischemic preconditioning: implications for cardioprotection

EXPERIMENTAL PROCEDURES

Animals, Chemicals & Reagents: Male Harlan Sprague-Dawley rats (200-250g) were housed under the recommendations of the *NIH Guide for the Care and Use of Laboratory Animals* (NIH publication #85-23, 1996), in a 12 hr. light/dark cycle with food and water available *ad libitum*. All chemicals were of the highest grade available from Sigma (St. Louis, MO) unless otherwise stated.

Pharmacologic agents were added to experiments dissolved in vehicle solvents as follows, with the concentrations of stock solutions and the final amounts (in %) of vehicle solvent added, listed in parentheses: In ethanol: ODQ (10 mM, 0.1%), cPTIO (30 mM, 0.166%), oligomycin (1 mg/ml, 0.1%). In methanol: LA (1-20 mM, 0.1%), LNO_2 (1-20 mM, 0.1%), E-SH (20 mM, 0.1%). In DMSO: CsA (5 mM, 0.04%), Glybenclamide (4 mM, 0.05%), Wortmannin (100 μM, 0.1%), UO126 (20 mM, 0.05%). In Krebs-Henseleit Buffer: 5-HD (300 mM, 0.1%). In mitochondrial proton leak assay buffer: GDP (100 mM, 1%), Genipin (10mM, 0.5%), BHT (400mM, 0.25%). In H_2O: CATr (1.5 mM, 0.33%), BKA (10 mM, 0.2%). The concentration of organic solvents (ethanol, methanol, DMSO) never exceeded 0.16%, and for all other reagents the stock solutions were prepared in the same buffer as experiments were performed in, or in pure H_2O, thus solvent addition did not pose any problems.

All procedures were performed in subdued light, purged with argon to prevent lipid oxidation. LNO_2 was synthesized from linoleic acid (LA), purified and quantified as previously [1, 2]. LNO_2 was solvated in CH_3OH and stored under argon at $^{-}80$ °C for up to 1

month, with fresh aliquots used for each experiment. Biotinylated LNO_2 (Bt-LNO_2) was synthesized and purified as previously [3]. All procedures were performed under subdued lighting conditions.

Mitochondrial Isolation & Measurements: Rat heart mitochondria were isolated and protein concentration measured as previously [4]. To determine mitochondrial H^+ leak, state 4 respiration rate and $\Delta\psi_m$ were measured using O_2 and $TPMP^+$ electrodes respectively, and succinate-energized respiration was titrated with malonate, as previously [4]. Fat-free BSA (0.25 % w/v) was present to chelate free fatty acids, nigericin was omitted to prevent H^+ leak secondary to K^+ fluxes, and oligomycin (1μg/ml) was present to enforce state 4 [4]. When present, LNO_2 (1-5 μM) was added 1 min. prior to energizing mitochondria. Optional inhibitors used were: 1 mM guanosine diphosphate (GDP) to inhibit uncoupling proteins (UCPs), 5 μM carboxyatractyloside (CATr, Calbiochem, San Diego CA) to inhibit adenine nucleotide translocase (ANT). Mitochondrial PT pore opening: was measured as the swelling-induced decrease in light scattering at 540 nm, in a Beckman DU800 spectrophotometer [5]. Mitochondria were suspended at 0.5 mg/ml protein in buffer comprising 120 mM KCl, 3 mM KH_2PO_4, 50 mM Tris, 5 mM succinate, and 1 μM rotenone, pH 7.35 at 37˚C. Swelling was initiated by addition of either $CaCl_2$ (100 μM) or LNO_2 (1-20 μM). Identity of the PT pore was verified by including 2 μM cyclosporin A (CsA) in incubations. In all mitochondrial experiments, non-nitrated linoleate (LA) served as a control. Mitochondrial state 4 respiration (energized with succinate) was also measured in the presence of oligomycin, as a surrogate marker for mitochondrial H^+ leak. The following inhibitors were used where indicated: 1mM GDP, 50 μM Genipin (Wako, Richmond VA) [6], 20 μM ethanethiol (E-SH),

30 μM 2-(4-Carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (c-PTIO, Axxora, San Diego CA), or 1 mM 2,6-di-tert-butyl-4-methyl-phenol (BHT).

Cardiomyocyte Isolation, Simulated IR (SIR) Injury, & Cellular Measurements: Ca^{2+} tolerant adult rat ventricular cardiomyocytes were isolated as previously [7], yielding ~$4x10^6$ cells/heart, >85% rod-shaped and excluding Trypan blue. $5x10^5$ cells were aliquoted in 5 ml of Krebs-Henseleit buffer plus 2% (w/v) BSA (KHB), in a shaking water bath (120 cycles/min.), and were treated under the following protocols: (i) Normoxia, comprising 1.5 hr. gassed with 95% O_2 plus 5% CO_2, pH 7.4; (ii) Simulated IR (SIR), comprising 1 hr. gassed with 95% N_2 plus 5% CO_2 in glucose-free KHB, pH 6.5, followed by 30 min. in KHB gassed with 95% O_2 plus 5% CO_2, pH 7.4; (iii) SIR plus LNO_2 or LA (0.25-5 μM) added 20 min. before hypoxia. (iv) SIR plus LNO_2 (as in iii), plus inhibitors of either soluble guanylate cyclase mitochondrial K_{ATP} channels, extracellular signal regulated kinase, or phosphoinositide 3 kinase. To switch conditions, cells were centrifuged (31 x *g*, 2 min.) and pellets resuspended in appropriate buffers. Thus, added components (LNO_2 etc.) that did not enter cells were nominally absent from extracellular media during ischemia. An exception to this was applied in the case of wortmannin and UO-126, which were also added to both the hypoxia and reperfusion incubation buffers. At the end of all protocols, cell viability was measured by Trypan blue exclusion. The Trypan blue assay can sometimes overestimate cell viability because mitochondria remain viable inside cells scored as dead [7]. Thus, post-SIR recovery of intra-cellular mitochondrial function ($\Delta\psi_m$) was also measured.

Cellular state 4 respiration was measured at 37°C using an O_2 electrode [7], with 10^5 cells suspended in 1 ml KHB plus oligomycin (1 μg/ml). After stabilization of respiration rate, LNO_2 or LA (0.25-5 μM) was added. At the end of incubations KCN (1

mM) was added to inhibit respiration and correct for non-mitochondrial O_2 consumption. To measure intracellular $\Delta\psi_m$, cells (2 x 10^5 in 2 ml KHB) were loaded with 20 nM TMRE (Molecular Probes, Eugene OR) for 20 min. at 37°C, with fluorescence (λ_{ex} 555 nm, λ_{em} 577 nm) measured throughout using a Cary Eclipse fluorimeter (Varian, Australia). Uncoupler (FCCP, 5 μM) was then added to dissipate $\Delta\Psi_m$, and Δ fluoresence recorded [7].

Langendorff Perfused Heart: Hearts were perfused as previously [4, 7], and subjected to one of the following protocols: (i) 45 min. normoxic perfusion, (ii) IPC comprising 20 min. equilibration followed by 3 x 5 min. ischemia interspersed with 5 min. reperfusion, (iii) Ischemia comprising 20 min. normoxic equilibration followed by 25 min. global ischemia, (iv) IPC plus ischemia, comprising 20 min. normoxic equilibration, then IPC (3 x 5 min) as in protocol (ii), then ischemia (25 min.) as in protocol 3. Ischemia was attained by stopping flow to the aortic cannula and submerging the heart in degassed Krebs-Henseleit buffer at 37°C. Mitochondria were immediately isolated at the end of each perfusion protocol. Experiments were also performed in which the $NO^{\bullet}$ synthase (NOS) inhibitor L-NAME (L-nitro-arginine methyl ester, 100μM) was present in the perfusion medium for 5 min. prior to and throughout IPC (i.e. protocol ii plus L-NAME).

Lipid Extraction & Detection by LC-MS: Lipids were extracted from 5 mg mitochondrial protein as previously [8]. Synthetic $[^{13}C_{18}]LNO_2$ internal standard (500 pg) was added to all samples to correct for any loss of analyte due to extraction. Nitroalkenes were detected by HPLC electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) using an Applied Biosystems API 5000 triple quadrupole mass spectrometer coupled to a Shimadzu Prominence HPLC system [2]. HPLC separations were performed on a C_{18} reverse phase column (150 x 2 mm, 3 μm particle size) with a mobile

phase consisting of **A** (0.1% acetic acid in H_2O) and **B** (0.1 % acetic acid in ACN). Nitroalkenes were resolved from the column using the gradient program: 45% **B** for 1 min., 45-80% **B** over 44 min., 80-100% **B** over 1 min., 100% **B** for 7 min., 100-45% **B** over 6 s., 45% **B** for 10 min. to re-equilibrate the column. Mass spectrometric analysis was performed in negative ion mode, using multiple MS scan modes including multiple reaction monitoring (MRM), product ion analysis (MS/MS), neutral loss and precursor ion loss. Based on product ion spectra of synthetic standards, optimal MRM transitions for LNO_2 and Bt-LNO_2 were identified as *m/z* 324/46 and 564/517, respectively. Biologically-derived nitroalkene derivatives of LNO_2 were identified by their retention time, precursor ion mass, MS/MS fragmentation pattern, and thiol reactivity [2]. Quantitation was achieved using $[^{13}C_{18}]LNO_2$ as internal standard during extraction and monitoring for the MRM transition *m/z* 342/46 during analysis. Peak area ratios between analyte and internal standard were determined and applied to an internal standard curve. Concentration of analyte was calculated using Analyst 1.4.2 quantitation software (Applied Biosystems).

Immunoprecipitation & Western Blotting: Bt-LNO_2 treated mitochondria (1 mg protein) or cardiomyocytes (10^5) were extracted as previously [4], and extracts incubated for 2 hr. at 4 °C with 20 µl of a 50% slurry of neutravidin-agarose (Pierce, Rockford IL). Beads were pelleted and washed as previously [4], then samples were separated by electrophoresis on 12% SDS-PAGE gels, blotted to nitrocellulose, and membranes were probed with antibodies against Biotin (Pierce), ANT (Santa-Cruz Biotech, Santa Cruz, CA) or UCP-2 (Santa-Cruz, or ADI, San-Antonio TX). In simple western-blot experiments, samples were extracted directly into SDS-PAGE loading buffer. Blots were developed using HRP-linked secondary antibodies, and enhanced chemiluminescence (GE

Biosciences). Additional western blots (supplemental data) were performed using antibodies against the 70kDa subunit of mitochondrial complex II (MitoSciences, Eugene OR), glycogen synthase kinase 3α (GSK-3α, Cell Signaling Technology, Danvers MA), GSK-3β (BD-Transduction Laboratories, San Jose CA), or the serine-9 phosphorylated form of GSK-3β (Cell Signaling Technology).

Statistics: All experiments were performed 4-8 times, with N referring to independent mitochondria, cell or heart preparations (separate animals). Significance between groups was established using ANOVA.

RESULTS

Figure 6 of the main manuscript presents evidence supporting the modification of both ANT and UCP-2 by LNO_2. The data in Fig. 6C and 6D also show that a wide variety of proteins are modified by LNO_2. While complete characterization of the nitroalkylation proteome is underway, and is beyond the scope of the current manuscript, we have used western blotting to elucidate several additional targets for LNO_2 modification during the course of unrelated studies. Several such proteins are shown in Supplement Figure 1. Panels 1A—1C show western blots performed on biotin immuprecipitates of Bt-LNO_2 treated mitochondria or cardiomyocytes. Panel A shows that both the 70 kDa subunit of complex II (succinate dehydrogenase, inner membrane) and the voltage dependent anion channel (VDAC) of the mitochondrial outer membrane are pulled down by Bt-LNO_2, suggesting these proteins are modified by nitroalkylation. Panel B shows that GSK-3β but not GSK-3α was also pulled down by Bt-LNO_2, again suggesting nitroalkylation of the former. The phosphorylation and inhibition of GSK-3β has been heavily implicated in cell signaling during IPC, and thus it was decided to investigate whether LNO_2 affects GSK-3β

phosphorylation status. Panel C shows that doses of LNO_2 from 0.5 to 5.0 μM did not affect either the level of GSK-3β or its phosphorylation status. The effects of LNO_2 on the activity of GSK-3β are currently unknown, although to date the only known mechanism for regulation of this enzyme is by phosphorylation. Thus it is unlikely that the cardioprotective efficacy of LNO_2 can be assigned to GSK-3β phosphorylation and inhibition.

In the main manuscript, Figure 6G showed a western blot for UCP-2, in mitochondria treated with varying doses of LNO_2, in the presence or absence of the UCP inhibitor GDP. LNO_2 dose-dependently depleted UCP-2 from the blot, and this was somewhat prevented by GDP. There could be several explanations for this phenomenon, including: (i) LNO_2 modification of UCP-2 leading to its degradation (e.g. by proteolysis) in the sample prior to analysis, (ii) LNO_2 modification of UCP-2 rendering the protein epitope unrecognizable by the antibody, or (iii) LNO_2 modification of UCP-2 rendering the protein more hydrophobic, thereby preventing its migration in SDS-PAGE. Hypothesis (ii) is precluded by the finding that multiple antibodies against different regions of UCP-2 gave the same result (data not shown). Support for hypothesis (iii) is presented in Supplemental Figure 1D. This panel shows a similar western blot to Figure 6G of the main manuscript, except that the entire gel including the stacker and comb was blotted, thus preserving any proteins that did not migrate properly on the gel. The results show that increasing doses of LNO_2 led to a loss of UCP-2 from the main gel at the molecular weight of 32 kDa (c.f. Fig. 6G), in parallel with an accumulation of UCP-2 immuno-reactivity at the border of the stacking and separating gels. It is therefore concluded that LNO_2 modification of UCP2 may increase its hydrophobicity and thus decrease its

motility in SDS-PAGE. The implications of such a finding for the field of protein modification by reactive lipids are profound; attempts to characterize the electrophilic lipid reactive proteome may have underestimated the total number of target proteins, because the modification itself may render proteins incompatible with common proteomic separation technologies (i.e., 2D gels).

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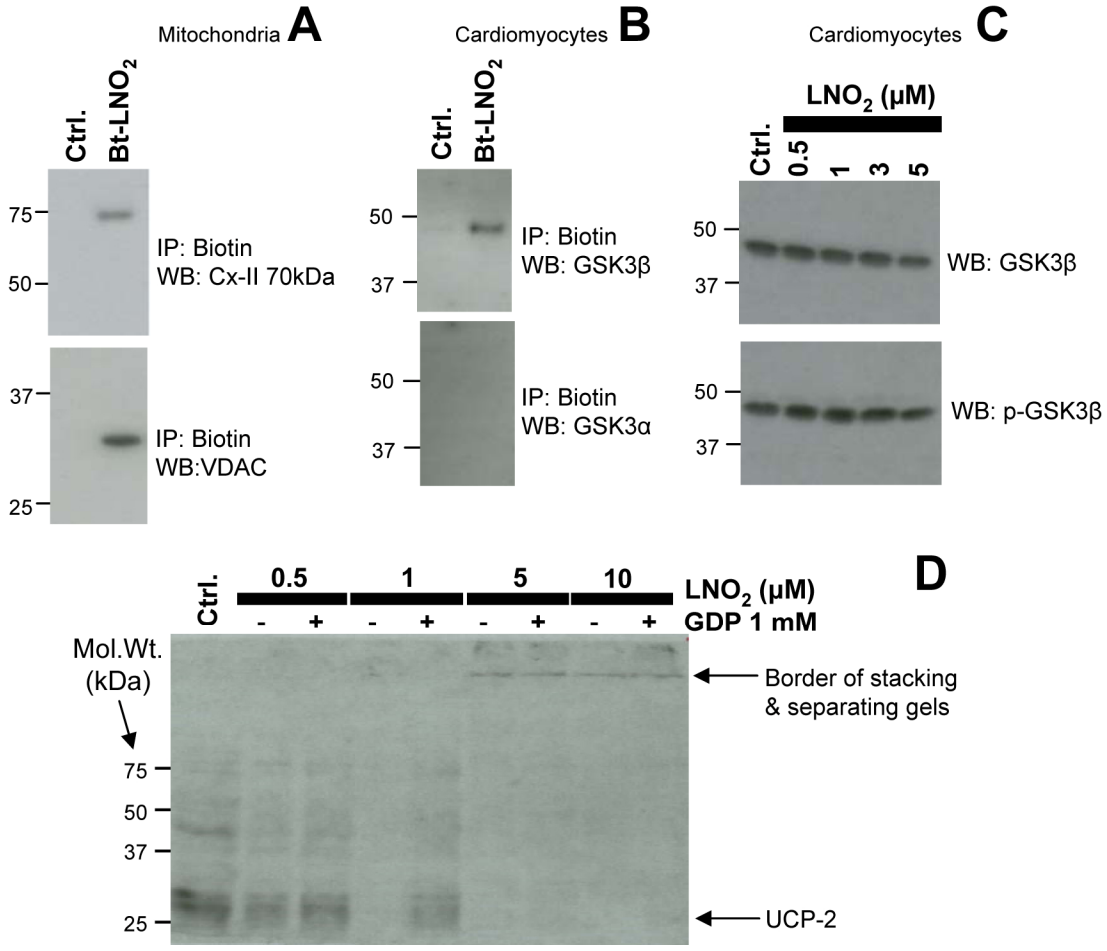
FIGURE LEGEND:

Supplement Figure 1: Identification of additional LNO_2 target proteins by western blotting. (A,B): Mitochondria or cells were treated with 1 μM Bt-LNO_2 for 5 min., proteins were immunoprecipitated using neutravidin-agarose beads, and western blotted with antibodies against complex II 70 kDa subunit, VDAC, GSK-3α and GSK-3β, as described in the methods (c.f. Fig. 6C—6G). Blots are representative of at least 3 independent experiments. **(C):** Cardiomyocytes were treated with the indicated doses of LNO_2 for 5 min. followed western blotting for GSK-3β or the serine-9 phosphorylated form of GSK-3β. **(D):** Mitochondria were treated with the indicated doses of LNO_2, in the presence or absence of 1 mM GDP, followed by western blotting for UCP-2 (c.f. Fig. 6G). The entire gel including stacker and comb were blotted, and the interface between the stacker and separating gels is indicated at right. Additional bands at non-native molecular weight for UCP-2 are thought to be due to non-specific antibody reactivity.


Mitochondria A
Ctrl.
Bt-LNO2
75
50
IP: Biotin
WB: Cx-II 70kDa
37
25
IP: Biotin
WB:VDAC
Cardiomyocytes B
Ctrl.
Bt-LNO2
50
37
IP: Biotin
WB: GSK3β
50
37
IP: Biotin
WB: GSK3α
Cardiomyocytes C
LNO2 (μM)
Ctrl.
0.5
1
3
5
50
37
WB: GSK3β
50
37
WB: p-GSK3β
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LNO2 (μM)
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GDP 1 mM
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(kDa)
75
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37
25
Border of stacking
& separating gels
UCP-2