Research paper

Fatty acid nitroalkenes induce resistance to ischemic cardiac injury by modulating mitochondrial respiration at complex II

Jeffrey R. Koenitzer [a], Gustavo Bonacci [a], Steven R. Woodcock [a], Chen-Shan Chen [a], Nadiezhda Cantu-Medellin [b], Eric E. Kelley [b], Francisco J. Schopfer [a,*]

[a] Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA 15261, USA
[b] Department of Anesthesiology, University of Pittsburgh, Pittsburgh, PA 15261, USA

ARTICLE INFO

ABSTRACT

Nitro-fatty acids (NO_2-FA) are metabolic and inflammatory-derived electrophiles that mediate pleiotropic signaling actions. It was hypothesized that NO_2-FA would impact mitochondrial redox reactions to induce tissue-protective metabolic shifts in cells. Nitro-oleic acid (OA-NO_2) reversibly inhibited complex II-linked respiration in isolated rat heart mitochondria in a pH-dependent manner and suppressed superoxide formation. Nitroalkylation of Fp subunit was determined by BME capture and the site of modification by OA-NO_2 defined by mass spectrometric analysis. These effects translated into reduced basal and maximal respiration and favored glycolytic metabolism in H9C2 cardiomyoblasts as assessed by extracellular H^+ and O_2 flux analysis. The perfusion of NO_2-FA induced acute cardioprotection in an isolated perfused heart ischemia/reperfusion (IR) model as evidenced by significantly higher rate-pressure products. Together these findings indicate that NO_2-FA can promote cardioprotection by inducing a shift from respiration to glycolysis and suppressing reactive species formation in the post-ischemic interval.

1. Introduction

Nitric oxide (•NO) and nitrite (NO_2^-)-mediated inflammatory reactions promote the generation of nitrogen dioxide (•NO_2), that in turn nitrates unsaturated fatty acids to form electrophilic nitroalkene derivatives (NO_2-FA) [1]. The β-carbon of the reactive nitroalkenyl substituent of NO_2-FA readily undergoes reversible Michael addition with functionally significant Cys and His residues

on transcription regulatory proteins and enzymes. In aggregate, the post-translational protein modifications (PTM) by low concentrations of NO_2-FA and other biological electrophiles promote adaptive signaling responses in a variety of metabolic and inflammatory disease models, including myocardial ischemia/reperfusion (IR) injury [1–4].

By virtue of their intrinsic reactivity, electrophilic fatty acids both activate and inhibit transcriptional regulatory programs and acutely alter protein catalytic activity (e.g., glyceraldehyde 3-phosphate dehydrogenase, xanthine oxidoreductase, matrix metallo-proteinases) [5–7]. Inflammatory and metabolic gene expression is modulated by NO_2-FA reaction with the redox-sensitive transcriptional regulatory factors Kelch-like ECH-associated protein 1/Nuclear factor (erythroid-derived 2)-like 2 (Keap1/Nrf2), p65 subunit of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and peroxisome proliferator activating receptor gamma (PPARγ) [6–8]. These Michael addition reactions are in turn modulated by a) reactions with low molecular weight nucleophiles such as glutathione (GSH) and hydrogen sulfide (H_2S) and b) reduction of the reactive nitroalkene by prostaglandin reductase-1 [9–12].

The multiplicity of protein targets indicates that pleiotropic signaling mechanisms underlie the actions of NO_2-FA and motivates the identification of functionally-significant molecular targets of biological electrophiles. Mitochondria provide a platform

and substrate for fatty acid nitration and thus may be significant in manifesting downstream responses to NO_2-FA-induced PTMs [1,13]. Respiratory chain components autoxidize to yield superoxide ($O_2^{\bullet-}$) and subsequently hydrogen peroxide (H_2O_2) resulting from spontaneous or enzymatically-catalyzed dismutation. This process is amplified during tissue IR events, promoting increased levels of secondary reactive species [14]. For example, the hydrophobic nature and small molecular radius of $\bullet$NO allows this radical species to readily diffuse into mitochondria, where it can either directly modulate respiratory chain function or react with $O_2^{\bullet-}$ to yield the nitrating species peroxynitrite ($ONOO^-$) [15–17]. In addition, mitochondrial NO_2^- can undergo either reduction to $\bullet$NO or mediate nitrosation and nitration reactions via dinitrogen trioxide or $\bullet NO_2$ formation respectively [18–21]. Nitrogen dioxide undergoes rapid radical addition reactions with abundant mitochondrial conjugated linoleic acid present in cardiolipin, yielding both oxidized and nitrated products [22].

Formation of nitrated fatty acids has been reported in cardiac mitochondria following cycles of hypoxia-reoxygenation that induce ischemic preconditioning [13]. In addition, the organelle supports the β-oxidation of NO_2-FA to electrophilic dinor and tetranor product later detected in plasma and urine [23]. The role of the mitochondria in cardiac IR injury remains an active area of discovery and a promising pharmacological target for cardioprotection [24]. In this regard, NO_2-FA-reactive mitochondrial protein targets have already been identified, including the mitochondrial K (ATP) channel and the adenine nucleotide transporter-1 channel [25,26].

Herein, complex II is established as a sensitive, reversible, and functionally-significant target of NO_2-FA reaction in cardiac mitochondria, resulting in respiratory inhibition, enhancement of glycolysis, suppression of superoxide production and the induction of acute cardioprotection in an isolated perfused rat heart model of global IR.

2. Experimental procedures

2.1. Materials

Nitro-oleic ($OA-NO_2$) and nitro-linoleic acid ($LA-NO_2$) were synthesized as previously [27]. Fatty acids were obtained from NuCheck (Elysian, MN) and other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Animals were housed in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication no. 85-23, revised 1996). All rodent and clinical studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Approval 12070398).

2.2. Mitochondrial isolation and respirometry

Fresh mitochondria were obtained from rat hearts. Following anesthesia with isoflurane, hearts were rapidly excised from male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) and placed into cold mitochondrial isolation buffer (10 mM HEPES, 250 mM sucrose, 0.2 mM EDTA). Hearts were minced into $\sim$2 mm fragments, then placed in clean isolation buffer, washed on filter paper and moved to a fresh beaker containing isolation buffer and 0.1% trypsin (dissolved in 1 mM HCl). After stirring for 5 min, tissue was homogenized with a Dounce homogenizer. Following addition of protease inhibitor to the homogenate, it was centrifuged at $600 \times g$ and the pellet discarded. The supernatant was retained and centrifuged at $8000 \times g$, the pellet washed with isolation buffer and again centrifuged at $8000 \times g$. The pellet was resuspended in a minimal volume ($\sim$500 μl) of isolation buffer to yield $\sim$20 mg/ml protein. For respirometry studies, this preparation was diluted to 0.5 mg/ml in respiration buffer (120 mM KCl, 25 mM sucrose, 10 mM HEPES, 1 mM EGTA, 1 mM KH_2PO_4, 5 mM $MgCl_2$) at pH 7.5 in a stirred chamber containing a Clark-type O_2 electrode. Respiratory control ratios (RCR) were determined with 8 mM malate/4 mM glutamate as substrates for state 4 respiration and 10 mM ADP was added to initiate state 3 respiration. Preparations with RCR < 4 were discarded. $OA-NO_2$ and oleic acid (OA) were diluted from methanol stocks and incubated with mitochondria for 10 min before addition of state 4 substrates (either 8 mM glutamate/4 mM malate or 8 mM succinate). O_2 concentration in the chamber was recorded for 15 min and maximal rates were determined from the linear portion of the curve after substrate addition. When mitochondria were used following freeze-thaw, oxidized cytochrome c ($cyt\ c$)(50 μM) was added just before substrate. FCCP (500 nM) was added 5 min prior to $OA-NO_2$ addition.

2.3. Measurement of respiratory chain complex activities

Rat heart mitochondrial preparations ($\sim$20 mg/ml) were subjected to one cycle of freeze thaw and measurements were made using 50 μg mitochondrial protein in 10 mM HEPES buffer containing 2.5 mM $MgCl_2$ and 2 mM KCl. Complex II activity was determined by following the decrease in absorbance at 600 nm (DCPIP, ε 19.1 $mM^{-1}\ cm^{-1}$) in buffer supplemented with 0.1 mM EDTA, 75 μM DCPIP, 50 μM decylubiquinone and 20 mM succinate [28]. Complex II+III activity was determined in the presence of 20 mM succinate, 50 μM decylubiquinone and 50 μM oxidized $cyt\ c$, by following the increase in absorbance at 550 nm (reduced $cyt\ c$ ε 21 $mM^{-1}\ cm^{-1}$) [29]. To determine the effect of NO_2-FA treatment on complex activity, mitochondria were incubated with $OA-NO_2$ or OA at pH 9.0 for 10 min, centrifuged at $14,000 \times g$, pellet resuspended at pH 7.4, and complex activity determined under the conditions described above. To test reversibility of the inhibition, 1 mM BME was added for an additional 10 min before centrifugation.

2.4. Characterization of $OA-NO_2$ addition reactions to complex II and Fp subunit

Blue native gel electrophoresis resolution of mitochondrial respiratory complexes was performed as described [30] with minimal modifications. Briefly, 400 μg of rat heart mitochondrial protein was treated with 0 or 20 μM $OA-NO_2$, extracted with lauryl maltoside and separated by tricine-SDS-PAGE. The complex II band was excised, sliced into 2 mm segments, dehydrated in 200 μl ACN for 10 min at 25 °C, and gel pieces dried under vacuum. Trans nitroalkylation of $OA-NO_2$ was achieved by rehydration in 50 mM phosphate buffer (pH 8.0) containing 500 mM BME for 1 h at 37 °C. The reaction was quenched by adjusting pH to 5 with 1 M formic acid. BME-$OA-NO_2$ adducts were determined by LC/MSMS, following the neutral loss of BME (404.3–326.2 transition in negative ion mode) as described [31]. Fp-$OA-NO_2$ adducts were determined using denaturing gels using a similar procedure, with TCEP used as reducing agent to avoid reversal of $OA-NO_2$ modifications as previously described [31]. The identification of the 70-kDa subunit was performed by running a parallel Western blot. Fp subunit was detected using a mouse monoclonal antibody (1:2000 dilution, Mitosciences, Eugene, OR) and an HRP-conjugated anti-mouse Ig antibody (1:5000 dilution, Cell Signaling Technologies, Danver, MA).

2.5. OA-NO$_2$ alkylation target residues in complex II Fp subunit

10 µg recombinant human complex II Fp subunit (MyBioSource, San Diego, CA) was treated with methanol or OA-NO$_2$ (1 to 50-fold molar excess) for 30 min at 37 °C and digested with sequencing grade trypsin (1:50) in 50 mM phosphate buffer, pH 7.4 at 37 °C for 16 h. Tryptic fragments were separated using a reverse-phase column (ReproSil C18 column, 3 µm, 75 µm × 100 mm) and peptides eluted with a linear gradient of solvent B (0.1% formic acid in acetonitrile, 2–60% in 46.5 min) over solvent A (0.1% formic acid in water) at a 300 nl/min flow rate. Peptides were detected in the positive ion mode using a linear ion trap mass spectrometer (LTQ-XL, Thermo Electron Corp., San Jose, CA). The electrospray voltage was set at 2 kV and the capillary temperature was 200 °C. Peptide sequencing was performed using Proteome Discoverer 1.1.0 (Thermo Scientific).

2.6. Extracellular flux analysis in cardiomyoblasts

H9C2 cells (ATCC, Manassas, VA) were maintained in DMEM (Mediatech, Manassas, VA) with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 ng/ml streptomycin (Invitrogen, Carlsbad, CA), and used between passages 17 and 30. For extracellular flux (XF) analysis, cells were plated at 20,000 per well in specialized Seahorse 24-well plates and left to adhere and grow overnight. The next day media was exchanged for DMEM supplemented with 25 mM glucose, 4 mM L-glutamine, and 1 mM pyruvate (600 µl final volume per well) 90 min prior to the start of assay. Optimal concentration of each modulator was determined in concentration response experiments.

Superoxide determination by DHE oxidation to 2-OH-E$^+$. Hydroethidine (HE) was purchased from Invitrogen. The standards of 2-hydroxyethidine (2-OH-E$^+$), and ethidium (E$^+$) were prepared as previously described [32]. Mitochondria (0.5 mg/ml in 500 µl respiration buffer) were incubated with OA-NO$_2$ (0–20 µM) for 10 min before addition of 8 mM succinate and 20 µM HE. Reactions were incubated at 37 °C for 30 min, followed by precipitation of mitochondria and pellet storage at −20 °C. Generation of 2-OH-E$^+$ was quantified by HPLC coupled to an electrochemical detector as previously reported [32]. The separation of the oxidized products of HE was performed using an ether-linked phenyl column (Phenomenex, 100 mm × 4.6 mm, 2.6 µm), an elution gradient from 25% to 60% B over A in 10 min at a flow rate of 0.75 ml/min. The following solvents were used: solution A, 50 mM phosphate buffer in 90% water and 10% acetonitrile and solution B, 50 mM phosphate buffer in 40% water and 60% acetonitrile. The peak's corresponding area were determined and concentrations calculated using an external standard curve containing synthetic 2-OH-E$^+$ and E$^+$ standards.

2.7. Langendorff-perfused heart preparation and global IR

Hearts were rapidly excised from male Sprague-Dawley rats following induction of anesthesia with ketamine/xylazine (80 mg/kg and 5 mg/kg), and retrograde perfused as described [33]. The perfusate was KH buffer containing 20 mM glucose, 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO$_4$, 1.25 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, and 25 mM NaHCO$_3$, equilibrated with 95% O$_2$, 5% CO$_2$ gas. Coronary flow was held constant at 8–12 mL/min at 37 °C. Hearts were equilibrated for 30 min before insertion of a balloon to the left ventricle, which was inflated to allow left ventricular developed pressure (LVDP) to be measured by a force transducer. Hearts which failed to surpass exclusion criteria (outlined elsewhere [33]) were discarded. Then, 15 min after balloon insertion LA-NO$_2$ (100 nM final concentration) or vehicle (methanol, at 1/1000 final dilution) were infused for 15 min just above the heart using a syringe pump. At the end of this interval, hearts were subjected to 35 min of no-flow ischemia, maintained at 37 °C in the perfusate buffer. Flow was restored slowly to the original rate over 5 min to initiate reperfusion, and LVDP and heart rate recorded for 60 min.

2.8. Statistical analysis

Where appropriate, Student's *t* test or analysis of variance with Bonferroni post-test was applied to the data, with $p < 0.05$ used as the cutoff for significance.

3. Results

3.1. Nitro-fatty acids inhibit complex II-linked respiration in isolated mitochondria

To first define the effects of a fatty acid nitroalkene on overall respiratory metabolism, complex I- or II-linked respiration was measured in isolated rat heart mitochondria preincubated with OA-NO$_2$ or OA. No inhibition of respiration was observed by up to 10 µM OA-NO$_2$ concentrations in the presence of the complex I-linked substrates malate and glutamate (Fig. 1A). In contrast, significant inhibition (∼50%) of succinate-mediated respiration was observed at 3 µM OA-NO$_2$ (Fig. 1B). Similarly, incubation with OA-NO$_2$ inhibited State 3 inhibition when succinate was used as substrate (Fig. 1C).

Inhibition of complex II-linked O$_2$ consumption could be bypassed by the addition of ascorbate/TMPD, substrates which donate electrons to complex IV via cyt *c* (not shown), indicating that complex II is a target of OA-NO$_2$. To test if inhibition was dependent on coupling state, the effect of OA-NO$_2$ was tested on freeze-thawed mitochondria supplemented with cyt *c* (Fig. 1D) and after FCCP uncoupling (Fig. 1E). Both treatments blunted the inhibitory effect of the nitroalkene, indicating that the respiratory state modulates the OA-NO$_2$ inhibition of complex II-linked respiration.

3.2. Inhibition of complex II activity by OA-NO$_2$ is reversible and pH-dependent

Given that uncoupled mitochondria (FCCP, freeze-thaw) protected from respiratory inhibition by OA-NO$_2$, and that Michael addition reactions are favored under basic conditions that promote thiol ionization, it was proposed that matrix pH may control OA-NO$_2$ covalent binding to its target residue. The pH-dependence of OA-NO$_2$ inhibition on complex II+III activity was first assayed in mitochondria subjected to a freeze-thaw cycle, followed by addition of 10 µM OA-NO$_2$ or OA over a range of pH values. While OA-NO$_2$ did not alter activity at pH 6.5 relative to control, inhibition became significant with higher pH values, with a maximum inhibition of 82% reached at pH 9.0 (Fig. 2A).

To further characterize the inhibition and measure the complex activities at their optimal pH, thawed mitochondria were incubated with OA-NO$_2$ or OA at pH 9.0 (where substantial inhibition of complex II+III activity had been observed, Fig. 2A), sedimented by centrifugation, and resuspended in buffer at pH 7.4. Independent activity assays showed that complex II was significantly inhibited by OA-NO$_2$ (∼80%, Fig. 2B) while complex III activity remained unchanged (Fig. 2C), confirming complex II as the target of pH-dependent respiratory inhibition by OA-NO$_2$. The potent inhibition by OA-NO$_2$ of complex II activity strongly contrast the observed inhibition of oxygen consumption in whole mitochondria (OA-NO2 at 10 µM leads to ∼25% inhibition in FCCP-uncoupled mitochondria in Fig. 1E *versus* ∼45% in Fig. 2A). This apparent conflicting data most likely originates from complex II activity not being a limiting factor in mitochondrial oxygen
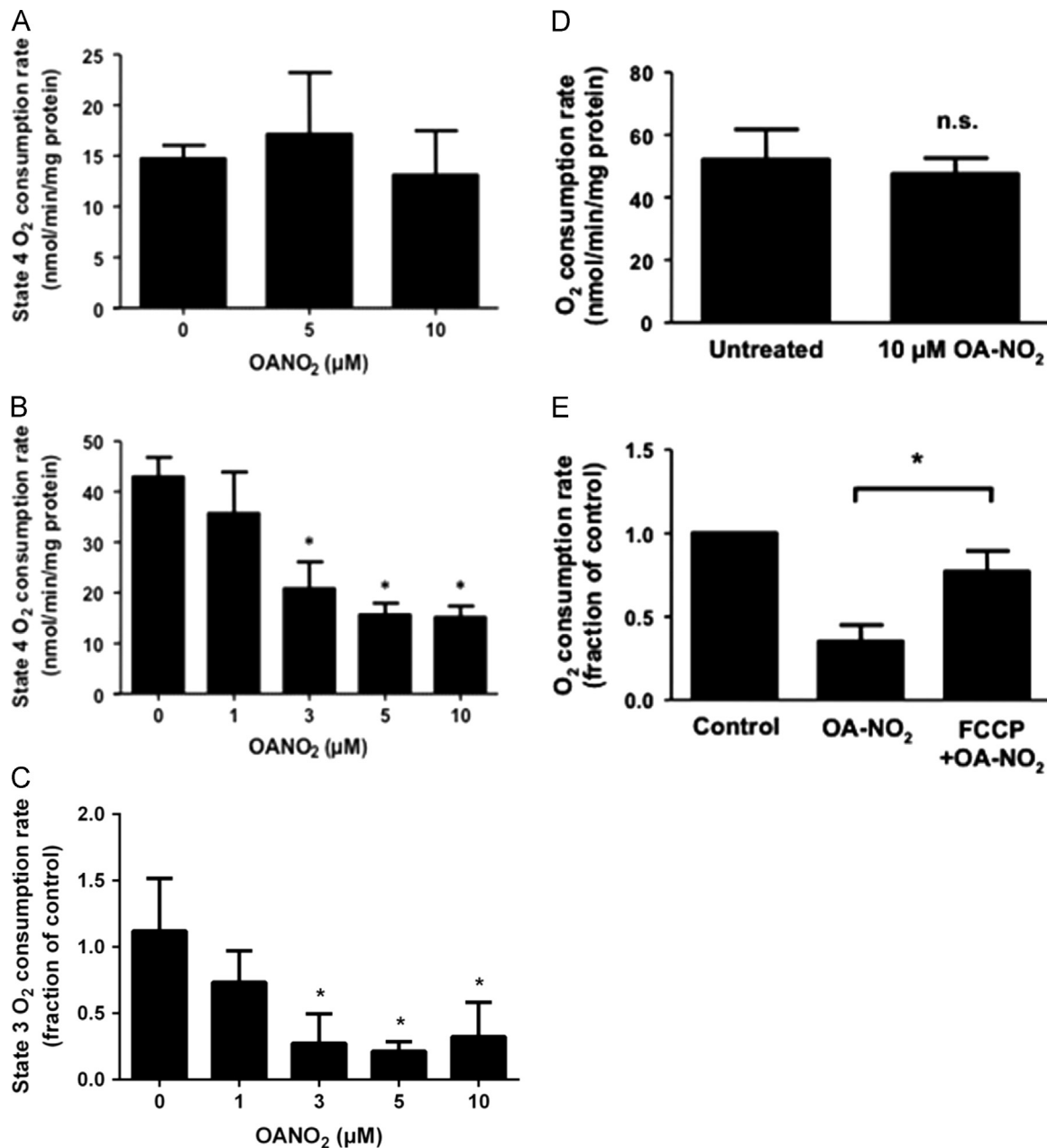
Fig. 1. OA-NO$_2$ inhibition of mitochondrial respiration. Rat heart mitochondria (0.5 mg) were incubated in respiration buffer (pH 7.5) for 5 min with OA-NO$_2$ (0–10 μM) prior to substrate addition. (A) Average O$_2$ consumption rates following addition of the complex I-linked substrates glutamate (8 mM) and malate (4 mM). (B) Average O$_2$ consumption rates following succinate (10 mM) addition. (C) Ratio of average State 3 O$_2$ consumption rates (OA-NO$_2$ *vs* OA) following succinate (10 mM) and ADP (10 mM) addition. (D) Uncoupling protects mitochondrial respiration from OA-NO$_2$ inhibition. Mitochondria were subjected to one cycle of freeze-thaw and incubated with OA-NO$_2$ (0–10 μM) in the presence of exogenous cyt *c* for 5 min prior to succinate (10 mM) addition. (E) FCCP (500 nM) protects from OA-NO$_2$ (10 μM) inhibition of succinate driven state 4 respiration. Values represent the mean $\pm$ SD of three independent experiments. $p < 0.05$, one-way ANOVA *vs* control.

consumption rates, contrasting the measurement of complex II − III activity.

NO$_2$-FA display signaling actions via reversible addition reactions to target Cys residues [7]. To determine the reversibility of nitroalkene inhibition of complex II, the restoration of activity was evaluated in the presence of β-mercaptoethanol (BME). Addition of this low molecular weight thiol to OA-NO$_2$–treated mitochondria fully reversed the loss of complex II+III activity to untreated control levels (Fig. 2D) while the concentration of BME had no effect on the respiratory activity of mitochondria treated with native OA (Fig. 2D). It was concluded that OA-NO$_2$ reversible inhibits complex II through Michael addition reactions and that mitochondrially-relevant pH changes modulate inhibition by OA-NO$_2$.

3.3. OA-NO$_2$ directly modifies the Fp subunit of complex II

To verify a Michael addition reaction between OA-NO$_2$ and complex II, a trans-nitroalkylation reaction to capture protein-adducted OA-NO$_2$ was first employed on intact complex II isolated following blue native electrophoresis [31]. No BME-OA-NO$_2$ adducts were observed in control conditions, while OA-NO$_2$-BME adducts from treated mitochondria were detected and co-eluted with synthetic standards (Fig. 3A and B). The complex II catalytic 70 kDa Fp subunit contains critical thiols that when modified modulate its activity [34–36]. To determine if this subunit of complex II was susceptible to OA-NO$_2$ addition, the Fp band was subjected to a trans-nitroalkylation reaction using BME as accepting nucleophile. The OA-NO$_2$ treatment resulted in dose-
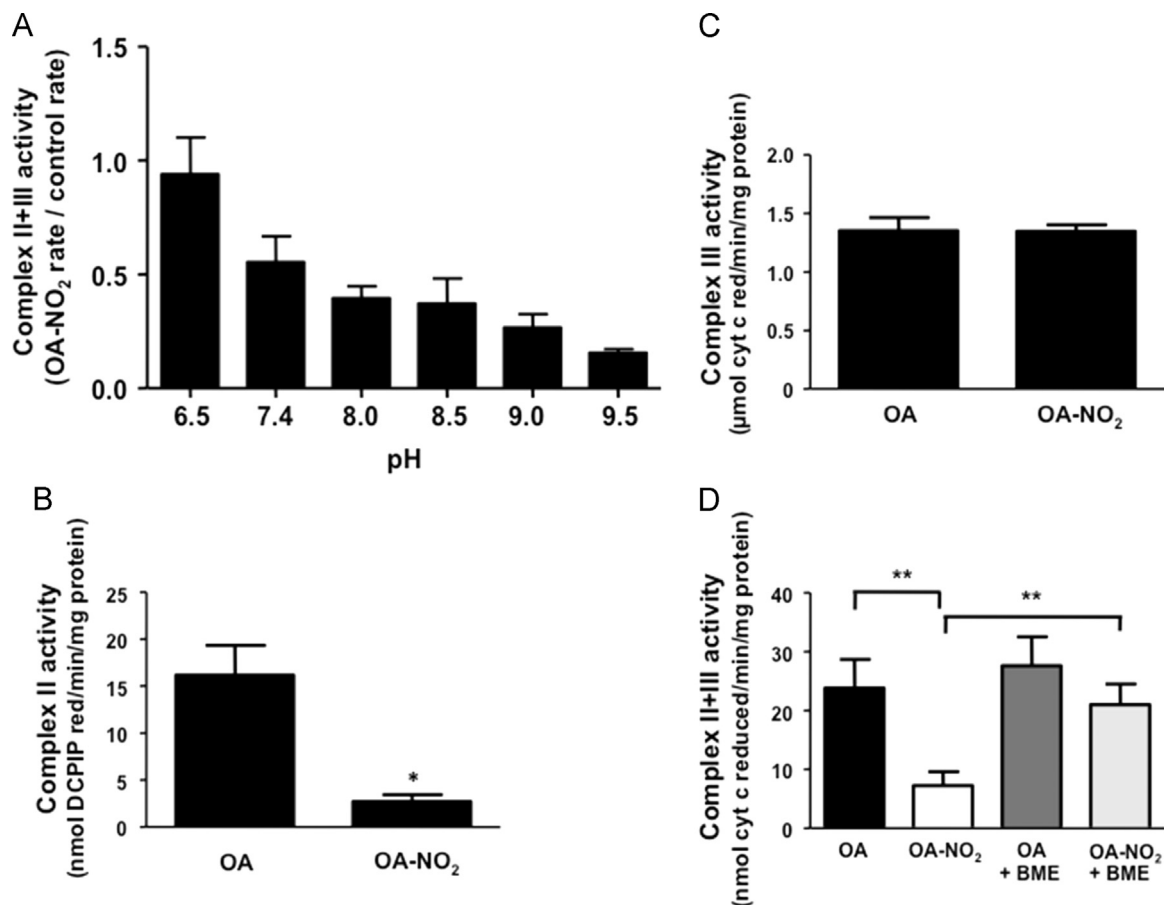
Fig. 2. pH and alkylation dependence of complex II inhibition by OA-NO$_2$. (A) Effect of pH on complex II+III activity (reduction of cytochrome c with succinate and decylubiquinone substrates added) of freeze-thaw mitochondria incubated with 10 μM OA or OA-NO$_2$. (B,C) Mitochondria incubated with OA or OA-NO$_2$ at pH 9.0 for 10 min were pelleted by centrifugation, resuspended and assayed for (B) complex II or (C) complex III activity. (D) Complex II inhibition by NO$_2$-FA is reversed by low molecular weight thiols. Mitochondria treated with OA or OA-NO$_2$ (10 μM) at pH 9.0 were subsequently incubated with $+/-1$ mM BME and complex II+III activity determined at pH 7.4. All data represent mean $\pm$ SD of three independent experiments; *$p < 0.05$ by Student's t test, two-tailed. **$p < 0.05$, one-way ANOVA.

dependent specific increases in BME-OA-NO$_2$ adducts that were absent in non-treated controls, indicating that complex II, and particularly the Fp subunit, is directly modified by OA-NO$_2$ (Fig. 3C and D).

3.4. Mass spectrometric analysis of the Fp subunit of complex II

To define specific reaction sites of OA-NO$_2$ within the Fp subunit, purified recombinant protein was trypsinized and peptides analyzed for adducts by mass spectrometry obtaining 64% sequence coverage. The analysis covered 11 of the 17 cys found in the 70 kDa Fp subunit. The OA-NO$_2$-treated digests revealed 7 OA-NO$_2$ target peptides (Table 1) identified by a 327.3 m/z increase and a characteristic shift in hydrophobicity and retention times when compared to native unmodified peptide. Two OA-NO$_2$ modifications corresponded to Cys residues and 5 to His residues (Table 1 and Supplementary data).

In particular all OA-NO$_2$-alkylated peptides displayed retention times ranging from 34 to 41 min while the corresponding native peptides eluted between 19 and 28 min. Five of the 7 modified peptides were identified with the same charge as the native peptides and two displayed a charge 1 unit higher (peptides SHTVAAQGGINAALGNMEEDNWR and KPFEEHWR) (Table 1 and Supplementary data).

3.5. Nitro-fatty acids induce metabolic shifts in cardiac muscle cells

Alterations in respiratory metabolism are associated with

cardioprotection in models of ischemia-reperfusion [37]. To determine if the inhibition of complex II by OA-NO$_2$ observed in isolated mitochondria occurred in intact cells, metabolic flux analysis using a Seahorse XF24 analyzer was performed on H9C2 cardiomyoblasts. Basal respiration and maximal respiration were significantly inhibited at low concentrations of OA-NO$_2$, while proton leak was unaffected (Fig. 4A).

Separately, the acute effects of OA-NO$_2$ on OCR were observed by addition of 10 μM OA-NO$_2$ to basally respiring cells. This concentration lead to a ~30% decrease in OCR over 15 min (Fig. 4B). This decrease was followed by an increase in ECAR, supporting the concept that cells shifted from respiratory to glycolytic metabolism in response to OA-NO$_2$. Because these effects mirrored those observed in isolated mitochondria, it was hypothesized that uncoupling mitochondria and lowering matrix pH may also abrogate respiratory inhibition by OA-NO$_2$ in intact cells. Myoblasts exposed to 500 nM FCCP displayed a 60% increase in basal OCR that was not influenced by addition of 10 μM OA-NO$_2$ (Fig. 4C), further affirming the role of pH in OA-NO$_2$-mediated cellular respiratory inhibition.

3.6. OA-NO$_2$ suppresses basal superoxide production in respiring mitochondria

One way in which respiratory chain inhibition may promote cardioprotection is by limiting formation of partially-reduced reactive oxygen species. To determine if OA-NO$_2$ would modulate $(O_2^{\bullet-})$ production in succinate respiring mitochondria, oxidation of the superoxide-sensitive probe hydroethidine was followed.
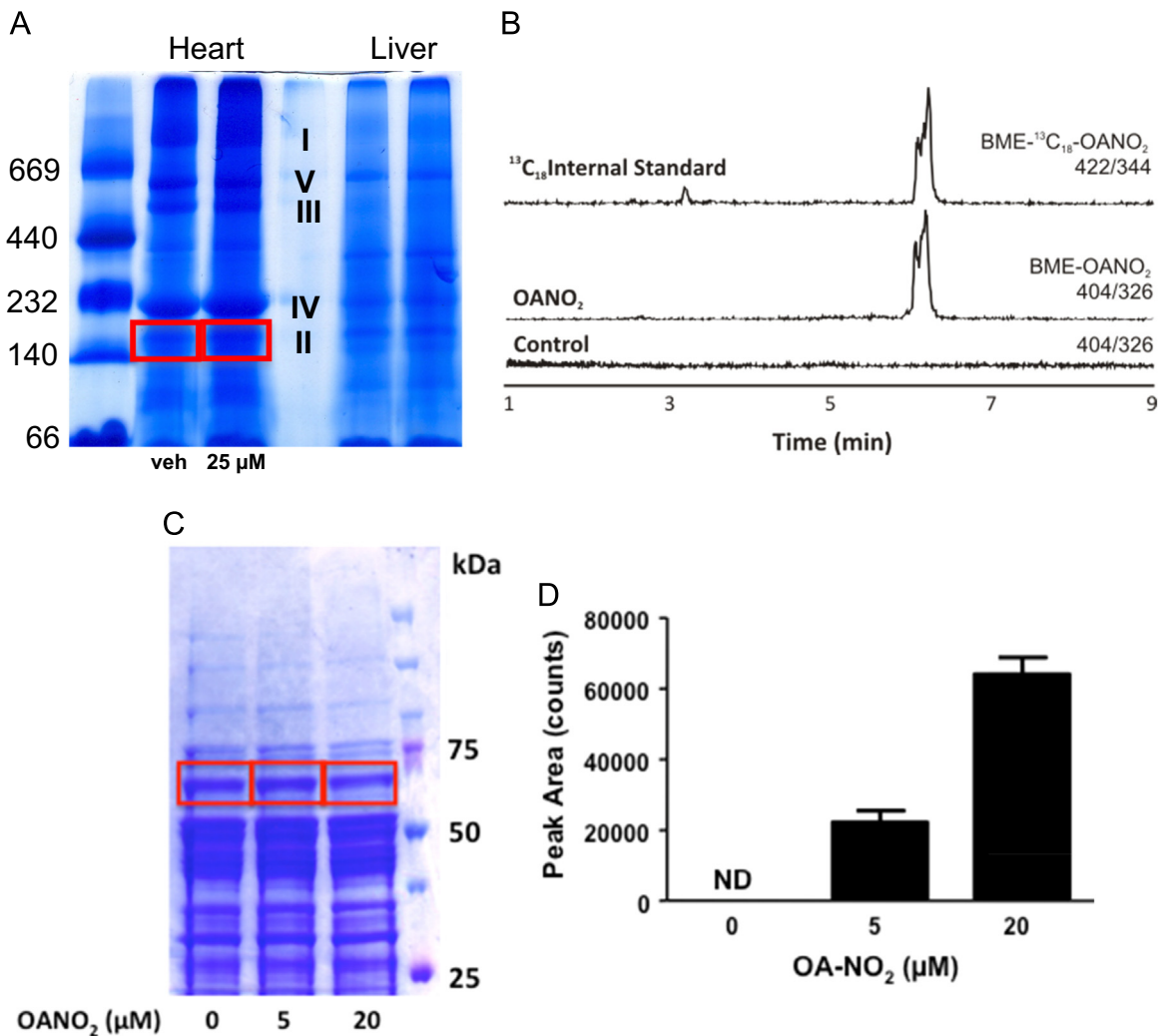
Fig. 3. OA-NO$_2$ modifies the Fp subunit of complex II. (A) Blue native gel of 400 µg of mitochondrial protein incubated with 0 or 20 µM OA-NO$_2$, the band corresponding to complex II highlighted. (B) LC/MS profile of the BME-OA-NO$_2$ adducts captured by BME induced trans nitroalkylation of the gel control and OA-NO$_2$ (20 µM) Complex II band. Elution of BME-^{13}C$_{18}$ OA-NO$_2$ internal standard shown. (C) SDS-PAGE Coomassie stain of 100 µg of mitochondrial lysate incubated with 0, 5 or 20 µM OA-NO$_2$ (D) OA-NO$_2$ content on the band corresponding to the Fp subunit of complex II was determined using BME transalkylation reaction in the presence of ^{13}C$_{18}$ OA-NO$_2$. Data represent mean $\pm$ SD of three independent experiments.

Mitochondrial exposure to 5 µM OA-NO$_2$ was sufficient to reduce $O_2^{\bullet-}$ production by 70%, with no additional decrease observed at higher concentrations (Fig. 5).

3.7. Nitro-fatty acids are cardioprotective in an ex vivo IR model

Compounds that shift cellular metabolic activity from respiration to glycolysis or limit reactive species formation are often protective in ischemia-reperfusion injury, and some complex II inhibitors induce cardioprotective effects. Therefore, the influence of fatty acid nitroalkenes on cardiac function was evaluated in a Langendorff-perfused heart model. Administration of 100 nM LA-NO$_2$ 15 min prior to induction of no-flow ischemia did not affect heart rate, coronary flow, and LVDP but resulted in a significantly higher rate-pressure product after reperfusion when compared to LA treated hearts (Fig. S1).

4. Discussion

Nitroalkene fatty acid derivatives are generated by mitochondria and specifically in the heart by the metabolic stress induced by ischemia-reperfusion [1,13]. These species, when added as synthetic homologs, mediate myocardial protection in IR [1,31]. Addition of NO$_2$-FA result in inhibition of mitochondrial respiration and reduction of superoxide anion formation as demonstrated using isolated mitochondria and whole cell flux analysis. Furthermore, cardioprotective effects upon mitochondrial inhibition by NO$_2$-FA preserved contractile function in an ex vivo model of acute IR and led to cardioprotection.

The evidence for a link between respiratory inhibition and cardioprotection in IR is extensive, though it is not clear if a common protective mechanism unites the inhibition of each complex [37]. Similarly, while many complex II inhibitors with distinct sites of action are protective in IR, the involvement of this complex in protection is not fully understood. Complex II inhibitors noted for their therapeutic efficacy in IR include malonate (a competitive inhibitor at the active site), TTFA and atpenin A5 (inhibitors of the quinone binding site), diazoxide (a non-competitive inhibitor whose binding site is unknown) and 3-nitropropionate (3-NP, a suicide inhibitor at the active site) [38–41]. Nonetheless, these inhibitors can display systemic toxicity as opposed to electrophilic nitroalkenes which are endogenously present and have been administered for up to 12 weeks in murine models of vascular disease with no apparent adverse effects [3].

A shared mechanism of cardioprotection for these structurally

Table 1

Mass spectrometry analysis of human recombinant complex II Fp subunit. Peptides detected to be modified by OA-NO_2 are shown with their respective retention times, $M+H^+$ mass and charge detected. Peptide coverage of Fp subunit after tryptic digest is shown underlined. Modified residues are shown.

Peptides targeted by OA-NO_2 in the Fp subunit of complex II

Peptide sequence	LC-Retention time (min)		Experimental mass $M+H^+$ (z detected)	
	Native	OA-NO_2	Native	OA-NO_2
SMQN**H**AAVFR	19.1	35.4	1160.7 (2)	1489.6 (2)
S**H**TVAAQGGINAALGNMEEDNWR	27.1	37.0	2443.1 (2)	2770.5 (3)
AAFGLSEAGFNTA**C**VTK	28.1	40.5	1688.2 (2)	2016.3 (2)
K**H**TLSYVDVGTGK	20.0	34.0	1406.2 (3)	1732.4 (3)
KPFEE**H**WR	19.7	34.1	1129.1 (2)	1457.2 (3)
VGSVLQEG**C**GK	20.2	39.3	1076.8 (2)	1404.0 (2)
W**H**FYDTVK	24.3	38.5	1095.8 (2)	1424.3 (2)

|	1	11	21	31	41	51
1	MSGVRGLSRL	LSARRLALAK	AWPTVLQTGT	RGFHFTVDGN	KRASAKVSDS	ISAQYPVVDH
61	EFDAVVVGAG	GAGLRAAFGL	SEAGFNTACV	TKLFPTRSHT	VAAQGGINAA	LGNMEEDNWR
121	WHFYDTVKGS	DWLGDQDAIH	YMTEQAPAAV	VELENYGMPF	SRTEDGKIYQ	RAFGGQSLKF
181	GKGGQAHRCC	CVADRTGHSL	LHTLYGRSLR	YDTSYFVEYF	ALDLLMENGE	CRGVIALCIE
241	DGSIHRIRAK	NTVVATGGYG	RTYFSCTSAH	TSTGDGTAMI	TRAGLPKCQDL	EFVQFHPTGI
301	YGAGCLITEG	CRGEGGILIN	SQGERFMERY	APVAKDLASR	DVVSRSMTLE	IREGRGCGPE
361	KDHVYLQLHH	LPPEQLATRL	PGISETAMIF	AGVDVTKEPI	PVLPTVHYNM	GGIPTNYKGQ
421	VLRHVNGQDQ	AACASVHGAN	RLGANSLLDL	VVFGRACALS	IEESCRPGDK	VPPIKPNAGE
481	ESVMNLDKLR	FADGSIRTSE	LRLSMQKSMQ	NHHAAVFRVGS	VLQEGCGKIS	KLYGDLKHLK
541	TFDRGMVWNT	DLVETLELQN	LMLCALQTIY	GAEARKESRG	AHAREDYKVR	IDEYDYSKPI

diverse inhibitors is appealing, and several candidates have been proposed. A mitochondrial K_{ATP} channel of undetermined molecular identity is activated by many complex II inhibitors and is considered crucial in preconditioning, with complex II proposed to have a structural role in this channel. In this regard, nitroxyl (HNO) is a thiol-modifying compound which inhibits complex II and activates the mitoK$_{ATP}$ channel, promoting cardioprotection when administered prior to ischemia [35,42]. While the potential role of this channel in NO_2-FA-mediated cardioprotection was not directly addressed, protection of primary ventricular myocyte cultures by LA-NO_2 in simulated IR was not affected by the mitoK$_{ATP}$ inhibitor 5-hydroxydecanoate [13]. Independent of mitoK$_{ATP}$ effects, a recent study indicates that succinate buildup during ischemia and its rapid metabolism during reperfusion is an important mechanism of ischemic injury. In this model it is reverse electron transfer to complex I which drives reactive species production and tissue damage [43]. This injury is inhibited by the prodrug dimethylmalonate, which is converted to the flavin-binding complex II inhibitor malonate, and a similar mechanism is possible with OA-NO_2 through specific binding to the flavin-containing Fp subunit [43,44].

In addition to reduced radical formation, the metabolic shift from cellular respiration to glycolysis observed in cell culture as a result of complex II inhibition can promote cell survival in IR [45]. Notably, enzymes in the glycolytic pathway are tightly regulated by electrophiles, as reported for GAPDH [7], suggesting that mitochondrial NO_2-FA trafficking might be involved in directing mitochondrial protein PTMs.

The mechanism of inhibition of the 70 kDa Fp subunit flavin by 3-NP involves its oxidation to yield the electrophile 3-nitroacrylate, which inhibits complex II upon Michael addition [46]. Despite the structural and mechanistic similarities between fatty acid nitroalkenes and the 3-carbon nitroalkene 3-nitroacrylate, addition of 3-NP at 10-fold molar excess did not inhibit OA-NO_2 binding to the Fp subunit, likely indicating that these compounds target different nucleophilic amino acids (not shown) [47,48]. Furthermore, 3-NP causes pronounced neurodegeneration in animal models independent of its cardioprotective effects [49], an effect that is not evident in acute or long term OA-NO_2 treatments. The apparent reversibility of NO_2-FA adduction of complex II by low molecular weight thiols is another important distinguishing feature, as 3-NP adducts are considered irreversible [46].

An earlier study linked acute cardioprotection by OA-NO_2 to mild uncoupling induced by nitroalkylation of the adenine nucleotide transporter (ANT1) in mitochondria [26]. However, this study used biotinylated NO_2-FA (derivatized at the carboxylic acid), whose intracellular metabolism and trafficking differs from that of the native fatty acid, to demonstrate ANT1 modification in isolated perfused hearts. These metabolic and trafficking differences are salient when studying the modification of mitochondrial proteins by NO_2-FA, as the entry of fatty acids to the mitochondrion requires the formation of carnitine and CoA derivatives. In addition, mitochondrial beta oxidation of NO_2-FA generates shorter chain electrophilic species that may have different targets than the parent compound, with biotinylated NO_2-FA not subject to this metabolic regulation [23]. Finally, the addition of biotin doubles the size of the fatty acid and neutralizes the carboxylic acid which may impact half-life and molecular targets. Of note, despite the differences in experimental approach, biotinylated nitrated linoleic acid has been reported to modify the Fp subunit of
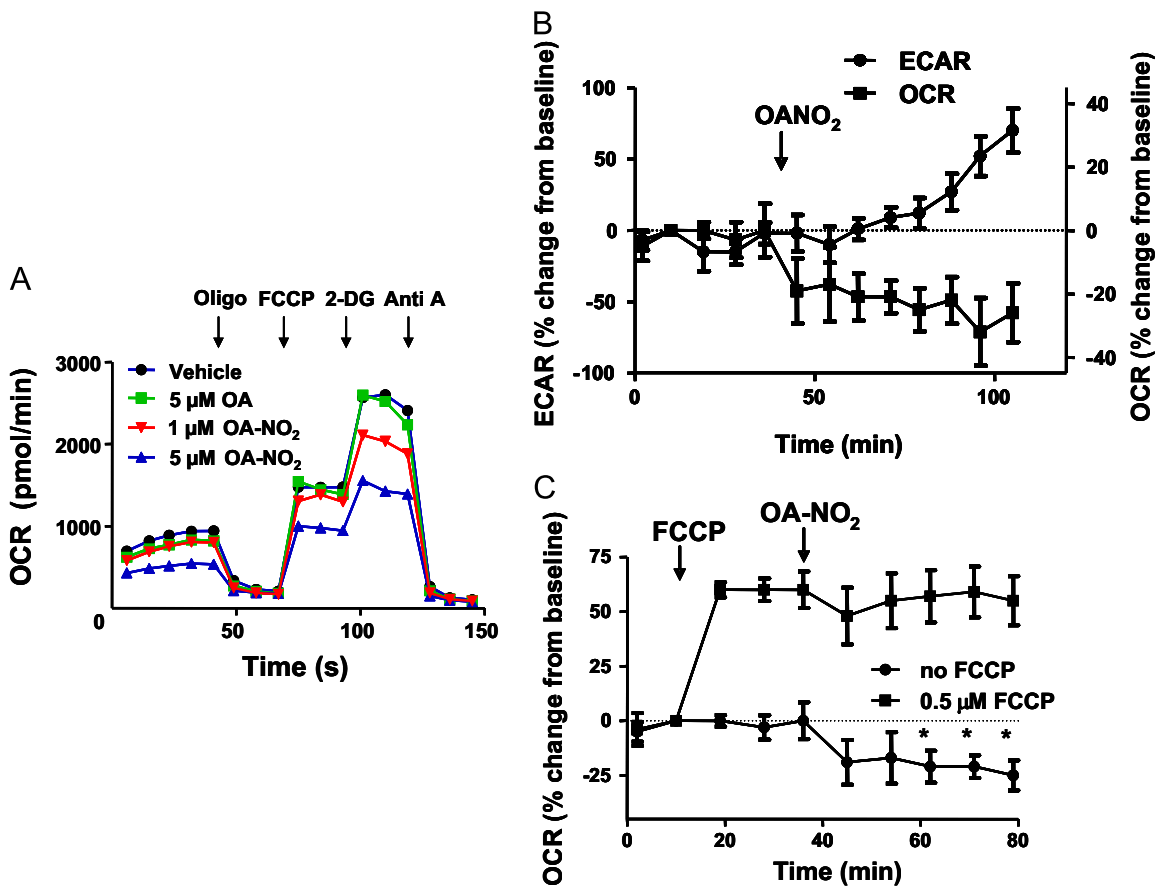
Fig. 4. OA-NO$_2$ inhibits respiration and promotes glycolysis in H9C2 cardiomyoblasts. O$_2$ consumption and extracellular acidification rates (OCR and ECAR) were measured in H9C2 cells (A) Following 2 h of incubation with 0–5 μM OA-NO$_2$, basal OCR was measured, followed by proton leak, maximal respiration, and non-mitochondrial O$_2$ consumption (induced by oligomycin, FCCP and 2-DG, and antimycin A, respectively). A representative experiment is shown. (B) 10 μM OA-NO$_2$ was added to respiring cells and the percent change in OCR and ECAR recorded. (C) Addition of 10 μM OA-NO$_2$ after uncoupling of respiration with 500 nM FCCP. For all experiments, $n=5$ wells per group, data represents mean ± SD.
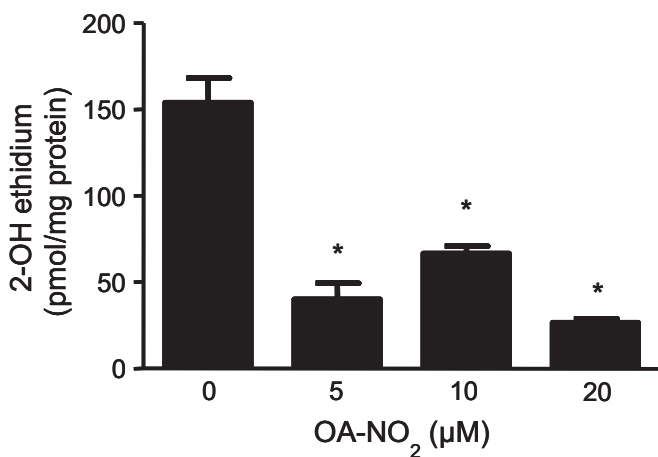
Fig. 5. OA-NO$_2$ suppresses basal superoxide production in isolated mitochondria. Superoxide was determined by the oxidation of DHE (20 μM) to 2-OHE in mitochondria respiring on succinate (10 mM) preincubated for 10 min with OA-NO$_2$ (0–20 μM). 2-OHE content in the mitochondrial pellet was determined by HPLC. A representative experiment is shown. $n=4$ reactions per group, mean ± SD.

complex II in mitochondria [13].

Additional insights from the present study in the context of previous reports of the mitochondrial effects of NO$_2$-FA include the observation of uncoupling and the concentrations used to elicit respiratory inhibition. In one study, a small (though significant) increase in O$_2$ consumption rate was noted by extracellular flux

analysis in H9C2 cells at 1 μM LA-NO$_2$ [26]. This was a minor effect relative to previous data showing robust uncoupling of primary rat ventricular myocytes at concentrations between 0.25 and 1 μM LA-NO$_2$, and in isolated mitochondria at concentrations up to 10 μM, with 20 μM required for inhibition [13]. In the present study, increased respiration in OA-NO$_2$-treated cardiomyoblasts relative to controls was not observed at nitroalkene concentrations below 10 μM.

The present data indicates that inhibition of complex II by NO$_2$-FA may depend in part on matrix pH, since respiratory coupling was a requirement for inhibition in both isolated mitochondria and cells. The dissipation of the membrane potential in uncoupled mitochondria reduces the delta psi and the pH differential across the membrane, lowering the pool of available thiolate anion on target proteins required for nitroalkylation reactions. Consistent with this, inhibition of complex II+III activity by NO$_2$-FA in isolated mitochondria after freeze-thaw was strongly dependent on pH, with maximal inhibition occurring near pH 9.0. The pH-dependent response of respiratory inhibition by OA-NO$_2$ suggests that modification of a thiol on complex II with a pKa of approximately 8.0 is responsible for NO$_2$-FA-mediated inhibition. Estimates of *in vivo* matrix pH using pH-sensitive GFP mutants indicates that this compartment in resting cardiomyocytes maintains ∼pH 7.9, and a higher local pH would be expected near the respiratory complexes, where protons are actively exported from the matrix [50]. Thus, physiologically relevant changes in matrix pH might tightly control nitroalkene-dependent inhibition of respiration. In this regard, cytochrome oxidase is highly sensitive to

nitric oxide, a condition associated with high levels of nitrated fatty acids, mitochondrial hyperpolarization and local higher pH levels.

The administration of nitrite mediates the post-translational modification of complex I thiols and leads to cardiac protection from ischemic insult [51]. Herein, no effect of OA-NO$_2$ on NADH-linked respiration in isolated mitochondria was observed, consistent with an earlier report showing no effect of LA-NO$_2$ on complex I activity [13]. The biochemical basis for complex I-specific inhibition by S-nitrosation but not S-nitroalkylation is unclear, and might relate to local spatial and charge constrains. With regards to complex II, it is a target for various reactive species. For example, a) Cys[90] in the Fp subunit of bovine complex II (corresponding to Cys[81] in the rat) is S-glutathionylated *in vitro* upon addition of oxidized glutathione GSSG, [52] b) nitroxyl, the one electron reduction product of ●NO preferentially inhibits complex II over complex I [35] and c) 4-HNE covalently modifies and inhibits the Fp subunit [36].

Prior studies have employed MS analysis to monitor redox modifications of the 70 kDa Fp subunit of complex II in post-ischemic myocardium. In the absence of reducing agents, a coverage of 64.9% was obtained, in agreement with the 64% obtained in our study [52]. A difference between studies is the coverage of cysteines, as we characterized 11 of the 17 cysteines present in human succinate dehydrogenase and that study covered 5 of 18 present in the bovine-derived enzyme, a coverage that increased to 88% after incubation with reducing agents. In this regard, the presence of OA-NO$_2$ adducts requires non-reducing conditions to stabilize modifications, as these agents induce β-elimination of OA-NO$_2$. In particular, Cys89, the target residues of OA-NO$_2$ in the human subunit is basally S-glutathionylated in bovine hearts (Cys90), and loss of this modification in IR is associated with decreased electron transfer efficiency and increased production [52] underscoring its role in mitochondrial respiration control. In addition, the role of different PTMs becomes evident in the control of $O_2^{\bullet-}$ formation as OA-NO$_2$ has an inhibitory effect and S-glutathionylation increases the formation of this radical. Additionally, OA-NO$_2$ targets Cys526 (homologous to bovine Cys537) which was reported to undergo oxidation to sulfonic acid in the bovine myocardium during IR further demonstrating the sensitivity of cysteine residues in Fp subunit to oxidative modifications [34]. Reversible nitroalkylation of these residues could further prevent their irreversible oxidation during injury. While exogenous OA-NO$_2$ and purified recombinant Fp subunit were employed here to map sites of adduction, future studies will seek to identify modified peptides in complex II following *in vivo* physiologic or pathophysiologic stresses, such as ischemic preconditioning or IR.

Signaling by individual electrophilic species involves effects on multiple protein targets in various cellular compartments, and it is likely that several mechanisms account for the acute *ex vivo* cardioprotection conferred by NO$_2$-FA. Overall our data support a role for respiratory inhibition in the cardioprotective mechanism of NO$_2$-FA, likely via inhibition of complex II, promoting glycolysis, suppressing reactive species formation, and preserving myocardial function in the post-ischemic interval. Endogenous production of fatty acid nitroalkenes during ischemic stress links inflammatory stress with beneficial metabolic responses, providing a potential target for future pharmacological intervention.

Conflict of interest

FJS and SRW acknowledge financial interest in Complexa, Inc.

Author contributions

JRK designed, performed and analyzed experiments, and wrote the manuscript. G.B. contributed to experimental design, data interpretation and mitochondrial measurements. S.R.W. provided nitrated fatty acids. C.S.C. contributed to cell-based experiments. N.C.M and E.E.K. designed and performed superoxide measurements. F.J.S. contributed to the overall concept, experimental design, data analysis and interpretation and manuscript writing.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2015.11.002.

References

[1] V. Rudolph, T.K. Rudolph, F.J. Schopfer, G. Bonacci, S.R. Woodcock, M.P. Cole, P. R. Baker, R. Ramani, B.A. Freeman, Endogenous generation and protective effects of nitro-fatty acids in a murine model of focal cardiac ischaemia and reperfusion, Cardiovasc. Res. 85 (2010) 155–166.

[2] J. Zhang, L. Villacorta, L. Chang, Z. Fan, M. Hamblin, T. Zhu, C.S. Chen, M.P. Cole, F.J. Schopfer, C.X. Deng, M.T. Garcia-Barrio, Y.H. Feng, B.A. Freeman, Y.E. Chen, Nitro-oleic acid inhibits angiotensin II-induced hypertension, Circ. Res. 107 (2010) 540–548.

[3] T.K. Rudolph, V. Rudolph, M.M. Edreira, M.P. Cole, G. Bonacci, F.J. Schopfer, S. R. Woodcock, A. Franek, M. Pekarova, N.K. Khoo, A.H. Hasty, S. Baldus, B. A. Freeman, Nitro-fatty acids reduce atherosclerosis in apolipoprotein E-deficient mice, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 938–945.

[4] F. Schopfer, C. Cipollina, B.A. Freeman., Formation and signaling actions of electrophilic lipids, Chem. Rev. 111 (2011) 5997–6021.

[5] E.E. Kelley, C.I. Batthyany, N.J. Hundley, S.R. Woodcock, G. Bonacci, J.M. Del Rio, F.J. Schopfer, J.R. Lancaster Jr., B.A. Freeman, M.M. Tarpey, Nitro-oleic acid, a novel and irreversible inhibitor of xanthine oxidoreductase, J. Biol. Chem. 283 (2008) 36176–36184.

[6] G. Bonacci, F.J. Schopfer, C.I. Batthyany, T.K. Rudolph, V. Rudolph, N.K. Khoo, E. E. Kelley, B.A. Freeman, Electrophilic fatty acids regulate matrix metalloproteinase activity and expression, J. Biol. Chem. 286 (2011) 16074–16081.

[7] C. Batthyany, F.J. Schopfer, P.R. Baker, R. Duran, L.M. Baker, Y. Huang, C. Cervenansky, B.P. Branchaud, B.A. Freeman, Reversible post-translational modification of proteins by nitrated fatty acids in vivo, J. Biol. Chem. 281 (2006) 20450–20463.

[8] B.A. Freeman, P.R. Baker, F.J. Schopfer, S.R. Woodcock, A. Napolitano, M. d'Ischia, Nitro-fatty acid formation and signaling, J. Biol. Chem. 283 (2008) 15515–15519.

[9] R.L. Alexander, D.J. Bates, M.W. Wright, S.B. King, C.S. Morrow, Modulation of nitrated lipid signaling by multidrug resistance protein 1 (MRP1): glutathione conjugation and MRP1-mediated efflux inhibit nitrolinoleic acid-induced, PPARgamma-dependent transcription activation, Biochemistry 45 (2006) 7889–7896.

[10] M. Nishida, T. Sawa, N. Kitajima, K. Ono, H. Inoue, H. Ihara, H. Motohashi, M. Yamamoto, M. Suematsu, H. Kurose, A. van der Vliet, B.A. Freeman, T. Shibata, K. Uchida, Y. Kumagai, T. Akaike, Hydrogen sulfide anion regulates redox signaling via electrophile sulfhydration, Nat. Chem. Biol. 8 (2012) 714–724.

[11] D.A. Vitturi, C.S. Chen, S.R. Woodcock, S.R. Salvatore, G. Bonacci, J.R. Koenitzer, N.A. Stewart, N. Wakabayashi, T.W. Kensler, B.A. Freeman, F.J. Schopfer, Modulation of nitro-fatty acid signaling: prostaglandin reductase-1 is a nitroalkene reductase, J. Biol. Chem. 288 (2013) 25626–25637.

[12] S.R. Salvatore, D.A. Vitturi, P.R. Baker, G. Bonacci, J.R. Koenitzer, S.R. Woodcock, B.A. Freeman, F.J. Schopfer, Characterization and quantification of endogenous fatty acid nitroalkene metabolites in human urine, J. Lipid Res. 54 (2013) 1998–2009.

[13] S.M. Nadtochiy, P.R. Baker, B.A. Freeman, P.S. Brookes, Mitochondrial nitroalkene formation and mild uncoupling in ischaemic preconditioning: implications for cardioprotection, Cardiovasc. Res. 82 (2009) 333–340.

[14] S. Shiva, M.N. Sack, J.J. Greer, M. Duranski, L.A. Ringwood, L. Burwell, X. Wang,

P.H. MacArthur, A. Shoja, N. Raghavachari, J.W. Calvert, P.S. Brookes, D.J. Lefer, M.T. Gladwin, Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer, J. Exp. Med. 204 (2007) 2089–2102.

[15] K. Megyesi, C.R. Kahn, J. Roth, D.M. Neville Jr., S.P. Nissley, R.E. Humbel, E. R. Froesch, The NSILA-s receptor in liver plasma membranes. Characterization and comparison with the insulin receptor, J. Biol. Chem. 250 (1975) 8990–8996.

[16] J.J. Poderoso, M.C. Carreras, C. Lisdero, N. Riobo, F. Schopfer, A. Boveris, Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles, Arch. Biochem. Biophys. 328 (1996) 85–92.

[17] J.J. Poderoso, C. Lisdero, F. Schopfer, N. Riobo, M.C. Carreras, E. Cadenas, A. Boveris, The regulation of mitochondrial oxygen uptake by redox reactions involving nitric oxide and ubiquinol, J. Biol. Chem. 274 (1999) 37709–37716.

[18] L. Castro, J.P. Eiserich, S. Sweeney, R. Radi, B.A. Freeman, Cytochrome c: a catalyst and target of nitrite-hydrogen peroxide-dependent protein nitration, Arch. Biochem. Biophys. 421 (2004) 99–107.

[19] S. Shiva, Mitochondria as metabolizers and targets of nitrite, Nitric Oxide 22 (2010) 64–74.

[20] P.R. Castello, P.S. David, T. McClure, Z. Crook, R.O. Poyton, Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes, Cell Metab. **3** (2006) 277–287.

[21] D.A. Vitturi, L. Minarrieta, S.R. Salvatore, E.M. Postlethwait, M. Fazzari, G. Ferrer-Sueta, J.R. Lancaster Jr., B.A. Freeman, F.J. Schopfer, Convergence of biological nitration and nitrosation via symmetrical nitrous anhydride, Nat. Chem. Biol. 11 (2015) 504–510.

[22] G. Bonacci, P.R. Baker, S.R. Salvatore, D. Shores, N.K. Khoo, J.R. Koenitzer, D. A. Vitturi, S.R. Woodcock, F. Golin-Bisello, M.P. Cole, S. Watkins St, C. Croix, C. I. Batthyany, B.A. Freeman, F.J. Schopfer, Conjugated linoleic acid is a preferential substrate for fatty acid nitration, J. Biol. Chem. 287 (2012) 44071–44082.

[23] V. Rudolph, F.J. Schopfer, N.K. Khoo, T.K. Rudolph, M.P. Cole, S.R. Woodcock, G. Bonacci, A.L. Groeger, F. Golin-Bisello, C.S. Chen, P.R. Baker, B.A. Freeman, Nitro-fatty acid metabolome: saturation, desaturation, beta-oxidation, and protein adduction, J. Biol. Chem. 284 (2009) 1461–1473.

[24] R.K. Dongworth, A.R. Hall, N. Burke, D.J. Hausenloy, Targeting mitochondria for cardioprotection: examining the benefit for patients, Future Cardiol. 10 (2014) 255–272.

[25] B.B. Queliconi, A.P. Wojtovich, S.M. Nadtochiy, A.J. Kowaltowski, P.S. Brookes, Redox regulation of the mitochondrial K(ATP) channel in cardioprotection, Biochim. Biophys. Acta 1813 (2011) 1309–1315.

[26] S.M. Nadtochiy, Q. Zhu, W. Urciuoli, R. Rafikov, S.M. Black, P.S. Brookes, Nitroalkenes confer acute cardioprotection via adenine nucleotide translocase 1, J. Biol. Chem. 287 (2012) 3573–3580.

[27] S.R. Woodcock, A.J. Marwitz, P. Bruno, B.P. Branchaud, Synthesis of nitrolipids. All four possible diastereomers of nitrooleic acids: (E)- and (Z)-, 9- and 10-nitro-octadec-9-enoic acids, Org. Lett. 8 (2006) 3931–3934.

[28] J.M. Brown, M.S. Quinton, B.K. Yamamoto, Methamphetamine-induced inhibition of mitochondrial complex II: roles of glutamate and peroxynitrite., J. Neurochem. 95 (2005) 429–436.

[29] S.J. Heales, A. Menzes, G.P. Davey, Depletion of glutathione does not affect electron transport chain complex activity in brain mitochondria: implications for Parkinson disease and postmortem studies, Free. Radic. Biol. Med. 50 (2011) 899–902.

[30] I. Wittig, H.P. Braun, H. Schagger, Blue native PAGE, Nat. Protoc. 1 (2006) 418–428.

[31] F.J. Schopfer, C. Batthyany, P.R. Baker, G. Bonacci, M.P. Cole, V. Rudolph, A. L. Groeger, T.K. Rudolph, S. Nadtochiy, P.S. Brookes, B.A. Freeman, Detection and quantification of protein adduction by electrophilic fatty acids: mitochondrial generation of fatty acid nitroalkene derivatives, Free. Radic. Biol. Med. 46 (2009) 1250–1259.

[32] J. Zielonka, J. Vasquez-Vivar, B. Kalyanaraman, Detection of 2-hydroxyethidium in cellular systems: a unique marker product of superoxide and hydroethidine, Nat. Protoc. 3 (2008) 8–21.

[33] R.M. Bell, M.M. Mocanu, D.M. Yellon, Retrograde heart perfusion: the Langendorff technique of isolated heart perfusion, J. Mol. Cell. Cardiol. 50 (2011)

940–950.

[34] L. Zhang, C.L. Chen, P.T. Kang, V. Garg, K. Hu, K.B. Green-Church, Y.R. Chen, Peroxynitrite-mediated oxidative modifications of complex II: relevance in myocardial infarction, Biochemistry 49 (2010) 2529–2539.

[35] S. Shiva, J.H. Crawford, A. Ramachandran, E.K. Ceaser, T. Hillson, P.S. Brookes, R. P. Patel, V.M. Darley-Usmar, Mechanisms of the interaction of nitroxyl with mitochondria, Biochem. J. 379 (2004) 359–366.

[36] O.M. Lashin, P.A. Szweda, L.I. Szweda, A.M. Romani, Decreased complex II respiration and HNE-modified SDH subunit in diabetic heart, Free. Radic. Biol. Med. 40 (2006) 886–896.

[37] L.S. Burwell, S.M. Nadtochiy, P.S. Brookes, Cardioprotection by metabolic shutdown and gradual wake-up, J. Mol. Cell. Cardiol. 46 (2009) 804–810.

[38] A.P. Wojtovich, P.S. Brookes, The endogenous mitochondrial complex II inhibitor malonate regulates mitochondrial ATP-sensitive potassium channels: implications for ischemic preconditioning, Biochim. Biophys. Acta 1777 (2008) 882–889.

[39] A.P. Wojtovich, P.S. Brookes, The complex II inhibitor atpenin A5 protects against cardiac ischemia-reperfusion injury via activation of mitochondrial KATP channels, Basic Res. Cardiol. 104 (2009) 121–129.

[40] K.D. Garlid, P. Paucek, V. Yarov-Yarovoy, H.N. Murray, R.B. Darbenzio, A. J. D'Alonzo, N.J. Lodge, M.A. Smith, G.J. Grover, Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K+ channels. Possible mechanism of cardioprotection, Circ. Res. 81 (1997) 1072–1082.

[41] R.A. Ockaili, P. Bhargava, R.C. Kukreja, Chemical preconditioning with 3-nitropropionic acid in hearts: role of mitochondrial K(ATP) channel, Am. J. Physiol. Heart Circ. Physiol. 280 (2001) H2406–H2411.

[42] X.L. Ma, F. Gao, G.L. Liu, B.L. Lopez, T.A. Christopher, J.M. Fukuto, D.A. Wink, M. Feelisch, Opposite effects of nitric oxide and nitroxyl on postischemic myocardial injury, Proc. Natl. Acad. Sci. USA 96 (1999) 14617–14622.

[43] E.T. Chouchani, V.R. Pell, E. Gaude, D. Aksentijevic, S.Y. Sundier, E.L. Robb, A. Logan, S.M. Nadtochiy, E.N. Ord, A.C. Smith, F. Eyassu, R. Shirley, C.H. Hu, A. J. Dare, A.M. James, S. Rogatti, R.C. Hartley, S. Eaton, A.S. Costa, P.S. Brookes, S. M. Davidson, M.R. Duchen, K. Saeb-Parsy, M.J. Shattock, A.J. Robinson, L. M. Work, C. Frezza, T. Krieg, M.P. Murphy, Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS, Nature 515 (2014) 431–435.

[44] S. Drose, L. Bleier, U. Brandt, A common mechanism links differently acting complex II inhibitors to cardioprotection: modulation of mitochondrial reactive oxygen species production, Mol. Pharmacol. 79 (2011) 814–822.

[45] V.M. Gohil, S.A. Sheth, R. Nilsson, A.P. Wojtovich, J.H. Lee, F. Perocchi, W. Chen, C.B. Clish, C. Ayata, P.S. Brookes, V.K. Mootha, Nutrient-sensitized screening for drugs that shift energy metabolism from mitochondrial respiration to glycolysis, Nat. Biotechnol. 28 (2010) 249–255.

[46] C.J. Coles, D.E. Edmondson, T.P. Singer, Inactivation of succinate dehydrogenase by 3-nitropropionate, J. Biol. Chem. 254 (1979) 5161–5167.

[47] T.M. Tomasiak, T.L. Archuleta, J. Andrell, C. Luna-Chavez, T.A. Davis, M. Sarwar, A.J. Ham, W.H. McDonald, V. Yankovskaya, H.A. Stern, J.N. Johnston, E. Maklashina, G. Cecchini, T.M. Iverson, Geometric restraint drives on- and off-pathway catalysis by the *Escherichia coli* menaquinol:fumarate reductase, J. Biol. Chem. 286 (2011) 3047–3056.

[48] L.S. Huang, G. Sun, D. Cobessi, A.C. Wang, J.T. Shen, E.Y. Tung, V.E. Anderson, E. A. Berry, 3-nitropropionic acid is a suicide inhibitor of mitochondrial respiration that, upon oxidation by complex II, forms a covalent adduct with a catalytic base arginine in the active site of the enzyme, J. Biol. Chem. 281 (2006) 5965–5972.

[49] M.F. Beal, E. Brouillet, B.G. Jenkins, R.J. Ferrante, N.W. Kowall, J.M. Miller, E. Storey, R. Srivastava, B.R. Rosen, B.T. Hyman, Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid, J. Neurosci. 13 (1993) 4181–4192.

[50] J. Llopis, J.M. McCaffery, A. Miyawaki, M.G. Farquhar, R.Y. Tsien, Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins, Proc. Natl. Acad. Sci. USA 95 (1998) 6803–6808.

[51] S. Shiva, M.T. Gladwin, Nitrite mediates cytoprotection after ischemia/reperfusion by modulating mitochondrial function, Basic Res. Cardiol. 104 (2009) 113–119.

[52] Y.R. Chen, C.L. Chen, D.R. Pfeiffer, J.L. Zweier, Mitochondrial complex II in the post-ischemic heart: oxidative injury and the role of protein S-glutathionylation, J. Biol. Chem. 282 (2007) 32640–32654.