ELECTROPHILIC NITRO-FATTY ACID REGULATION OF MITOCHONDRIAL FUNCTION

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Nitro-fatty acids (NO_2-FA) are endogenous mediators generated by reactions of nitrogen dioxide with unsaturated fatty acids. They are electrophilic and signal by reversibly reacting with nucleophilic cysteine and histidine residues on proteins, thus altering protein function. NO_2-FA mediate cardioprotection in *in vivo* models of ischemia-reperfusion (IR) through not fully defined mechanisms. Mitochondria play a central role in both IR injury and redox signaling, with respiratory inhibition a common pathway in cardioprotective signaling. It was hypothesized that NO_2-FA induce tissue-protective metabolic shifts through mitochondrial interactions.

Respirometry in isolated mitochondria demonstrated that complex II-linked, but not complex I-linked, respiration was inhibited by nitro-oleic acid ($OANO_2$). Activity assays showed that inhibition of complex II was pH-dependent and reversible by the low molecular weight thiol β-mercaptoethanol (BME). Modification of the Fp subunit of complex II was confirmed following electrophoresis of mitochondrial proteins from $OANO_2$-treated lysates: addition of BME displaced protein-bound $OANO_2$ and led to the formation of BME-$OANO_2$ adducts as measured by liquid chromatography/mass spectrometry (LC/MS). Extracellular flux analysis, where O_2 consumption and media acidification are measured as surrogates for respiratory and glycolytic activity, was employed to determine the effects of $OANO_2$ on bioenergetics in cardiomyoblasts. Pre-incubation with $OANO_2$ inhibited basal and maximal respiration, while acute $OANO_2$ injection inhibited respiration and promoted glycolysis, a tissue-protective shift in IR. Protection against IR injury to the heart was observed in a Langendorff-perfused heart model as improved cardiac output recovery during reperfusion in the presence $OANO_2$.

Additional studies monitored metabolism of $OANO_2$ in cardiac tissue and cells. NO_2-FA are metabolized by prostaglandin reductase-1 (PGR1) and β-oxidation to yield non-electrophilic nitroalkanes and shorter chain nitroalkenes, respectively. PGR1 is inhibited by indomethacin and

β-oxidation by etomoxir, so these compounds were used to inhibit $OANO_2$ metabolism. Indomethacin treatment attenuated $OANO_2$ reduction and enhanced protein adduction by $OANO_2$ and its electrophilic metabolites, while etomoxir favored the formation of non-electrophilic nitroalkanes and reduced β-oxidation products. Indomethacin also significantly enhanced $OANO_2$ signaling in cardiomyoblasts, elevating heme oxygenase-1 expression compared to $OANO_2$ alone. This work identifies mitochondrial actions of NO_2-FA relevant to cardioprotection, and illuminates NO_2-FA metabolic pathways relevant to signaling in the myocardium.

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1.0 INTRODUCTION

Reactive species derived from oxygen and nitric oxide are produced during inflammation and promote oxidation and nitration of biomolecules, including unsaturated fatty acids. Among the products of these reactions are α,β-unsaturated carbonyl and nitro derivatives of fatty acids, electrophilic species whose reactivity with nucleophilic amino acids provides a means of post-translational protein modification and signaling. These electrophilic fatty acids activate cytosolic and nuclear stress-response pathways (through Nrf2/Keap1 and PPARγ, for example). There is also growing evidence that mitochondria generate electrophilic species. This appreciation, when combined with the role of mitochondrial dysfunction in conditions where exogenously delivered electrophiles exhibit therapeutic benefit, suggests that mitochondrial electrophile targets are also important in the resolution and prevention of inflammatory injury. Cardioprotective signaling pathways in particular appear to converge on mitochondria, with nitro-fatty acids recently shown to protect against cardiac ischemia/reperfusion injury in a murine model. While numerous mitochondrial proteins are subject to modification by electrophiles, defining the targets most relevant to cytoprotection during inflammatory stress remains a clinically-relevant goal.

1.1 CARDIAC REPERFUSION INJURY

Cardiovascular disease has been the leading cause of death in the United States every year since 1919, and myocardial ischemia in particular imposes a severe morbidity and mortality burden. 16.8 million Americans are estimated to have coronary heart disease, and each year over one million experience a new or recurrent coronary attack (1). Timely restoration of arterial patency is required for tissue preservation following acute myocardial infarction, and thrombolysis and percutaneous angioplasty have emerged as the therapeutic gold standards. However, the benefit of these approaches is limited by reperfusion injury, in which additional tissue damage accompanies the return of oxygenated blood to the ischemic heart. No clinical strategies for abrogating reperfusion injury currently exist, though there is ample interest: agents currently under study include calcium channel blockers, free radical scavengers, and nitric oxide donors (2). The recent observation that nitrated fatty acids, endogenous electrophilic species produced during inflammation, are cardioprotective in murine ischemia-reperfusion suggests that these compounds and their cellular targets may open new clinical avenues toward the prevention of reperfusion injury (3).

1.2 REACTIVE GASES (O_2, $^{\bullet}NO$), CELL SIGNALING AND INFLAMMATION

When first characterized as a signaling molecule, the gaseous free radical nitric oxide ($^{\bullet}NO$) exhibited classical high-affinity ligand-receptor binding behavior with its specific receptor, the heme iron of soluble guanylate cyclase (sGC). Observations over the ensuing 20 years have augmented this view, revealing an expansive biochemical and physiological role for $^{\bullet}NO$ in mammalian biology, with an ever-growing cast of molecular, cellular, and tissue targets.

Chemically, $^{\bullet}NO$ reacts with molecular oxygen (O_2) and its derivatives (ROS) to generate a range of oxidation products (NO_x) whose downstream reactivities greatly increase the breadth of what is termed "$^{\bullet}NO$ signaling". These reactive nitrogen species (RNS) differentially induce biomolecule oxidation, nitration (addition of NO_2), and nitrosation (addition of NO), each of which has implications in cell signaling (4). One well-characterized RNS-forming reaction is that of $^{\bullet}NO$

with superoxide ($O_2^{\bullet-}$), which occurs at nearly diffusion-limited rates to produce peroxynitrite ($ONOO^-$) (5). $ONOO^-$ is itself strongly oxidizing, and when protonated undergoes homolytic scission to produce hydroxyl radical ($^{\bullet}OH$) and nitrogen dioxide ($^{\bullet}NO_2$), the latter a nitrating agent and the former the most potent biological oxidant (5). Moreover, $ONOO^-$ reacts avidly with CO_2 to produce nitrosoperoxycarbonate ($ONOOCO_2^-$), a reaction of particular importance in acidic milieus which tips the balance in successive reactions toward nitration versus oxidation, and leads to the formation of a carbonate radical ($HCO_3^{\bullet}$) (6). Through its own activity and that of its byproducts, $ONOO^-$ engages in lipid and protein oxidation and nitration, enabling signal transduction and changes in cellular function (7). Radical reactions of $^{\bullet}NO$ with molecular oxygen lead to an additional set of RNS with distinct properties. The reaction of $^{\bullet}NO$ with one of the unpaired electrons of O_2 forms $^{\bullet}NO_2$, which may react further with $^{\bullet}NO$ to yield N_2O_3 or N_2O_4—nitrosating and nitrating compounds, respectively (8). Ultimately, this array of reactions provides the palette from which $^{\bullet}NO$ draws its myriad cGMP-independent *in vivo* effects (Figure 1).

Though many RNS facilitate pro-oxidant reactions, $^{\bullet}NO$ predominantly mediates protective effects during inflammation (9). The selective modification of biomolecules by RNS to engage signaling pathways provides an appealing explanation for the inflammation-resolving effects of $^{\bullet}NO$. In particular, RNS (and ROS) interact with unsaturated fatty acids to form electrophilic lipid derivatives, a class of molecules gaining recognition for their anti-inflammatory effects.

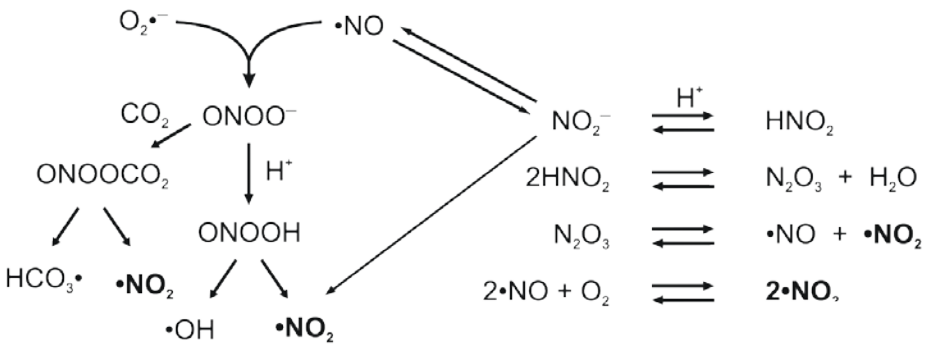

Figure 1. Redox reactions of nitric oxide in inflammation and formation of nitrogen dioxide. The nitrating species nitrogen dioxide is generated by a variety of reactions in the inflammatory milieu. Following the rapid reaction of nitric oxide and superoxide, peroxynitrite (ONOO-) may react with carbon dioxide or be protonated, and in each case will decompose to form $^{\cdot}NO_2$ along with another radical species. Oxidation of nitric oxide to nitrite can be followed by $^{\cdot}NO_2$ formation as well in acidic compartments through the decomposition of N_2O_3. Finally, nitric oxide reaction with O_2 can yield $^{\cdot}NO_2$ directly.

1.3 CHARACTERISTICS AND REDOX-DEPENDENT GENERATION OF ELECTROPHILES

An electrophile is a compound which forms a bond with a nucleophile by accepting an electron pair. This reaction occurs at an electron-poor region of the molecule whose presence can typically be attributed to a nearby electron-withdrawing substituent, most commonly a carbonyl (C=O). Electrophiles may be produced endogenously, obtained from the diet, or arise during metabolism of xenobiotics. Here we concern ourselves with endogenous electrophiles generated as by-products of lipid oxidation.

Olefins conjugated to electron-withdrawing groups constitute a major portion of endogenously-produced electrophiles. Such compounds are products of cellular redox reactions, and while important in inflammation, are also produced in a tightly regulated fashion during normal metabolism. In the latter case production is predominantly enzymatic, while free radical-based non-enzymatic pathways become significant under oxidizing conditions. Notably, polyunsaturated fatty acids (PUFA) are highly susceptible to oxidation and readily undergo peroxidation by enzymatic or free radical chain reaction mechanisms, yielding numerous electrophilic species (10).

Non-enzymatic peroxidation of unsaturated fatty acids is initiated by hydrogen abstraction from a methylene carbon along the lipid backbone, leaving an unpaired electron. Under aerobic conditions, this newly formed radical ($L^{\bullet}$) reacts rapidly with oxygen to form a lipid peroxyl radical ($LOO^{\bullet}$), which can be reduced to a hydroperoxide (LOOH), propagate the chain reaction by abstracting a proton from an adjacent PUFA or form an endoperoxide via cyclization (11). $L^{\bullet}$ and $LOO^{\bullet}$ may also react with other radical species such as $^{\bullet}NO$ or $^{\bullet}NO_2$ (12). Hydroperoxides and endoperoxides are unstable and decompose to form a variety of carbonyl-containing compounds, some with electrophile functionality, while nitro-fatty acids formed by $^{\bullet}NO_2$ addition acquire electrophilic character due to the strong electron-withdrawing activity of the nitro group (10,13). PUFA oxidation and peroxidation also occur enzymatically via cyclooxygenase (COX) and lipoxygenase (LOX) activities, and when followed by dehydrogenase metabolism can yield α,β-unsaturated carbonyl-containing electrophiles (10). Arachidonic acid (a ω-6 fatty acid) and longer-chain ω-3 fatty acids can be modified to produce electrophilic species by these enzymatic pathways. In brief, fatty acid-derived electrophiles can be considered in two groups: α,β-unsaturated carbonyls and nitro-fatty acids.

1.3.1 α,β-unsaturated carbonyls

α,β-unsaturated carbonyls are a class of electrophiles whose ranks include the reactive aldehydes 4-hydroxynonenal (4-HNE), 4-oxononenal (4-ONE), and acrolein (2-propenal), as well as ω-3 and ω-6 fatty acid derivatives such as the cyclopentenone prostaglandins and oxo-eicosatetraenoic acids (oxo-ETEs) (14). 4-HNE and 4-ONE are non-enzymatically derived, largely from decomposition of peroxidized linoleic acid. By this model, autoxidation of lineoleate forms 13-hydroperoxy-9,11-octadecaenoic acid (13-HPODE), which oxidatively decomposes to 4-hydroperoxynonenal (HPNE). HPNE can be converted to either 4-HNE or 4-ONE by reduction or dehydrogenase activity, respectively (Figure 2) (15). Acrolein is produced during lipid peroxidation by poorly elucidated mechanisms, and is also formed during oxidation of carbohydrates and amino acids (16).

ω-6-derived electrophiles are produced enzymatically via COX and LOX as products of the ω-6 fatty acid arachidonate. These include the A and J series of cyclopentenone prostaglandins, such as 15-deoxy-prostaglandin J2 (15d-PGJ_2), along with the LOX-derived 5-, 12-, and 15-oxo-ETEs (14). However, non-enzymatic pathways can yield similar or identical products to the above. Endoperoxides formed as intermediates during non-enzymatic peroxidation can undergo a series of internal rearrangements and reactions to form cyclopentenone structures containing α,β-unsaturated carbonyls (17). oxo-ETEs can also be generated through non-enzymatic lipid oxidation (18). ω-3 fatty acids are subject to many of the same oxidation pathways as ω-6 fatty acids, though their corresponding electrophilic species have distinct biological properties. It has been proposed that oxidation products of ω-3 fatty acids mediate their protective cardiovascular effects (19).

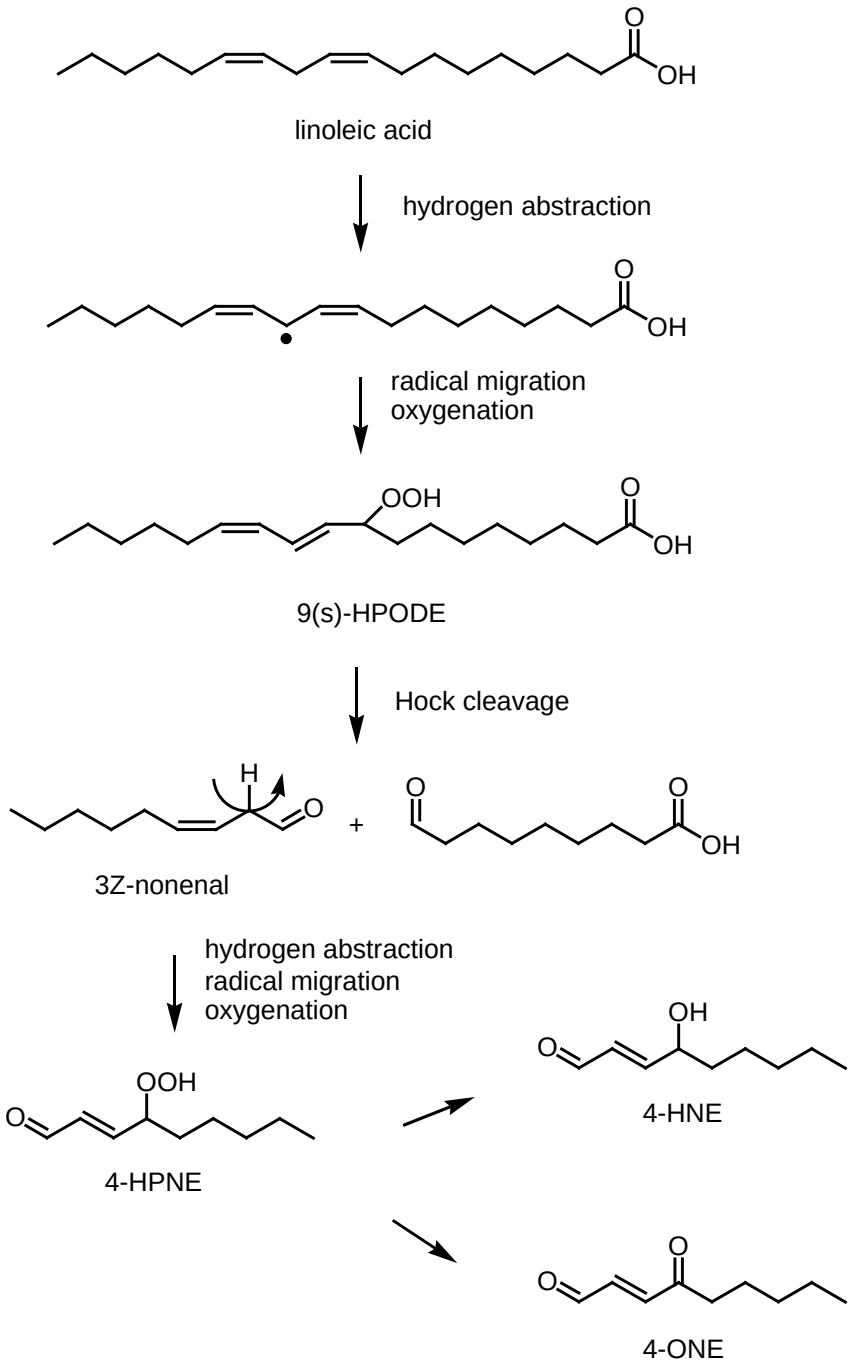

Figure 2. Non-enzymatic generation of electrophiles from linoleic acid. Following peroxidation of linoleic acid, 9(s)-hydroperoxyoctadecadienoic acid (HPODE) undergoes Hock fragmentation to form 3Z-nonenal. Peroxidation of this substrate yields the α,β-unsaturated carbonyl-containing electrophiles 4-hydroxynonenal and 4-oxononenal.

1.3.2 Nitro-fatty acids

Nitro-fatty acids (NO_2-FA) are electrophilic fatty acid derivatives that are a byproduct of lipid oxidation reactions involving reactive O_2 and $^{\bullet}NO$-derived species. NO_2-FA consist of an electron-withdrawing vinyl NO_2 group present in an unsaturated fatty acid. $^{\bullet}NO$ is lipophilic and tends to concentrate (roughly 20-fold) in lipid microenvironments, including the interiors of membranes and lipoproteins. Molecular O_2 compartmentalizes similarly and consumes $^{\bullet}NO$, creating a molecular “lens” effect that accelerates $^{\bullet}NO$ oxidation several hundred-fold and yields an array of oxidizing, nitrosating and nitrating species (20). Multiple RNS ($^{\bullet}NO_2$, $ONOO^-$, HNO_2, NO_2^+) facilitate oxidation and nitration of PUFA. The nitrating compound $^{\bullet}NO_2$ may form NO_2-FA by hydrogen abstraction and reaction with $L^{\bullet}$ radicals, while NO_2^+ mediates nitration by ionic addition mechanisms. $ONOO^-$ serves as a membrane-diffusible PUFA-nitrating agent. HNO_2, which is the protonated form of nitrite (NO_2^-), has a pKa of 3.4, rendering it a possible player in NO_2-FA formation in acidic tissues or organelle (21). Nitrite may also participate in nitration reactions indirectly, via its conversion to $^{\bullet}NO$ and $^{\bullet}NO_2$ by the nitrite reductase activity displayed by many heme-containing proteins. While all of these mechanisms may have a role in NO_2-FA production *in vivo*, it remains unknown which predominate. It is clear, however, that NO_2-FA production occurs *in vivo* in response to pathologic stress. A recent study demonstrated that mice that have undergone cardiac ischemia via a coronary artery ligation procedure generate increased levels of NO_2-FA compared to sham controls (3).

Thus, during oxidative stress and inflammation, ROS, RNS, and unsaturated fatty acids combine to generate various reactive electrophiles. Due to the signaling characteristics of electrophiles, the generation of these species has implications in the propagation and resolution of inflammatory disease.

1.4 SIGNALING ACTIONS OF ELECTROPHILES

Like other reactive species since shown to be important in normal physiology, electrophiles were first recognized for their cytotoxicity. Indeed, electrophiles may have deleterious effects: alkylation of DNA by electrophilic compounds is a well-characterized phenomenon that promotes carcinogenesis, and large-scale or irreversible disruptions of protein function may lead to apoptotic or necrotic cell death. However, this view changed as various adaptive mechanisms for countering electrophilic stress were elucidated, and it is now appreciated that electrophiles at sub-toxic levels induce protective or adaptive responses to stress (22). Electrophilic lipids engage in cell signaling via Michael addition reactions with cellular nucleophiles (Figure 3). As these include cysteine thiols, the imidazole of histidine, and the ε-amino group of lysine, post-translational modification is a major mode of electrophile signaling, leading to changes in protein trafficking, function and catalytic activity (23). Importantly, despite their similar chemical reactivities, different electrophiles may target distinct protein pools (24). Of particular importance are reactions with transcription factors, several of which are involved in coordinating cellular responses to electrophiles.

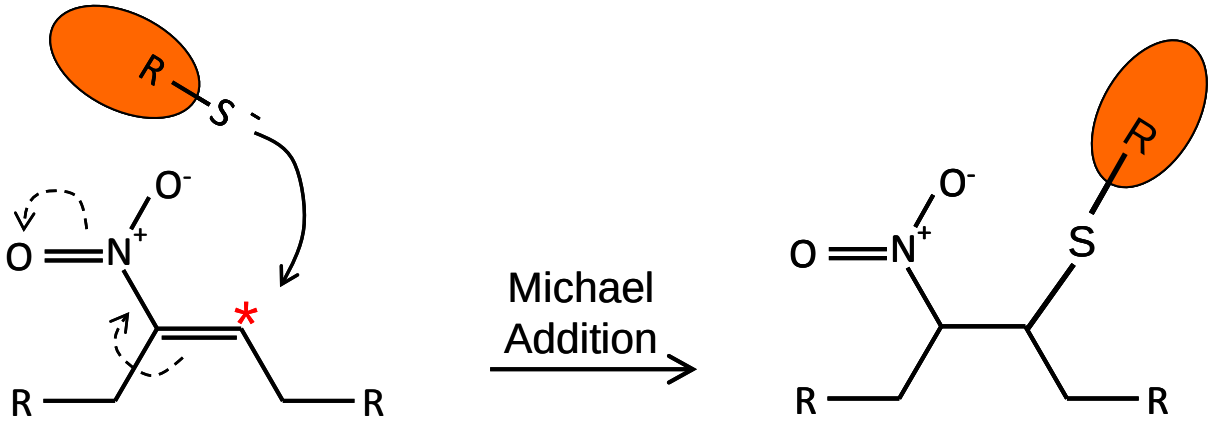

Figure 3. Thiol adduction by nitro-fatty acids. The electron-withdrawing nitro-group reduces electron density at the carbon indicated by an asterisk. This carbon is then subject to attack by a nucleophile (in this example, a cysteine thiolate). The Michael adduct thus formed represents a covalent, reversible modification.

1.4.1 Electrophile-activated signaling pathways

A canonical example of genomic regulation common to most electrophiles is the Nrf2/Keap1 (Nuclear erythroid 2-related factor 2/Kelch ECH associating protein) pathway. Under basal conditions, the transcription factor Nrf2 is held in the cytoplasm and tagged for proteasomal degradation through its regulator, Keap1, which possesses several reactive and evolutionarily conserved cysteine residues critical in maintaining its interaction with Nrf2. Covalent adduction of these cysteines releases Nrf2, which translocates to the nucleus and activates the *cis*-acting electrophile response element (ERE), which regulates the expression of phase-II genes involved in electrophile detoxification and restoration of redox balance, including glutathione S-transferases (GSTs), heme oxygenase-1 (HO-1), thioredoxin, and components of the glutathione synthesis pathway (25). Another target of electrophiles, peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor involved in regulation of lipid metabolism and cell differentiation, and the target of the thiazoladinedione (TZD) class of anti-hyperglycemic medications. In recent years various endogenous electrophiles have been promoted as endogenous PPARγ ligands, including the cyclopentenone prostaglandin 15d-PGJ_2 and NO_2-FA. Unlike TZDs, these electrophiles bind the receptor covalently, and ultimately lead to a gene expression profile distinct from that of their synthetic counterparts (26). Electrophiles, notably 15d-PGJ_2, 4-HNE, and NO_2-FA, also signal through the heat-shock factor (HSF) family of transcription factors, leading to upregulation of chaperones (heat-shock proteins) involved in protein folding, trafficking, and turnover (27). In addition to the activation of cytoprotective signaling by these mechanisms, electrophiles downregulate the pro-inflammatory gene expression induced by nuclear factor κB (NF-κB), a multi-subunit transcription factor whose nuclear translocation is inhibited by its association with inhibitor of κB (IκB) under basal conditions. The NF-κB p50 and p65 subunits and IκB possess electrophile-reactive residues of functional significance, which when modified suppress NF-κB transcription of target genes by inhibiting translocation or impairing DNA binding (14). While these pathways illustrate the variety of cytoplasmic and nuclear signaling targets of electrophiles, mitochondrial transduction of electrophile signaling will be addressed in particular.

1.5 THE MITOCHONDRION AS A SOURCE AND TARGET OF ELECTROPHILES

1.5.1 Electrophile generation by mitochondria

As noted above, the generation of electrophiles frequently involves the interaction of oxidizing species and PUFA. Mitochondria are a prominent source of reactive oxygen and nitrogen species and possess an abundance of unsaturated fatty acids on the vast surface area of their inner membranes, thus representing a fertile environment for lipid peroxidation and electrophile production (28). In addition, cytochrome *c*, along with its involvement in electron transport, functions as a peroxidase when structurally destabilized by oxidation or upon interactions with anionic phospholipids. The result of this activity is primarily formation of hydroxy and hydroperoxy derivatives of cardiolipin, a phospholipid found exclusively in mitochondrial membranes. These oxidized species are critical factors in the induction of apoptosis through cytochrome *c* release (29). Oxidized fatty acids are preferentially hydrolyzed by mitochondrial phospholipase A2 (PLA_2) activity, and may be modulated by ROS production—one mitochondrial PLA_2 isoform is activated by superoxide (30). Combined, these activities may provide a mechanism for the generation of free electrophilic fatty acid derivatives by mitochondria.

Mitochondria are also involved in the production of NO_2-FA. For example, mitochondrial NO_2-FA levels increase following ischemic preconditioning of *ex vivo* Langendorff-perfused mouse hearts, to an estimated concentration of several hundred nanomolar within the organelle (31). Additional evidence of mitochondrial NO_2-FA production comes from recent studies using isolated rat liver mitochondria, which produce [^{15}N] nitro-oleic and nitro-linoleic acid when supplied with only [^{15}N] NO_2^-, as demonstrated by LC/MS analysis. NO_2-FA production increases only slightly in the presence of substrate, but is abolished by addition of sodium cyanide. However, inhibition of complex I with rotenone has no effect on NO_2-FA levels, suggesting that function of only downstream respiratory chain components is required (32). The mechanism of NO_2-FA synthesis under these conditions remains undefined, and work to determine factors that modulate NO_2-FA production from NO_2^- is ongoing.

1.5.2 Mitochondrial targets of electrophiles

In light of these observations, mitochondria likely experience high local concentrations of electrophilic fatty acid derivatives, and electrophile effects within mitochondria should be relevant in normal and oxidatively perturbed physiology. Importantly, mitochondria are enriched in thiol-containing proteins that are subject to Michael addition reactions, and the relatively high matrix pH increases the amount of thiolate anion available for these reactions. One broad proteomic analysis identified 809 independent protein targets of electrophiles in mitochondria, with diverse metabolic and signaling roles (24).

Effects on oxidative phosphorylation are not consistent among electrophiles, or even with identical chemical species in different experimental contexts. For example, 4-HNE inhibits respiration at cytochrome *c* oxidase in mitochondria isolated from rat hearts, but inhibits complex III more potently in rat brain mitochondria and may also inhibit NADH-linked respiration via α-ketoglutarate dehydrogenase (33,34). However, intact cardiac myocytes increase their respiration rates upon exposure to low micromolar concentrations of 4-HNE (35). Similarly, 15d-PGJ_2, which inhibits complex I in isolated mitochondria, leads to increased complex I activity in endothelial cells, possibly by upregulating ROS production (36). A similar pattern may emerge with NO_2-FA: in a study on cardiac myocytes, exposure to nitro-linoleic acid precipitated mild uncoupling via activation of uncoupling protein 2 (UCP2) and the adenine nucleotide transporter (ANT), seen as an increase in oligomycin-clamped state 4 respiration, while preliminary data from isolated mitochondria indicate that NO_2-FA inhibit respiration (31,32). These studies highlight the importance of assessing electrophile effects in cell and organ systems along with subcellular fractions. Mitochondrial ROS production is also affected by many electrophiles. In addition to its respiratory effects via complex I, 15d-PGJ_2 induces ROS formation in intact cells, even at non-toxic concentrations. The mitochondrial origin of ROS was confirmed by dichlorofluorescein (DCF) imaging studies in endothelial and Rho0 cells and reversal of ROS production with rotenone (37). 4-HNE, despite having distinct effects on the respiratory chain from 15d-PGJ_2, also initiates ROS generation by mitochondria in various cell lines (38,39). Importantly, pathological levels of electrophiles may also deplete mitochondrial GSH stores through the activity of GSTs, impairing the organelle's antioxidant capacity (40).

Current data suggest that the influence of electrophilic species on mitochondria can limit the initiation and progression of diseases whose pathogenesis involves mitochondrial dysfunction.

1.6 MITOCHONDRIA AS MEDIATORS AND MITIGATORS OF TISSUE RESPONSES TO METABOLIC AND INFLAMMATORY STRESS

In addition to their critical role in ATP synthesis, mitochondria are central coordinators of stress responses, integrating diverse cellular signals regarding energy balance, apoptosis, oxygen tension, and redox balance while driving downstream signaling events.(41) The dysregulation of mitochondrial function observed in human diseases such as diabetes and cardiac ischemia/reperfusion exemplifies the importance of this regulatory ability.

1.6.1 Metabolic stress—diabetes

Mitochondrial dysfunction is involved in the pathogenesis of diabetes. Type 2 diabetics have impaired aerobic capacity at the whole-organism level, and examination of specific insulin-resistant tissues has revealed impaired oxidative phosphorylation with decreased expression of respiratory chain proteins and numerous other aberrant metabolic behaviors (42). Adipose tissue and skeletal muscle display dramatic changes in mitochondrial morphology in diabetics, including smaller size and lower numbers (43). The molecular explanations for these changes are still under investigation, but include downregulation of PPAR-γ coactivator 1α (PGC-1α), which is a key activator of respiratory complex expression and mitochondrial biogenesis (42). In addition, muscle tissues in T2DM exhibit “metabolic inflexibility,” or an inability to switch between glucose and lipid oxidation, resulting in preferential uptake and oxidation of lipids and decreased glucose metabolism. In cardiac muscle this effect is partially mediated by activation of PPAR-α, likely through interactions with long-chain fatty acid ligands (44). Enhanced fatty acid oxidation leads to redox imbalance via an increased NADH:NAD^+ ratio, which further inhibits glucose oxidation by impeding pyruvate dehydrogenase activity and promotes mitochondrial ROS formation. Mitochondrial ROS are implicated in many disease-related complications, including insulin resistance, microvascular dysfunction, and diabetic cardiomyopathy. In the latter case, one model proposes that ROS and lipid peroxidation byproducts lead to uncoupling protein (UCP) activation, lowering the inner mitochondrial membrane (IMM) potential, increasing O_2 consumption, and decreasing contractile efficiency (45).

1.6.2 Cardiac ischemia/reperfusion

Aberrant mitochondrial function is central to the pathology of cardiac ischemia/reperfusion injury. Severe ischemia depletes myocardial ATP, leading to ion pump failure and ionic imbalance, including an excess of cytosolic Ca^{2+} following loss of SERCA activity (46). The permeability transition pore (PTP) remains closed due to the low pH and low intramitochondrial Ca^{2+} concentrations during ischemia. These conditions are reversed upon reperfusion: pH increases and the IMM potential is restored, leading to Ca^{2+} influx to mitochondria, favoring PTP opening and ultimately apoptotic cell death (47). An additional injurious factor during reperfusion is ROS generation, which occurs at high levels. ROS and their reactive byproducts contribute to apoptosis and necrosis, as evidenced by effective antioxidant therapeutic interventions in animal models (48).

Though energetic failure is a precipitating event in I/R injury, pharmacologic or physiologic disruption of mitochondrial metabolism is associated with cardioprotection. Electron transport chain inhibitors acting at each of the respiratory complexes have been shown to improve outcomes in I/R, and ischemic preconditioning leads to ETC inhibition at multiple levels. The mechanisms underlying this seemingly paradoxical effect are not well understood, but it is proposed that respiratory chain inhibition at the time of reperfusion weakens the accompanying ROS burst and Ca^{2+} influx to mitochondria (49). Another metabolic pathway relevant to cardioprotection is fatty acid oxidation. Glucose oxidation is more efficient than fatty acid oxidation per mole O_2 consumed, a fact which becomes especially salient in situations of low O_2 supply, and which has been pharmacologically exploited: the 3-ketoacyl-coenzyme A thiolase inhibitor class of heart failure medications, which includes trimetazidine and ranolazine, inhibit FAO and shift myocardial substrate usage from fatty acids to glucose (50). The reasons for this change in efficiency are complex, but are due to the lower P/O ratio for fatty acid oxidation versus glucose oxidation, mitochondrial uncoupling (via UCP shuttling of fatty acid anions or activation of UCPs by ROS), and futile cycling of fatty acid metabolic intermediates (51).

1.7 CONCLUSION

Mitochondria are a critical nexus of cell signaling processes in inflammation. The increased redox activity of mitochondria during metabolic stress and cardiac ischemia/reperfusion favors formation of electrophilic lipid derivatives through PUFA interactions with ROS and RNS. These species adduct proteins at nucleophilic cysteine, histidine, and lysine residues, and activate adaptive signaling pathways by regulating transcription factor activity. In addition, mitochondrial targets of electrophiles are numerous, and electrophiles can be cytoprotective in settings of mitochondrial dysfunction (NO_2-FA in models of type 2 diabetes and cardiac I/R, for instance). The electrophile targets in mitochondria which promote protection are incompletely characterized, but may include respiratory chain components and UCPs (modulating ATP and ROS production and O_2 consumption), matrix metabolic enzymes (altering the contribution of fatty acid oxidation to cellular bioenergetics), and apoptotic machinery. Improving our understanding of electrophile signaling in mitochondria will aid in the development of effective interventions for metabolic and inflammatory disease.

1.8 HYPOTHESIS

The overall hypothesis for the studies presented here is that electrophilic fatty acids generated by metabolic and inflammatory processes serve to induce adaptive tissue responses. More specifically, based on the information outlined above it was hypothesized that nitro-fatty acids covalently modify and alter the activity of respiratory chain components to induce tissue-protective metabolic alterations.

2.0 FATTY ACID NITROALKENES MODULATE MITOCHONDRIAL RESPIRATION AT COMPLEX II AND INDUCE RESISTANCE TO ISCHEMIC CARDIAC INJURY

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2.1 ABSTRACT

Nitro-fatty acids (NO_2-FA) are metabolic and inflammatory-derived electrophiles that promote tissue protection in renal and cardiac ischemia-reperfusion (IR). Since drugs which inhibit respiratory chain function and favor glycolysis can display cardioprotective actions, it was hypothesized that NO_2-FA impact mitochondrial electron transport and induce cardioprotective metabolic shifts in cells. Nitro-oleic acid ($OANO_2$) addition inhibited complex II-linked (but not complex I-linked) respiration in isolated rat heart mitochondria at low μM concentrations. Complex II activity analysis revealed $OANO_2$ inhibition was pH-dependent and reversible by the addition of the low molecular weight thiol β-mercaptoethanol (BME). Following electrophoretic separation of proteins from $OANO_2$-treated mitochondria, addition of BME to the Coomassie-stained band corresponding to the Fp subunit of complex II yielded $OANO_2$-BME adducts by reverse Michael addition, revealing post-translational protein modification by $OANO_2$. Extracellular flux analysis of H9C2 cardiomyoblasts confirmed the importance of respiratory inhibition by $OANO_2$, as low μM concentrations of $OANO_2$ reduced basal and maximal respiration and favored glycolytic metabolism. The addition of NO_2-FA also induced acute cardioprotection in an isolated perfused heart IR model. Together these findings indicate that NO_2-FA can promote cardioprotection by inducing a shift from respiration to glycolysis in the post-ischemic interval.

2.2 INTRODUCTION

The inflammatory stress associated with myocardial ischemia/reperfusion promotes the *in vivo* nitration of unsaturated fatty acids to yield electrophilic nitro-fatty acids (NO_2-FA) (3) that mediate adaptive signaling actions in a variety of models of metabolic and inflammatory disease (52,53). Broadly and reversibly reactive with protein Cys and His residues, these compounds activate and deactivate diverse cell signaling cascades, acutely altering protein catalytic activity (GAPDH, MMP9) and ultimately modulating inflammatory and metabolic gene expression (via reactions with the redox-sensitive transcriptional regulatory factors Keap1/Nrf2, NF-κB, HSF, and PPARγ) (13,54,55). This multiplicity of targets suggests that several independent mechanisms underlie the beneficial effects of NO_2-FA. Indeed, previous investigations

describing cardioprotection by NO_2-FA have implicated both the suppression of NF-κB-regulated gene expression in an *in vivo* murine coronary ligation model) and inhibition of the mitochondrial adenine nucleotide transporter (ANT, in an isolated perfused rat heart global ischemia model) (3,56).

Further interest in mitochondrial-based mechanisms of NO_2-FA -mediated cardioprotection arises from the contributions of this organelle to fatty acid nitration, attributable to the proximity of reactive species formation and a convoluted membrane rich in readily-oxidized polyunsaturated fatty acids. Separate studies have documented the generation of NO_2-FA during *in vivo* IR injury and in mitochondria following the cyclic hypoxia-reoxygenation episodes of ischemic preconditioning (3,31). Further, isolated mitochondria incubated with nitrite (NO_2^-) produce NO_2-FA even under normoxic conditions, after NO_2^- oxidation to, nitrogen dioxide ($^{\cdot}NO_2$) and subsequent addition to conjugated linoleic acid (57). As any *in vivo* cardioprotective role of NO_2-FA would be expected to involve targets proximal their site of production, redox-sensitive mitochondrial proteins represent appealing candidates.

The central role of the mitochondrion in cardiac ischemia/reperfusion injury remains an active area of discovery. The generation of partially reduced oxygen and nitric oxide (NO)-derived species, their reaction with critical constituents of the electron transport chain, calcium overload, loss of membrane potential, changes in permeability transition pore function and release of cytochrome *c* are all central events in the cardiomyocyte death that accompanies reperfusion injury (48,58-61). A number of therapeutic strategies targeting each of these events have proven capable of diminishing infarct size in experimental models. Notably, there is a statistically significant relationship between compounds that inhibit respiratory metabolism and the promotion of cardioprotection following IR, thus structurally-diverse and mechanistically-distinct inhibitors acting at each complex of the respiratory chain have proven effective in reducing infarct size (49). The predominant mechanisms underlying this phenomenon both remain a subject of debate, and motivate interest in identifying additional drug candidates that promote the metabolic shift from respiration to glycolysis. For example, high-throughput screening recently revealed a novel function for the FDA-approved motion sickness drug meclizine, which has proven cardio- and neuroprotective in IR models (62).

As the electron transport chain is exquisitely sensitive to reactive species, we hypothesized that NO_2-FA mediate cardioprotection, in part, through the post-translational modification and inhibition of respiratory chain components. Herein, complex II is established as a sensitive and reversible target of inhibition by NO_2-FA in heart mitochondria. This led to

respiratory inhibition and enhancement of glycolysis in a cardiomyoblast cell line and acute cardioprotection in an isolated perfused rat heart model of global IR.

2.3 EXPERIMENTAL PROCEDURES

2.3.1 Reagents

Nitro-oleic and nitro-linoleic acid were synthesized according to an existing method (63). Other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

2.3.2 Mitochondrial isolation and respirometry

Fresh mitochondrial isolates were obtained from rat hearts. Following anesthesia with isoflurane, hearts were rapidly excised from male Sprague-Dawley rats and placed into cold mitochondrial isolation buffer (10 mM HEPES, 250 mM sucrose, 0.2 mM EDTA). Hearts were minced to 2 mm slices, then placed in clean isolation buffer and 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 glass-teflon homogenizer. Following addition of protease inhibitor to the homogenate, it was centrifuged at 600x*g* and the pellet discarded. The supernatant was retained and centrifuged at 8000x*g*, the pellet washed with isolation buffer and again centrifuged at 8000x*g*. The pellet was resuspended in a minimal volume (~500 µl) of isolation buffer to yield a preparation of approximately 20 mg/ml protein. For respirometry studies, this preparation was diluted in respiration buffer (120 mM KCl, 25 mM sucrose, 10 mM HEPES, 1 mM EGTA, 1 mM KH_2PO_4, 5 mM $MgCl_2$) in a stirred chamber containing a Clark-type O_2 electrode. Respiratory control ratios (RCR) were determined at 1 mg/ml final protein concentration with 8 mM malate/4 mM glutamate as substrates for state 4 respiration and addition of 10 mM ADP to initiate state 3. Preparations with RCR values <4 were discarded. For NO_2-FA inhibition experiments, a final protein concentration of 350 µg/ml was employed. $OANO_2$ and OA were diluted from methanol stocks to final concentrations of 0-10 µM, 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 rates determined from the linear portion of the curve, after ~3 min. When mitochondria were used following freeze-thaw, oxidized cyt *c* was added just before substrate at 300 µg/ml. When FCCP was used, it was diluted from a methanol stock to achieve a final concentration of 500 nM and added 5 min before addition of $OANO_2$.

2.3.3 Respiratory chain complex activity measurements

Rat heart mitochondrial preparations described above (~20 mg/ml) were frozen until use. All activity measurements were made in the presence of 50 µg mitochondrial protein in 10 mM HEPES buffer containing 2.5 mM $MgCl_2$ and 2 mM KCl. Complex II activity was determined in a reaction mixture (1 mL) which also contained 0.1 mM EDTA, 75 µM DCPIP, 50 µM decylubiquinone and 20 mM succinate, following the decrease in absorbance at 600 nm. Complex II+III activity was determined in the same HEPES buffer with 50 µg mitochondrial protein, 20 mM succinate, 50 µM decylubiquinone and 50 µM oxidized cytochrome *c*, by following the increase in absorbance at 550 nm (using an extinction coefficient of 21 $mm^{-1}cm^{-1}$. In experiments where mitochondria were incubated with $OANO_2$ or OA before centrifugation and resuspension, preparations were incubated with 10 µM fatty acid in buffer at pH 9.0 for 10 min, followed by centrifugation at 14,000 x *g*. In some cases, 1 mM BME was added for an additional 10 min before centrifugation. The supernatant was discarded, the pellet resuspended at pH 7.4, and complex activity determined under the conditions described above.

2.3.4 Respiratory chain complex protein analyses

Blue native gel electrophoresis resolution of mitochondrial respiratory complexes was performed as described (64) with minimal modification. 400 µg of rat heart mitochondrial protein was treated with 0 or 20 µM $OANO_2$, extracted with lauryl maltoside and separated by tricine-SDS-PAGE. Because of the sensitivity of Complex II to inhibition by fatty acid nitroalkenes, the band corresponding to complex II was a primary subject of focus. It was excised, sliced into 2 mm segments, and incubated in 200 ul ACN for 10 min at 25^{o}C. ACN was removed and the gel pieces dried under vacuum while being centrifuged for 10 min, followed by rehydration in 50 mM phosphate buffer (pH 8.0) containing 500 mM BME for 1 h at 37^{o}C. Then, pH was adjusted to

5.0 with 1 M formic acid to quench trans-nitroalkylation reactions. BME-electrophilic lipid adducts were determined in the buffer by LC/MS, following the neutral loss of BME from the adduct as described (65). In denaturing gels, identification of the 70 kDa subunit was performed by Western blot following SDS-PAGE of 40 µg mitochondrial protein treated with 0, 5, or 10 µM $OANO_2$. Identically loaded gel lanes were excised, one set for Coomassie stain and one for WB analysis. Following transfer of the WB gel to a PVDF membrane and blocking with 5% milk, a mouse monoclonal antibody (Mitosciences, Eugene, OR) against the 70 kDa Fp subunit was added at 1:2000 dilution in TTBS. After washing, an HRP-conjugated anti-mouse Ig antibody (Cell Signaling Technologies, Danver, MA) was added at 1:5000, and film was developed following addition of ECL reagents after additional washing. The Coomassie stained bands corresponding to the 70 kDa subunit were excised, sliced into 2 mm segments, and incubated in 200 ul ACN for 10 min at 25^{o}C. ACN was removed and the gel pieces dried under vacuum while being centrifuged for 10 min, followed by rehydration in 50 mM phosphate buffer (pH 8.0) containing 500 mM BME for 1 h at 37^{o}C. pH was adjusted to 5 with 1 M formic acid to quench trans-nitroalkylation reactions. BME adducts were determined in the buffer by LC/MS, following the neutral loss of BME from the adduct as described (65) with minor modifications.

2.3.5 Extracellular flux analysis in H9C2 cells

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. Compounds for injection in the Seahorse analyzer were diluted into this medium at 10x final concentration and pipetted into the injection ports on the cartridge at appropriate volumes for 10x dilution (66 µl for port A, 73 µl for port B, etc.); other treatments were administered 90 min prior to XF analysis where indicated. A Seahorse XF24 analyzer (Seahorse Bioscience, North Billerica, MA), which allows real-time determinations of O_2 and proton concentrations in a transient 7 µl chamber in each well of the plate (66), was employed to simultaneously determine glycolytic and respiratory flux. Data are expressed as either pmol/min

(for O_2 consumption), mpH/min (for extracellular acidification), or as percentage of basal rate of either of these parameters.

2.3.6 Isolated, perfused Langendorff heart preparations

Hearts were rapidly excised from male Sprague-Dawley rats following induction of anesthesia with ketamine/xylazine (80 mg/kg and 5 mg/kg), and perfused in retrograde as described (67). 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_2PO_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^{o}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 (67)) were discarded. Then, 15 min after balloon insertion 100 nM LNO_2 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^{o}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.

2.3.7 Statistical analysis

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

2.4 RESULTS

2.4.1 Nitro-fatty acid inhibition of O_2 consumption by isolated mitochondria

Electrophile effects on respiratory chain components are varied, with some electrophiles targeting multiple complexes and other electrophiles preferentially modifying distinct proteins (68,69). To first define the effects of a fatty acid nitroalkene on overall respiratory metabolism, isolated rat heart mitochondria were preincubated with low μM concentrations of $OANO_2$ or oleic acid (OA) with substrates supporting either complex I- or II-linked respiration. No inhibition of respiration was observed by up to 10 μM $OANO_2$ concentrations in the presence of the complex I-linked substrates malate and glutamate (Figure 4A). In contrast, significant inhibition of succinate-mediated respiration was observed at 3 μM $OANO_2$. This concentration also induced an initial phase of rapid respiration upon addition of succinate, but this was not sustained and ultimately a linear rate of O_2 consumption only ~30% of control was achieved (Figure 4b). Inhibition of complex II-linked respiration could be reversed by the addition of ascorbate/TMPD, substrates which donate electrons to complex IV via cytochrome *c* (Figure 18, Appendix A). Together, these data suggest that complex II is a target of NO_2-FA. One freeze-thaw cycle was sufficient to eliminate the effect of $OANO_2$ on succinate-linked respiration having added cytochrome *c* (Figure 5A). Pretreatment with the uncoupling agent FCCP blunted the inhibitory effect of $OANO_2$ (Figure 5B). Thus, the respiratory state influenced the ability of $OANO_2$ to inhibit complex II-linked respiration.

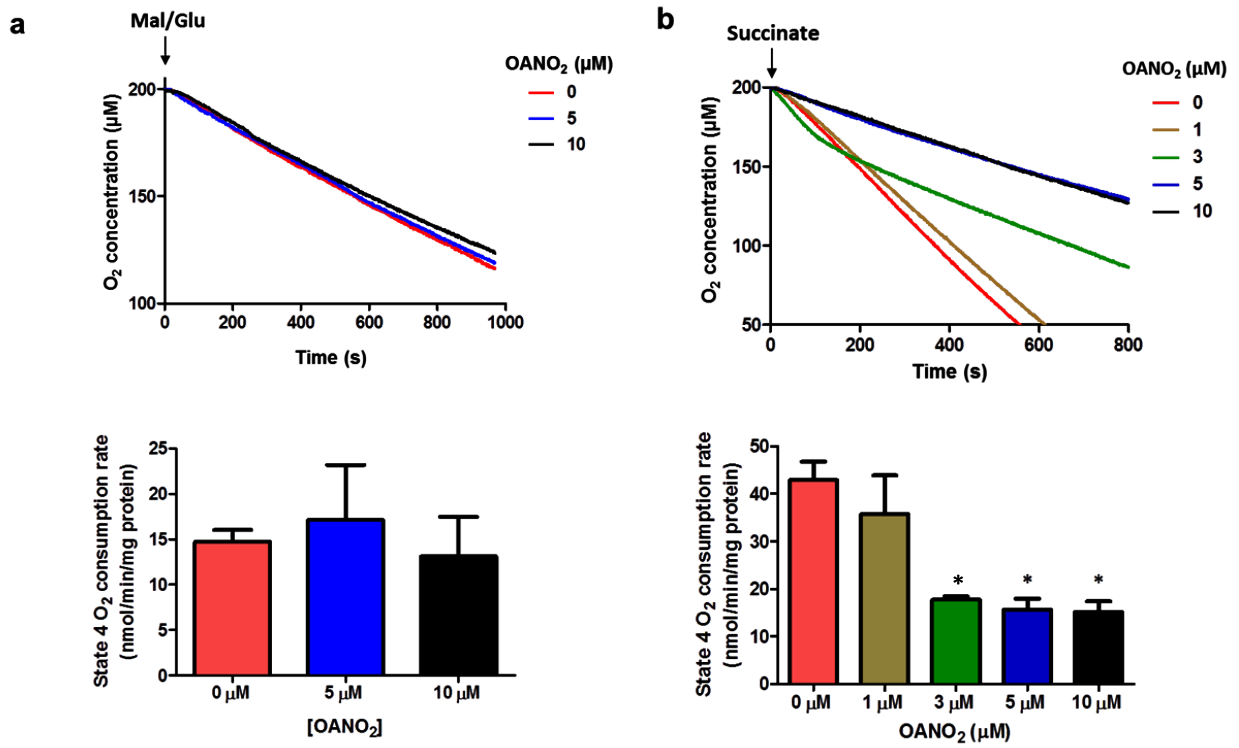

Figure 4. NO_2-FA inhibition of complex II-linked respiration. Freshly isolated rat heart mitochondria (0.5 mg, all RCR > 4) were incubated in respiration buffer (pH 7.2) for 5 min with $OANO_2$ (0-10 μM) prior to substrate addition. **a)** Representative O_2 concentration traces following addition of the complex I-linked substrates glutamate (8 mM) and malate (4 mM) (upper panel). Average rates from three independent experiments ± SD (lower panel). **b)** O_2 concentration traces following succinate (10 mM) addition (upper panel). Rates for bar graph (lower panel) were taken from the linear portion of the each trace (n=3, mean ± SD); *, $p < 0.05$ by one-way ANOVA, versus 0 μM $OANO_2$.

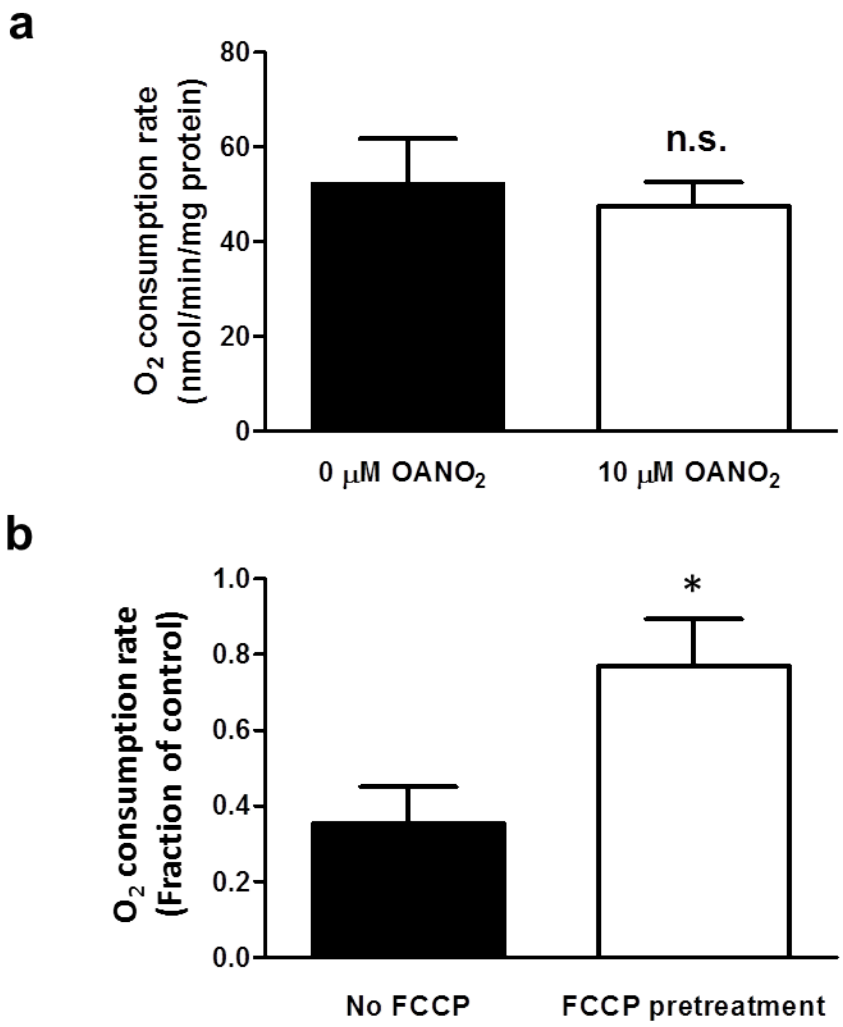

Figure 5. Effect of uncoupling on inhibition by NO_2-FA. a) Mitochondria, following one cycle of freeze-thaw, were supplied with 50 μM cytochrome *c* and incubated with 0 or 10 μM $OANO_2$ for 5 min before addition of 10 mM succinate. Values represent the linear O_2 consumption rates following succinate addition (n=3, mean ± SD). **b)** Freshly isolated mitochondria were exposed to 0 or 500 nM FCCP prior to 5 min of incubation with 10 μM $OANO_2$. O_2 consumption rates were determined following addition of 10 mM succinate (n=3, mean ± SD); *, $p < 0.05$ by Student's *t* test, two-tailed.

2.4.2 Inhibition of complex II activity by nitroalkenes is reversible and pH-dependent

Given that interventions which uncouple mitochondria (FCCP, freeze-thaw) led to a loss of respiratory inhibition by $OANO_2$, and that the availability of the Cys thiolate for Michael addition reactions is greater at high pH, the maintenance of a high matrix pH may be necessary for $OANO_2$ binding to its target residue. Preliminary experiments indicated that inhibition of complex II activity at pH 7.4 by $OANO_2$ was modest relative to the effects on succinate-linked respiration. To assess the pH-dependence of $OANO_2$ inhibition of complex II, complex II+III activity was first assayed in mitochondria subjected to a freeze-thaw cycle, followed by addition of 10 μM $OANO_2$ or OA over a range of pH values. While $OANO_2$ did not alter activity at pH 6.5 relative to control, inhibition became more pronounced with increasing pH, with a maximum inhibition of 82% reached at pH 9.0 (Figure 6).

Activity of complex II alone was not measured over this pH range, as the assay was sensitive to high pH values. Thus, to ensure that the observed inhibition was due to a loss of complex II activity, thawed mitochondria were incubated with $OANO_2$ or OA at pH 9.0, sedimented by centrifugation and resuspended in buffer at pH 7.4. Independent activity assays for complex II and complex III were then conducted. While complex II activity was significantly inhibited by $OANO_2$ (~80%, Figure 7a), complex III activity was unchanged (Figure 7b), confirming that complex II is the target of pH-dependent respiratory inhibition by $OANO_2$.

If the nitroalkene inhibition of complex II is a redox signaling event, this would be better facilitated if the Michael addition reaction were reversible. In this regard, the low molecular weight thiol β-mercaptoethanol (BME, 1 mM, pH 9.0) to $OANO_2$–treated mitochondria fully reversed the loss of complex II+III activity to levels reflective of untreated controls (Figure 8). This concentration of BME had no effect on the respiratory activity of mitochondria treated with native OA. It was concluded that $OANO_2$ inhibits complex II by thiol-reversible Michael and that the state-dependence of respiratory inhibition in freshly isolated mitochondria may be due to changes in matrix pH.

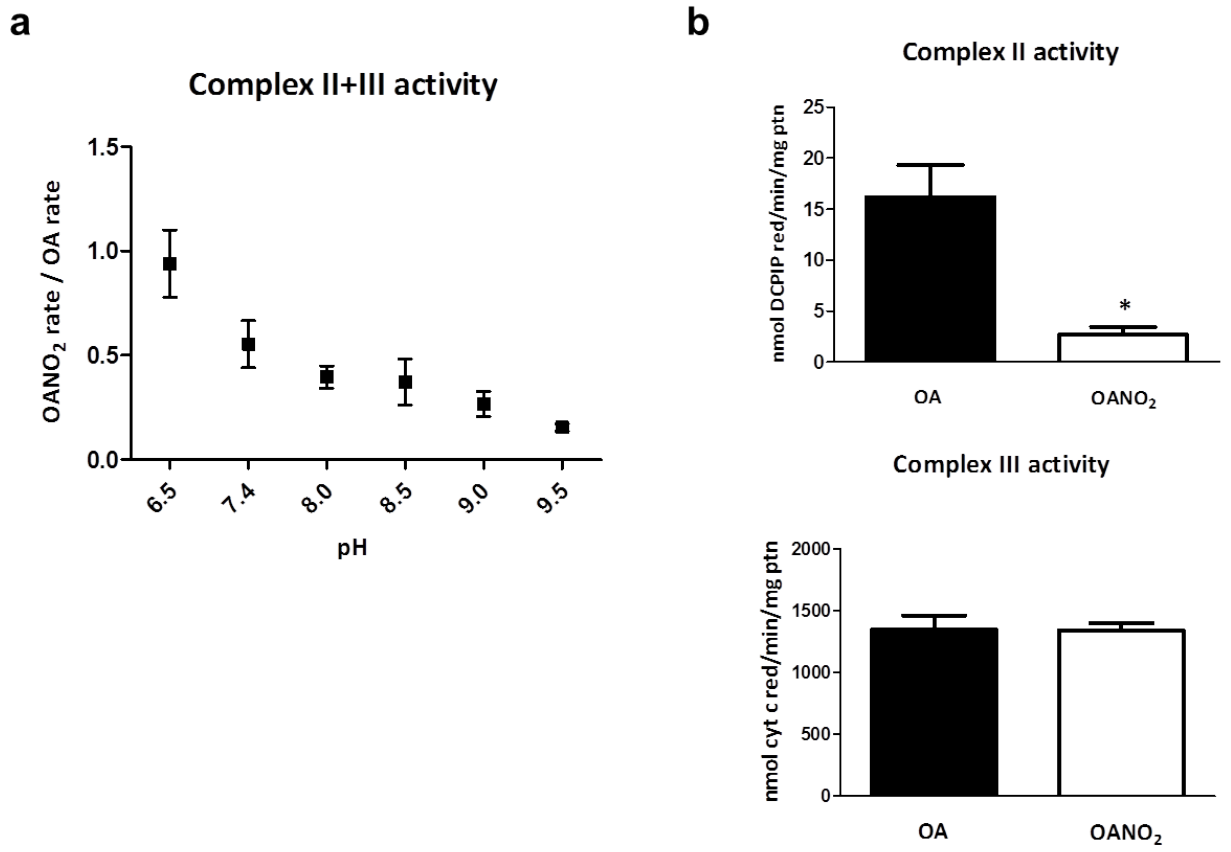

Figure 6. pH dependence of complex II inhibition by NO_2-FA. a) Following freeze-thaw, 50 µg of mitochondrial protein was incubated with 10 µM OA or $OANO_2$ at varied pH and assayed for complex II+III activity. Data represent first order rates over 240 s (n=3, mean ± SD). **b)** Mitochondria incubated with OA or $OANO_2$ at pH 9.0 for 10 min were pelleted by centrifugation, resuspended and assayed for complex II (upper panel) or complex III (lower panel) activity. All data represent n=3, mean ± SD; *, $p < 0.05$ by Student's *t* test, two-tailed, versus OA.

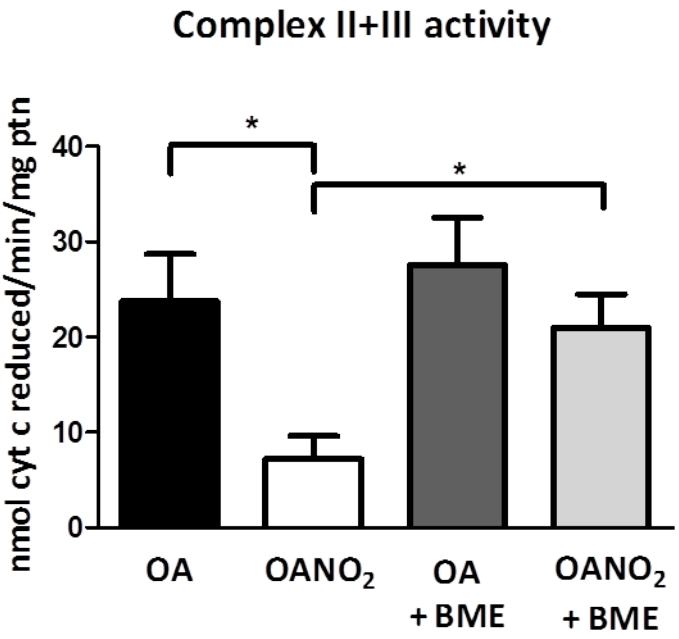

Figure 7. Complex II inhibition by NO_2-FA is reversed by low molecular weight thiols. Mitochondria treated with OA or $OANO_2$ at pH 9.0 were subsequently incubated with +/-1 mM BME, centrifuged, washed, and resuspended at pH 7.4. Complex II+III activity was then determined (n=3, mean ± SD for all groups); *, $p < 0.05$, one-way ANOVA.

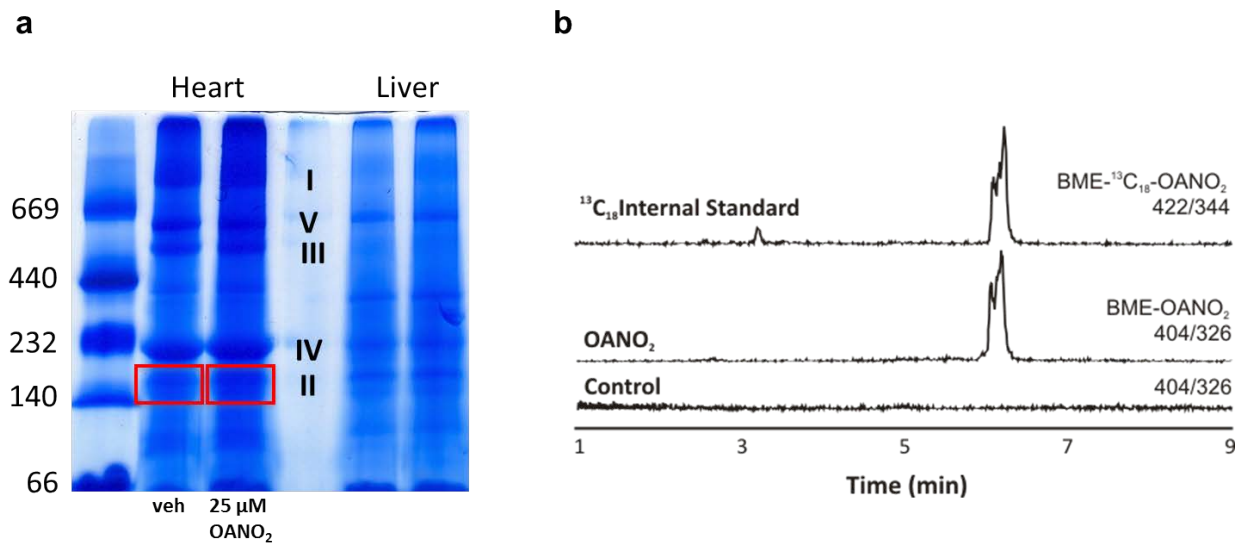

Figure 8. NO_2-FA modify complex II. 400 µg of mitochondrial protein from frozen stocks was incubated with 0 or 20 µM $OANO_2$, and respiratory complexes were solubilized with lauryl maltoside and separated by blue native PAGE. The complex II band was removed, dried with ACN and rehydrated with 500 mM BME. **a)** A representative blue native gel, with the band corresponding to complex II is highlighted. **b)** LC/MS profile of the BME-$OANO_2$ adducts observed in control and 20 µM $OANO_2$-treated conditions, with a ^{13}C internal standard shown.

2.4.3 Nitro-fatty acids directly modify the Fp subunit of complex II

The pH-dependence and thiol reversibility of complex II inhibition suggested that NO_2-FA modify Cys or His residues in this complex by Michael addition. Because other thiol-reactive species such as $ONOO^-$, nitroxyl, and HNE mediate inhibition of complex II through oxidation or adduction of critical cysteine residues on the catalytic 70 kDa Fp subunit (70-72), it was hypothesized that NO_2-FA would also modify this protein. To test this concept, a trans-nitroalkylation reaction to capture protein-adducted thiols was first employed (65). Mitochondrial lysates were incubated with $OANO_2$ (0-20 μM), proteins were denatured and then separated by SDS-PAGE. Following Coomassie staining, the band corresponding to the Fp subunit of complex II was confirmed by Western blot analysis and obtained. This was incubated with BME (500 mM) and BME-$OANO_2$ adducts representing previously protein-bound $OANO_2$ were determined by LC/MS, monitoring the neutral loss of BME from the adduct. No BME-$OANO_2$ adducts were observed in controls, while $OANO_2$ dose-dependent increases in the area of the 404/326 transition were observed, indicating that the Fp subunit of complex II is directly adducted by $OANO_2$ (Figure 9).

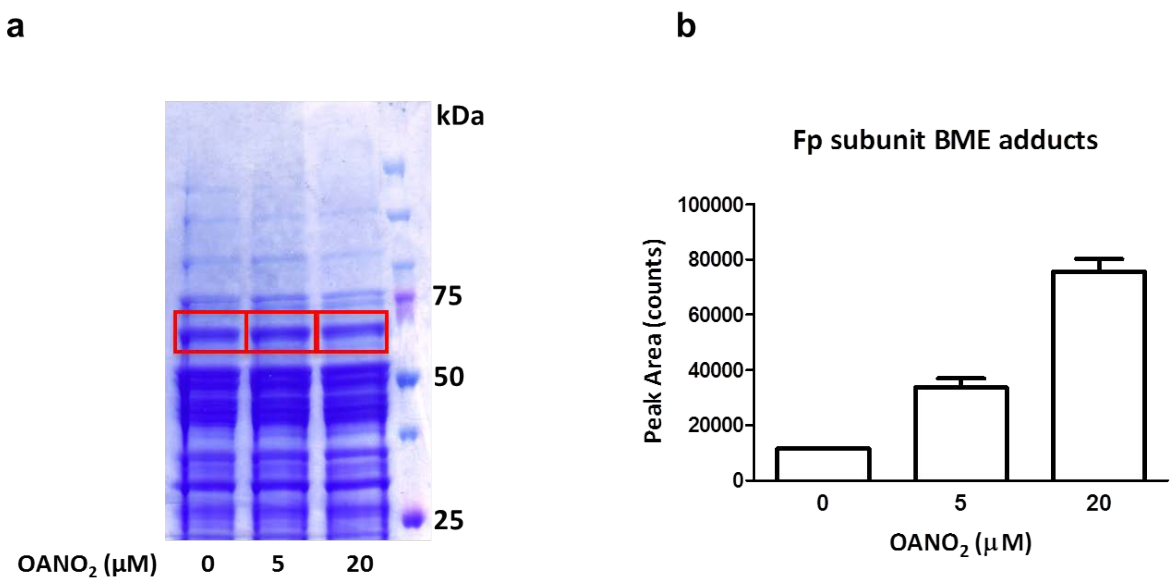

Figure 9. NO_2-FA directly modify the Fp subunit of complex II. 100 µg of mitochondrial lysate was incubated with 0, 5 or 20 µM $OANO_2$, followed by SDS-PAGE and protein labeling by Coomassie stain. The band corresponding to the 70 kDa subunit of complex II was removed, dried with ACN and rehydrated in the presence of 500 mM BME. **a)** A representative Coomassie-stained gel, with the 70 kDa Fp subunit highlighted. **b)** Peak areas for BME-$OANO_2$ adducts at 0, 5, and 20 µM $OANO_2$. n=3 per group, mean ± SD.

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

Alterations in respiratory metabolism are associated with cardioprotection in models of ischemia-reperfusion (49). To determine if the effects of NO_2-FA observed in isolated mitochondria occurred intact cells, H9C2 cardiomyoblasts were underwent metabolic analysis using a Seahorse XF24 analyzer. The O_2 consumption rate (OCR, representing respiratory activity) and the extracellular acidification rate (ECAR, reflective of glycolytic flux) were simultaneously measured and the effect of $OANO_2$ on a variety of bioenergetic parameters was determined using a previously described profiling approach (73). Basal respiration, O_2 consumption due to proton leak, and maximal respiration were determined following a 2 h incubation with low μM $OANO_2$ and OA concentrations. Basal and maximal respiration were significantly inhibited at low concentrations of $OANO_2$, while proton leak was unaffected (Figure 10a).

Separately, the acute effects of $OANO_2$ on OCR were observed by addition of 10 μM $OANO_2$ to basally respiring cells. This concentration lead to a ~30% decrease in OCR over 15 min (Figure 10b). Notably, this decrease in OCR was followed by an increase in ECAR, supporting that cells shifted from respiratory to glycolytic metabolism in response to respiratory inhibition by $OANO_2$. 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 $OANO_2$ in intact cells. Myoblasts exposed to 500 nM FCCP displayed a 60% increase in basal OCR and was not influenced addition of 10 μM $OANO_2$ (Figure 10c). This supports the concept that pH-dependent modification of respiratory components impacts the extent of $OANO_2$-mediated cellular respiratory inhibition.

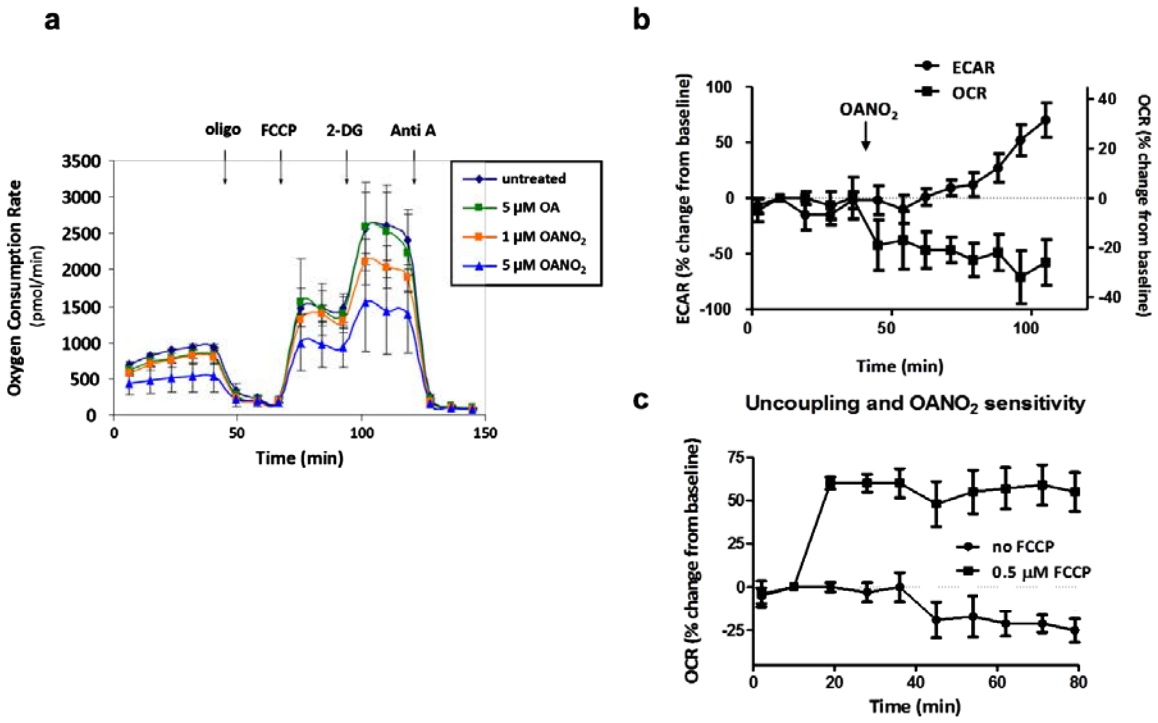

Figure 10. NO_2-FA inhibit respiration and promote glycolysis in H9C2 cells. O_2 consumption and extracellular acidification rates (OCR and ECAR) were measured in H9C2 cells in unbuffered DMEM by a Seahorse XF24 analyzer. **a)** Following 2 h of incubation with 0-5 µM $OANO_2$, basal OCR was measured, followed by proton leak, maximal respiration, and non-mitochondrial O2 consumption (induced by oligomycin, FCCP and 2-DG, and antimycin A, respectively). A representative experiment is shown. **b)** 10 µM $OANO_2$ was added to respiring cells and the change in OCR and ECAR recorded. **c)** Addition of 10 µM $OANO_2$ after uncoupling of respiration with 500 nM FCCP. For all experiments, n=5 wells per group, mean ± SD.

2.4.5 Nitro-fatty acids are cardioprotective in an *ex vivo* cardiac ischemia-reperfusion model

Because compounds which shift cellular metabolic activity from respiration to glycolysis are often protective in ischemia-reperfusion injury and some complex II inhibitors can induce cardioprotective effects, the influence of fatty acid nitroalkenes on cardiac function was evaluated in a Langendorff-perfused heart model. For this, 100 nM LNO_2 or LA was administered for 15 min prior to induction of no-flow ischemia. Following 35 min of ischemia, flow was restored for 1 h, during which recovery of cardiac output (rate-pressure product, RPP) was monitored. Heart rate, coronary flow, and LVDP were all unaffected by LNO_2 administration prior to ischemia. RPP recovery in LNO_2-treated hearts was significantly greater than that of LA-treated hearts during the reperfusion interval (Figure 11).

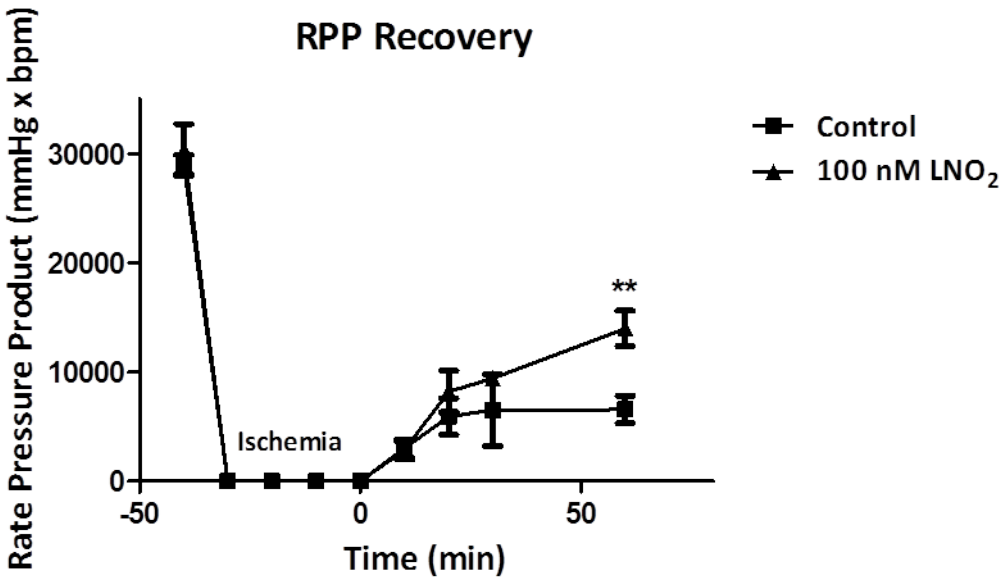

Figure 11. NO_2-FA preserve myocardial function *ex vivo* after global ischemia. Following 15 min of equilibration, a pressure balloon was inserted into the left ventricle, and pressure changes measured by a force transducer. Rate pressure product (RPP) was determined as the product of heart rate and LV developed pressure. The data represents the recovery of RPP following 35 min global, normothermic ischemia. All data points n=6, mean ± SD; **, $p < 0.01$, for Student's *t* test versus vehicle control at the time point indicated.

2.5 DISCUSSION

Nitroalkene fatty acid derivatives are generated by mitochondria and specifically in the heart by the metabolic stress induced by ischemia-reperfusion (31,74). These species, when added as synthetic homologs, mediate myocardial protection in IR (65,74). We therefore sought to define the predominant mitochondrial respiratory chain effects of NO_2-FA and whether this may contribute to the cardioprotective actions of electrophilic fatty acids. In this study complex II is established as the major respiratory chain target of in isolated mitochondria, and intact cardiomyoblasts were sensitive to respiratory inhibition by these compounds at similar concentrations. Further, consistent with previous reports of cardioprotection, NO_2-FA pretreatment preserved contractile function in an *ex vivo* model of acute IR.

The evidence for a link between respiratory inhibition and cardioprotection in IR is extensive, though it is not well defined whether a common protective mechanism unites the inhibition of each complex (49). Similarly, while many site II inhibitors with distinct sites of action are protective in IR, the involvement of this complex in protection is incompletely 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 noncompetitive inhibitor whose binding site is unknown), and 3-nitropropionate (3-NP, a suicide inhibitor at the active site) (75-78). In all cases, these inhibitors can display systemic toxicity as opposed to electrophilic nitroalkenes which have been administered for up to 12 weeks in murine models of vascular disease with no apparent adverse effects (53).

A shared mechanism of cardioprotection for these structurally 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. Nitroxyl (HNO) is a thiol-modifying compound which inhibits complex II and activates the mitoK_{ATP} channel, promoting cardioprotection when administered prior to ischemia (71,79). While the potential role of this channel in NO_2-FA-mediated cardioprotection was not directly addressed, protection of primary ventricular myocyte cultures by LNO_2 in simulated IR was not reversible by the mitoK_{ATP} inhibitor 5-hydroxydecanoate (31). Independent of mitoK_{ATP} effects, complex II inhibition also leads to the modulation of superoxide and secondary species by complexes I and III, and an increase in partially reduced oxygen species from complex III is viewed to promote myocardial protection through stress kinase modulation (80,81). Either in

tandem with or separately from these mechanisms, the promotion of glycolytic metabolism over cellular respiration can also be a result of complex II inhibition that promotes cell survival in IR (62). While effects of NO_2-FA on ROS generation was not explored herein, cells treated with low μM $OANO_2$ shifted to glycolytic metabolism, providing another potential mechanism of tissue protection by NO_2-FA in cardiac IR. This event is unique, given the abundance of regulatory thiols in glycolytic enzymes and the observation that GAPDH is a target in vivo for inhibition by electrophiles (54). In aggregate, this data may also suggest that fatty acid trafficking favors the modification of mitochondrial over cytosolic proteins by nitroalkenes.

Notably, 3-nitropropionate undergoes oxidation by the 70 kDa Fp subunit flavin to yield the electrophile 3-nitroacrylate, the reactive metabolite of 3-NP which inhibits complex II upon Michael addition (82). 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 $OANO_2$ binding to the Fp subunit, likely indicating that these compounds target different nucelophilic amino acids (Figure 17, Appendix A) (83,84). This may be explained by steric hindrance inherent in the bulkier 18 carbon $OANO_2$ acyl chain that might prevent access to 3-nitroacrylate-reactive centers. A distinct site of modification from 3-NP may be of toxicological significance, since the latter causes pronounced neurogenerative responses in animal models independent of its cardioprotective effects (85). 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 (82).

An earlier study linked acute cardioprotection by $OANO_2$ to mild uncoupling induced by nitroalkylation of the adenine nucleotide transporter (ANT1) in mitochondria (56). However, this study used biotinylated NO_2-FA, whose intracellular metabolism and trafficking differs from that of the native fatty acid, to demonstrate ANT1 modification in isolated perfused hearts. These 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 addition of carnitine and CoA to the carboxylate. 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 pathway (86). Finally, the addition of biotin greatly increases the size of the fatty acid and may lead to modification of proteins and residues distinct from unmodified NO_2-FA, with the differences in binding sites of 3-nitroacrylate and $OANO_2$ above underscoring the importance of structural differences in nitroalkene signaling.

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 LNO_2 (56). 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 LNO_2, and in isolated mitochondria at concentrations up to 10 μM, with 20 μM required for inhibition (31). Herein, increased respiration in $OANO_2$-treated cardiomyoblasts relative to controls was not observed unless added acutely at high >10 μM nitroalkene concentrations, which may be due to uncoupling or metabolism of the fatty acid. Similarly, while transient $OANO_2$–induced iincreases in respiration were observed in isolated mitochondria at low μM concentrations, respiratory inhibition then occurred within seconds. These previous studies used a synthetic strategy for LNO_2 or $OANO_2$ that yields a mixture of regioisomers (9-nitro-9-*cis*-octadecenoic and 10-nitro-9-*cis*-octadecenoic acids, in the case of $OANO_2$) (87), whereas the present study used a stereospecific synthesis giving pure 10-nitro-9-*cis*-octadecenoic acid (63). Other variables such as the timing of compound administration before analysis and the determination of NO_2-FA stock concentrations may also influence cellular and *in vitro* responses observed at particular doses (57). In summary, previous studies have provided significant perspective and a further understanding of the dose-dependence of bioenergetic fatty acid nitroalkene derivative effects *in vivo* will require further investigation.

The present data indicates that inhibition of complex II by NO_2-FA may depend in part on matrix pH, since respiratory coupling was an apparent requirement for inhibition in both isolated mitochondria and cells. The dissipation of the proton gradient in uncoupled mitochondria tends to decrease matrix pH as protons diffuse back from the intermembrane space, lowering the availability of thiolate anion for nitroalkylation by NO_2-FA on target proteins. 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 $OANO_2$ 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 (88). Thus, changes in matrix pH are a plausible mechanism accounting for the effect of uncoupling on fatty acid nitroalkene-induced respiratory inhibition.

The administration of nitrite mediates the post-translational modification of complex I thiols and leads to cardiac protection from ischemic insult (89). Herein, there was no effect of

$OANO_2$ on NADH-linked respiration in isolated mitochondria. This is consistent with an earlier report that reported no effect of LNO_2 on complex I activity (31). Why complex I is susceptible to inhibition by S-nitrosation and not S-nitroalkylation is unclear, but there is a precedent in that a number of reactive species display a predilection toward complex II cysteines. 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, b) the loss of S-glutathionylation in rat hearts occurs during IR–induced cardiac injury and allied succinate dehydrogenase inhibition (90) and nitroxyl preferentially inhibits complex II over complex I (71). Inhibition of complex II in heart mitochondria by the electrophile 4-HNE has also been reported, with modification of the Fp subunit observed by Western blot (72).

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 and preserving myocardial function in the post-ischemic interval. Endogenous production of fatty acid nitroalkenes during ischemic stress thus serves to link inflammatory stress with beneficial metabolic responses, a pathway which their pharmacological administration may exploit.

3.0 MYOCARDIAL FATTY ACID NITROALKENE METABOLISM BY PROSTAGLANDIN REDUCTASE-1 AND BETA OXIDATION

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3.1 ABSTRACT

Nitro-fatty acids (NO_2-FA) are electrophilic signaling mediators whose nitroalkene moiety enables reactivity with nucleophilic amino acid residues. Metabolism of NO_2-FA occurs by reduction to non-electrophilic nitroalkanes (by prostaglandin reductase-1 (PGR1)), mitochondrial β-oxidation, and protein adduction, though the relative contributions of these mechanisms to inactivation of NO_2-FA are unclear. An LC/MS-based approach allowed measurement of these metabolic reactions in H9C2 cardiomyoblasts and isolated perfused rat hearts. Inhibitors of PGR1 (indomethacin) and β-oxidation (etomoxir) revealed the contributions of these pathways to NO_2-FA metabolism and signaling. Additionally the effect of hypoxic conditions on reduction versus oxidation reactions was determined. Indomethacin preserved nitro-oleic acid ($OANO_2$) levels in the media of treated cells and abrogated the reduction of $OANO_2$ in isolated hearts, enabling increased downstream chemical and signaling reactions (thiol adduction and HO-1 expression). Etomoxir decreased the generation of shorter chain nitroalkenes and increased the levels of reduced metabolites. Hypoxic conditions in isolated and perfused ischemic hearts also increased reduction and reduced β-oxidation of $OANO_2$. In summary, inhibition of PGR1-mediated metabolism of NO_2-FA enhances electrophilic thiol adduction signaling, preventing saturation of the nitroalkene double bond.

3.2 INTRODUCTION

Fatty acid-derived electrophiles, including α,β-unsaturated ketone and nitroalkene derivatives, engage multiple adaptive signaling pathways and induce protective tissue responses in inflammatory pathologies (91). Nitro-fatty acids (NO_2-FA) in particular have cardioprotective activity in ischemia-reperfusion injury *in vivo*, *ex vivo*, and in cell-based models (3,56). Several mechanisms of action have evolved to transduce these beneficial effects of nitro and other electrophilic fatty acids in myocardial injury, including mitochondrial complex II inhibition, promoting a shift to glycolytic metabolism, respiratory uncoupling via the adenine nucleotide transporter (ANT1), and inhibition of NF-κB (56,74,92). Each of these diverse NO_2-FA-mediated signaling mechanisms relies fundamentally on the availability of either the parent nitroalkene or its electrophilic metabolites to modify cysteine residues on target proteins.

NO_2-FA are subject to a variety of metabolic reactions *in vivo* including β-oxidation, saturation by prostaglandin reductase-1 (PGR1), non-enzymatic adduction to proteins by Michael addition (thereby facilitating signaling activity), and conjugation to glutathione (86). PGR1 reduction and glutathione conjugation deactivate the electrophilic functionality of NO_2-FA, while β-oxidation leads to the formation of shorter electrophilic species, apparently unable to proceed past the position of the nitro group to remove the electrophilic moiety (86). The importance of these catabolic reactions in determining the potency and efficacy of NO_2-FA signaling is further characterized herein.

By inhibiting the metabolism of active signaling mediators, pharmacokinetics can be modulated to allow administration of lower and/or less frequent doses and providing an alternative to chemically modifying the parent compound. For example, the plasma half-life of peptide therapeutics may be extended by peptidase inhibitors (93). The recent emergence of electrophilic inflammatory modulators as potential therapeutics, such as bardoxolone methyl for treating chronic renal disease, suggests that other electrophilic compounds may also be of clinical utility (94). In this regard, it becomes important to understand mechanisms of electrophile inactivation and metabolism.

PGR1, which reduces the double bond proximal to the nitro group in NO_2-FA to form non-electrophilic nitroalkanes (95), is present in most mammalian tissues and is inhibited by cyclooxygenase (COX) inhibitors at a Ki for PGR1 that is ~50x lower than for COX (96). Recently, siRNA knockdown of transfected PGR1 was observed to limit nitroalkene reductase activity human embryonic kidney cells (95). Based on these data, it was hypothesized that

indomethacin pretreatment would enhance NO_2-FA signaling in the heart by preventing the inactivation of the electrophilic nitroalkene moiety. Also, β-oxidation results in the formation of shorter chain NO_2-FA, which may have a distinct signaling behavior compared with the parent compound, or be more or less susceptible to inactivation by PGR1. An agent that blocks β-oxidation, such as the carnitine palmitoyl transferase 1 (CPT1) inhibitor etomoxir, may therefore also impact signaling by NO_2-FA.

Of particular relevance to electrophile signaling during cardiac ischemia-reperfusion, redox changes associated with hypoxia may also influence NO_2-FA metabolism, since a) β-oxidation is inhibited by hypoxia and b) reducing equivalents such as NADH accumulate (97). Notably, NADPH, which is a cofactor for PGR1-mediated NO_2-FA reduction, can be depleted in cardiac tissue under low oxygen conditions via reactions that can also suppress reduced glutathione and protein thiol pools (97,98). In aggregate, these circumstances may enhance NO_2-FA availability or modulate other signaling responses and warrants evaluation.

In this study a targeted metabolic profiling approach is employed to determine the effects of pharmacological inhibitors and a physiological perturbation, hypoxia, on NO_2-FA metabolism and signaling activity in cardiomyoblasts and isolated perfused rat hearts.

3.3 EXPERIMENTAL PROCEDURES

3.3.1 Reagents

Nitro-oleic acid and its ^{13}C internal standard were synthesized according to an existing method (63). Solvents for extractions and HPLC/ESI-MS/MS were purchased from Burdick and Jackson (Muskegon, MI). Other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

3.3.2 Langendorff-perfused heart preparation

Hearts were rapidly excised from male Sprague-Dawley rats following induction of anesthesia with isoflurane, and perfused in retrograde as described (67). 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_2PO_4, and 25 mM $NaHCO_3$, equilibrated with a gas mixture of 95% O_2, 5% CO_2. Coronary flow was held constant at 8-12 mL/min at 37^oC. In experiments where indomethacin was used, it was diluted to 200 µM in 1 L of perfusate buffer in 1 mL dimethylsulfoxide (DMSO). In control experiments 1 mL DMSO was added to the perfusate as vehicle. Hearts were equilibrated for 30 min before infusion of $OANO_2$ (5 µM final concentration) or vehicle (methanol, at 1/1000 final dilution) via a syringe pump just above the heart. Infusion was maintained for 15 min followed by 30 min washout, and effluent fractions collected during this period. Following washout, hearts were flash-frozen in liquid N_2 for later analysis.

3.3.3 Metabolite extraction from heart and NEM displacement of protein adducts

For extraction of $OANO_2$ and its metabolites, a protocol previously described for the analysis of phospholipids was employed (99). Briefly, 100 mg heart tissue was mechanically ground to homogeneity in 1 ml PBS using a Tekmar Tissumizer (Cincinnati, OH). Protein concentration was determined by BCA assay (Pierce, ThermoFisher, Rockford, IL), and adjusted to 20 mg/ml with PBS, after which 1 ml of homogenate was transferred to a glass tube for extraction. 2.5 ml of a lipid extraction solvent consisting of 30% hexane, 20% isopropanol and 2% formic acid was

added to the homogenate and the mixture vortexed for 30 s. 2.5 ml hexane was then added followed by vortexing for an additional 30 s. To separate phases, this mixture was centrifuged at 2,000 rpm for 5 min. The upper hexane fraction was removed, dried in a RapidVap (Labconco, Kansas City, MO), resuspended in 500 µl MeOH, and transferred to a vial for LC/MS analysis. For determination of protein-adducted electrophiles, initial tissue homogenates were split into two fractions, and one treated with 20 mM N-ethylmaleimide (NEM) for 1 h at 37^{o}C. Following the incubation, ^{13}C-labeled internal standard for $OANO_2$ was added to each sample and hexane extraction performed as above.

3.3.4 Liquid chromatography/mass spectrometry (LC/MS) determination of nitro-fatty acid metabolites

Lipid extracts were applied to a reverse phase HPLC column (2 x 20 mm C18 Mercury column, Phenomenex, Torrance, CA) for resolution of $OANO_2$ and its metabolites, with a gradient consisting of solvents (A): H_2O with 0.1% HOAc and (B): acetonitrile with 0.1% HOAc at a flow rate of 0.75 ml/min. 10 µl of sample was applied to the column at 11% B (1 min) and eluted with a linear increase in B (11-100% in 6 min), followed by 1 min washing with 100% B and re-equilibration for 1.5 min at 11% B. Analytes were quantified by electrospray ionization tandem mass spectrometry ESI-MS/MS (API4000 or AB5000 triple quadrupole mass spectrometer, Applied Biosystems, San Jose, CA) in the negative ion mode using multiple reaction monitoring (MRM).

3.3.5 Metabolite determination in H9C2 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 metabolite analysis, cells were plated at 200,000 per well in 6 well plates and left to adhere and grow overnight in 2 ml of media. The following day media was exchanged and wells were treated with 50 µM etomoxir (diluted from aqueous stock), 50 µM indomethacin (diluted from DMSO stock), or the corresponding vehicle. All wells (except 3 reserved as untreated controls) were then treated with

2.5 μM $OANO_2$. 200 μl of media was removed and added to 800 μl methanol after 0, 30, 60, and 90 min of $OANO_2$ treatment, and at the time of cell harvest (24 h).

3.3.6 SDS-PAGE, Western blot for HO-1 and actin

After 24 h of treatment with $OANO_2$, cells were lysed in 200 μl RIPA buffer, centrifuged at 14,000 rpm for 5 min and supernatant protein determined by BCA assay. 20 μg of protein per well were separated by SDS-PAGE, followed by semi-dry transfer to PVDF membrane. The membrane was probed with a monoclonal anti-HO-1 antibody (Enzo Life Sciences, Farmingdale, NY) in 5% milk/TBST, dilution 1:1000. After washing, secondary antibody (anti-rabbit, Cell Signaling, Manassas, VA) was applied at 1:2000 dilution.

3.3.7 Statistical Analysis

Differences between groups were determined by Student's *t* test, with $p < 0.05$ used as a cutoff for significance.

3.4 RESULTS

3.4.1 Indomethacin treated hearts exhibit reduced saturation of NO_2-FA

The nitroalkene saturase prostaglandin reductase-1 (PGR1) is inhibited by a variety of cyclooxygenase inhibitors, with indomethacin exhibiting the greatest potency (96). It was hypothesized that isolated perfused hearts treated with indomethacin would tend to metabolize NO_2-FA by beta oxidation rather than reduction of the nitroalkene moiety. Indomethacin (or DMSO vehicle) was included in the perfusate at 200 µM, and lipids extracted from a flash-frozen heart after 15 min infusion and 30 min washout of 5 µM $OANO_2$. LC-MS/MS of the lipid extract resolved the parent compound (18:1) and its β-oxidation products (16:1, 14:1), as well as the saturated nitroalkanes at each chain length (18:0, 16:0, 14:0, and 12:0) (Figure 12a). Relative peak areas (unsaturated/saturated) in treated and untreated hearts were determined and a reduction in saturated metabolites at each chain length was noted in the indomethacin treated hearts (Figure 12b).

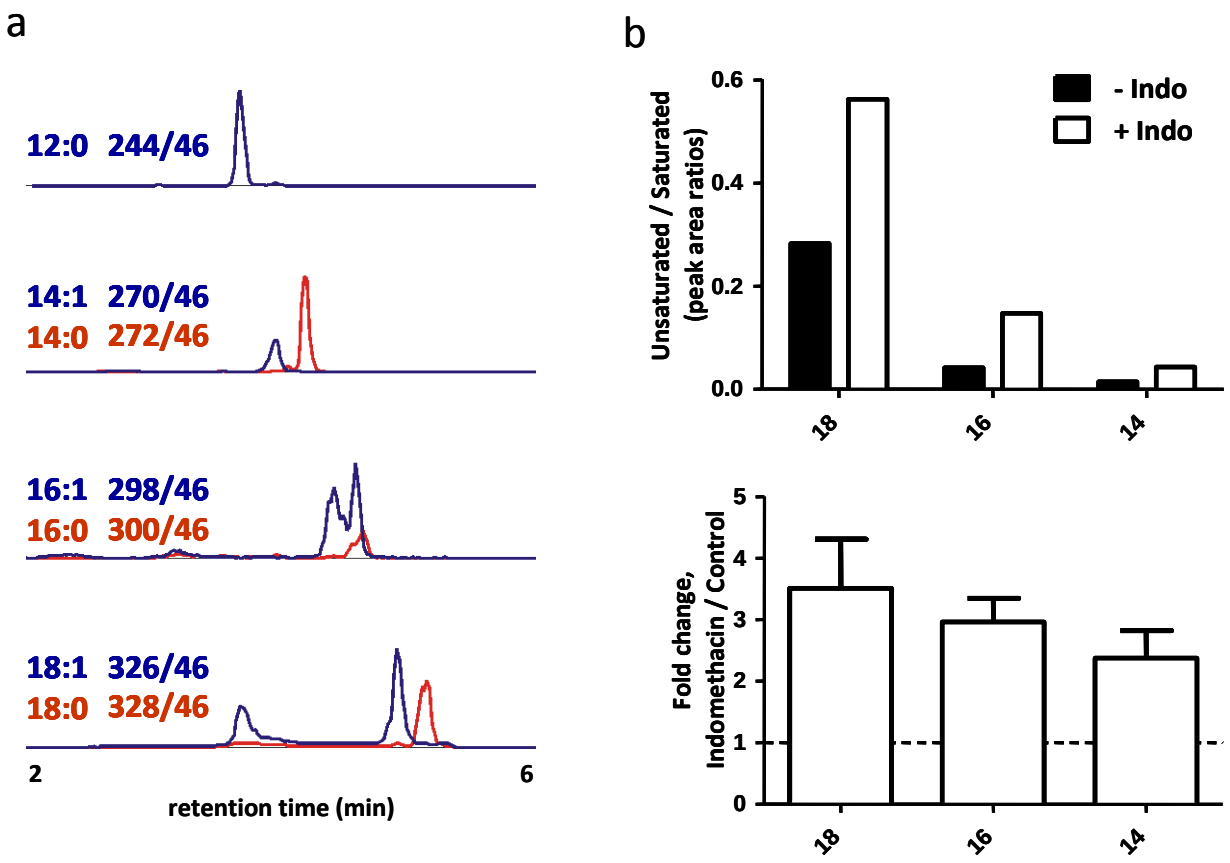

Figure 12. Impact of indomethacin on NO_2-FA metabolites in heart. Isolated perfused hearts were infused with 5 µM $OANO_2$ for 15 min followed by 30 min washout. Perfusate contained 200 µM indomethacin or DMSO vehicle at 1:1000. Following flash-freeze, lipids were extracted from homogenized heart tissue and determined by HPLC-MS/MS. **a)** Chromatogram for $OANO_2$ and saturation/β-oxidation products. **b)** Peak area ratios for unsaturated nitroalkenes to nitroalkanes at carbon chain lengths 18, 16 and 14. Representative data from one experiment (upper panel). Fold change in unsaturated/saturated ratio in indomethacin treatment versus control for 3 independent experiments (means ± SD) (lower panel).

3.4.2 Indomethacin promotes availability of electrophilic nitroalkenes for thiol adduction

Indomethacin- or vehicle-treated hearts were also subjected to analysis of total thiol adduction by NO_2-FA. 100 mg of flash frozen tissue was homogenized and split into two fractions, one of which was treated with 20 mM N-ethylmaleimide (NEM) for 1 h. NEM competes for thiol binding with NO_2-FA, which is found only in the free (non-thiol adducted state) after treatment with excess NEM. Following the NEM incubation, lipids from treated and untreated fractions were extracted in the presence of a ^{13}C internal standard for $OANO_2$. The normalized peak areas for each NO_2-FA chain length in the untreated fraction were subtracted from the corresponding peak areas in the NEM-treated fraction, with the difference corresponding to the protein- and low molecular weight thiol-adducted NO_2-FA pool. Nitroalkene β-oxidation products of $OANO_2$ were determined and peak areas normalized to a ^{13}C-$OANO_2$ internal standard (added prior to lipid extraction). Indomethacin pretreatment augmented the levels of 16:1 and 14:1 NO_2-FA in hearts (Figure 13a and 13b), likely reflecting both the decrease in saturation of these shorter chain species and increased availability of 18:1 for β-oxidation. 18-, 16-, and 14-carbon NO_2-FA all exhibited a greater degree of thiol binding in indomethacin- versus vehicle-treated hearts (Figure 13c), which reflects changes in glutathione (GSH) conjugate formation and protein binding. Finally, total fatty acid nitroalkene levels (adducted+free) were also elevated in the presence of indomethacin (Figure 13d). Together, these data suggest that indomethacin preserves the activating double bond in NO_2-FA, resulting in higher levels of free and thiol-adducted electrophilic nitroalkenes despite active β-oxidation.

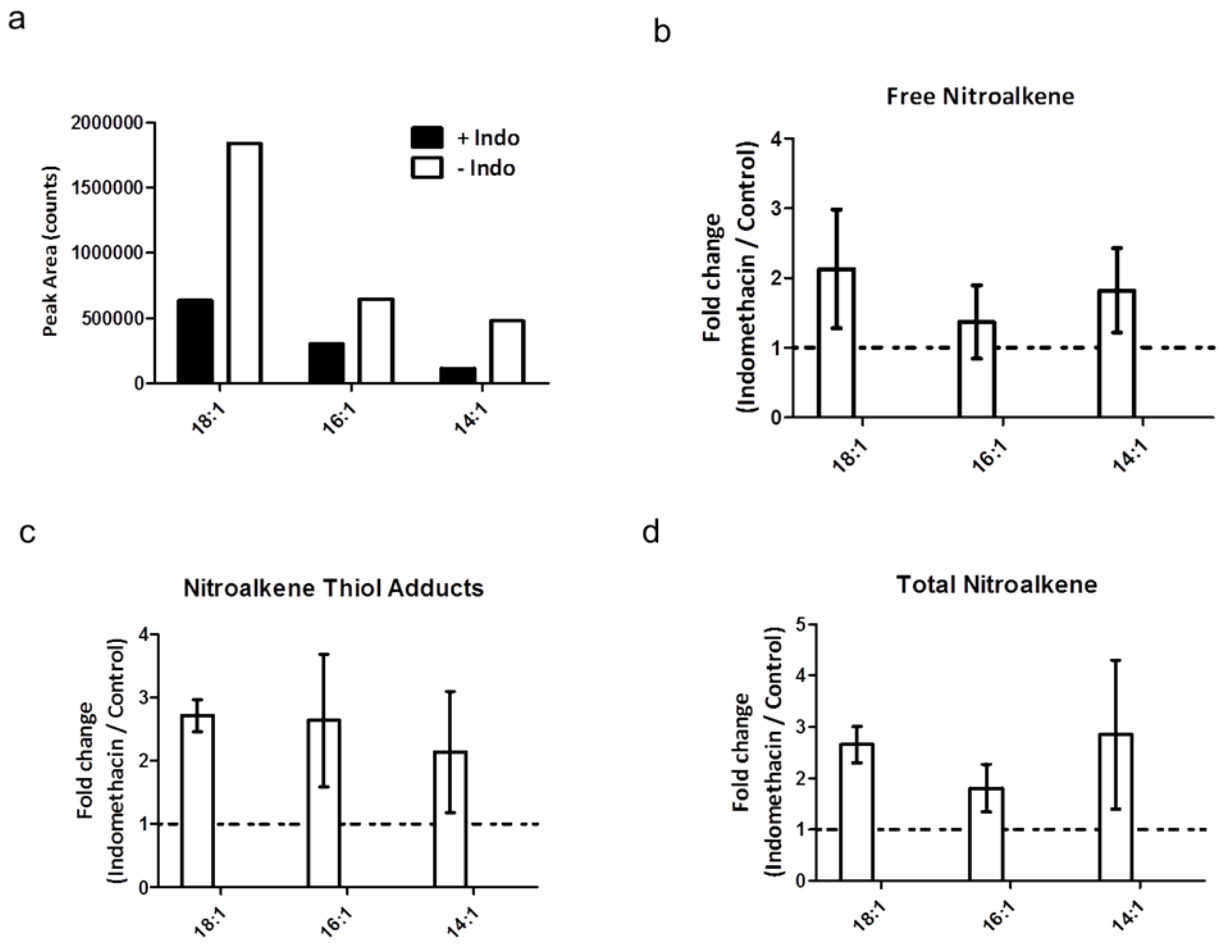

Figure 13. Indomethacin effects on free, thiol adducted and total nitroalkene levels. Following $OANO_2$ treatment with or without indomethacin as above, lipid extracts were performed on frozen heart tissue in the presence and absence of 20 mM N-ethylmaleimide (NEM), and the increase in nitroalkene with NEM incubation was considered the thiol-adducted pool. **a)** Nitroalkene metabolite peak areas from a representative experiment. **b)** Fold change in free nitroalkene levels for indomethacin treatment versus DMSO vehicle from 3 independent experiments (means ± SD). **c)** Fold-induction of thiol-adducted nitroalkenes of 18, 16, and 14 carbon chain lengths in the presence of indomethacin versus vehicle. Means ± SD for 3 independent experiments. **d)** Fold change in total (free+adducted) nitroalkene in indomethacin treated hearts versus control (3 independent experiments, means ± SD).

3.4.3 Indomethacin treatment prevents NO_2-FA metabolism and enhances signaling in H9C2 cells

Given the enhanced availability of and thiol adduction by NO_2-FA observed in indomethacin-treated hearts, it was hypothesized that this PGR1 inhibitor would promote signaling by NO_2-FA in cells. H9C2 rat cardiomyoblasts were treated with 2.5 μM $OANO_2$, which significantly induced HO-1 expression at 24 h. This induction was significantly increased in the presence of 50 μM indomethacin (Figure 14a). In addition, metabolites were determined from methanol extraction of the media at 30 min intervals for 90 min and again at the time of cell harvesting (24 h). These data demonstrate that $OANO_2$ is, at least initially, maintained at significantly elevated levels in the presence of indomethacin (Figure 14b). Further, the electrophilic metabolites of $OANO_2$ (16:1, 14:1) are significantly increased even at 24 h (Figure 14b). While there was a trend toward reduced levels of 18:0 and 16:0 with indomethacin treatment over the first 90 min, this did not achieve significance (Figure 14c). Overall, these analyses support the notion that indomethacin enhances the signaling efficacy of $OANO_2$ by preventing its metabolism into non-electrophilic species.

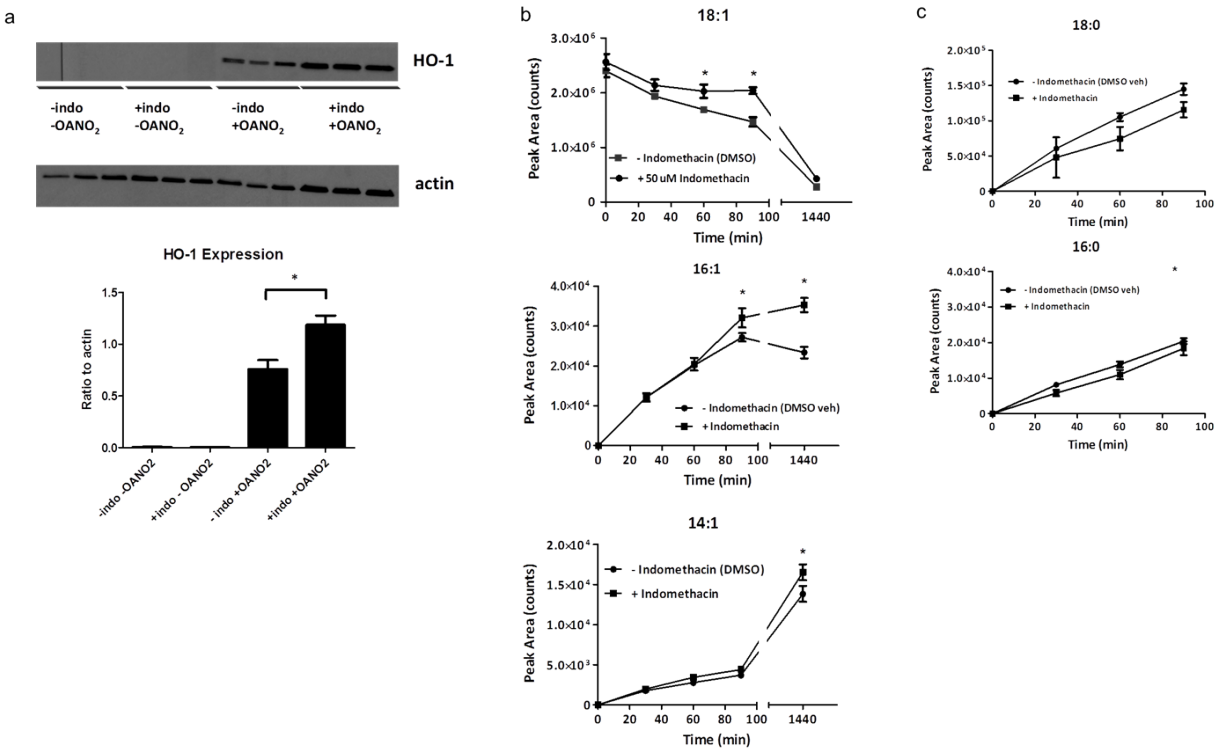

Figure 14. Indomethacin-induced changes in $OANO_2$ metabolism and signaling in H9C2 cells. H9C2 cells were grown to 80% confluency in 6 well plates and treated with 0 or 2.5 µM $OANO_2$, following addition of 50 µM indomethacin (or DMSO vehicle). Media was sampled every 30 min for 90 min, and at 24 h, with metabolites determined by LC/MS. Cells were harvested and lysed at 24 h, and Western blotting for HO-1 was performed on cell extracts. **a)** Western blot for HO-1, data in triplicate (upper panel). Densitometry analysis (lower panel). Means ± SD. **b)** Peak areas for $OANO_2$ over time from indomethacin and vehicle treated wells (upper panel), as well as electrophilic 16:1 (middle panel) and 14:1 (lower panel) nitroalkenes. **c)** Peak areas over time for nitroalkanes 18:0 (upper panel) and 16:0 (lower panel); *, $p < 0.05$ by Student's *t* test versus vehicle control.

3.4.4 Etomoxir reduces NO_2-FA beta oxidation, promoting reduction to the nitroalkane

NO_2-FA are metabolized by beta oxidation *in vivo*, and CoA and carnitine derivatives have been identified in tissue and plasma, respectively, of $OANO_2$-treated animals. How disruption of this signaling pathway impacts metabolism and signaling by NO_2-FA has not been shown, however. H9C2 cells were treated with 50 μM etomoxir and metabolites measured in media as above. While no difference in the disappearance of $OANO_2$ was observed compared to control, 16:1 levels were decreased over the first 90 min and 14:1 levels depressed at 24 h with etomoxir treatment (Figure 15a). Conversely, 18:0 levels were elevated during the first 90 min, while 16:0 was increased at 24 h (Figure 15b).

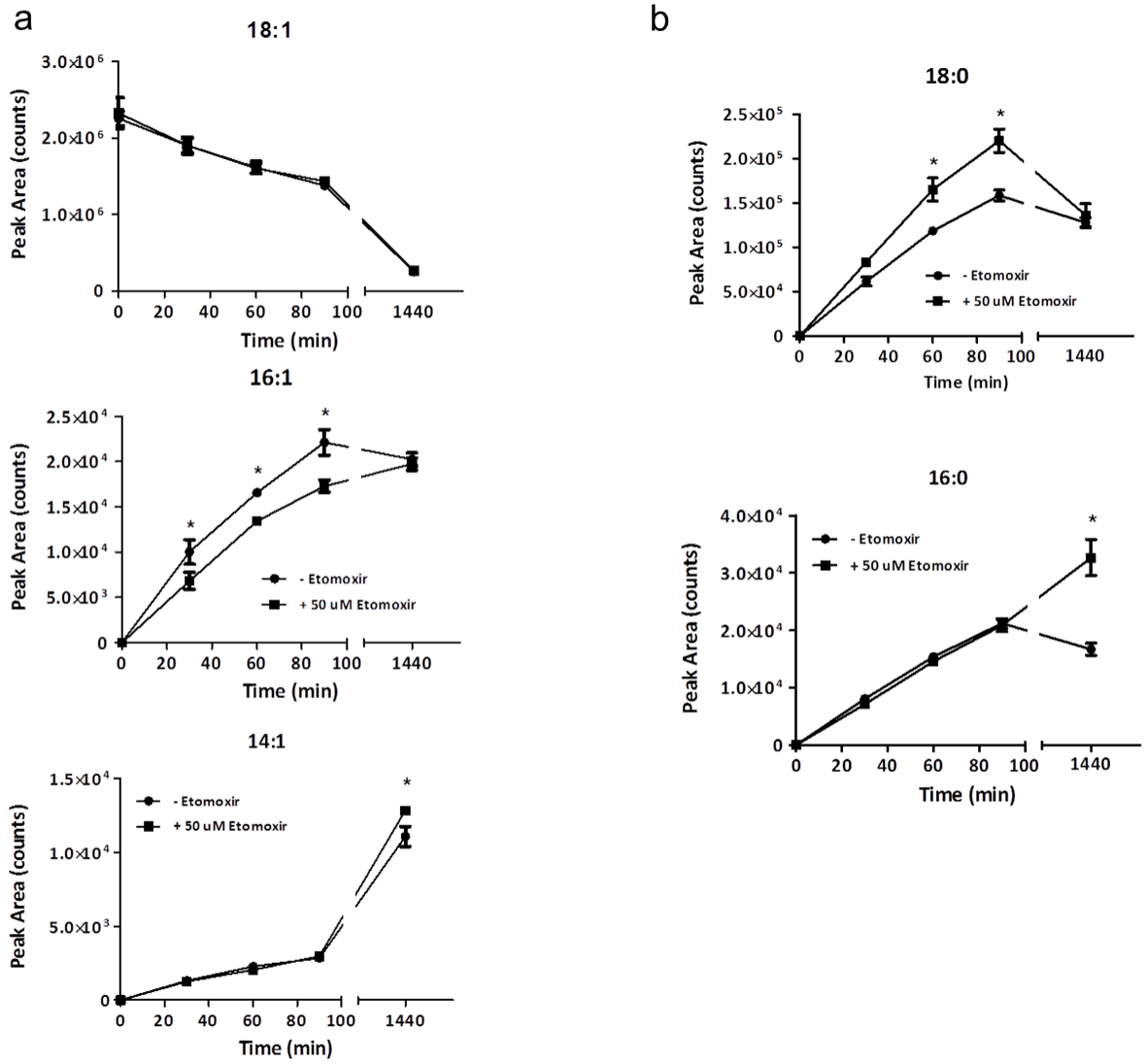

Figure 15. Etomoxir effects on $OANO_2$ metabolism in H9C2 cells. H9C2 cells were treated with 2.5 µM $OANO_2$, with or without prior addition of 50 µM etomoxir. Media was sampled every 30 min for 90 min and at 24 h, followed by LC/MS determination of $OANO_2$ and metabolites. **a)** $OANO_2$ peak area over time from wells treated with etomoxir versus control (upper panel), and peak areas for electrophilic nitroalkenes 16:1 (middle panel) and 14:1 (lower panel). **c)** Peak areas for nitroalkanes 18:0 (top panel) and 16:0 (lower panel). n = 3, mean ± SD; *, $p < 0.05$ by Student's *t* test versus vehicle control.

3.4.5 Hypoxia inhibits beta oxidation and promotes saturation of NO_2-FA

Hypoxic conditions may promote the availability of reducing equivalents (e.g. NADPH) for PGR1-mediated saturation of nitroalkenes, and also prevent efficient beta oxidation of NO_2-FA. We sought to determine the effect of hypoxia on NO_2-FA metabolism in the isolated perfused heart using perfusates equilibrated with 5% CO_2/95% N_2 or 5% CO_2 / 95% O_2. Hearts were treated as before with 5 μM $OANO_2$ for 15 min, with an additional 30 min washout, and flash frozen prior to lipid extraction. Peak area ratios of saturated to unsaturated species for 18, 16, and 14 carbon chains were elevated in the hypoxic hearts relative to high oxygen controls (Figure 16a). As expected, β-oxidation products of $OANO_2$ were decreased under hypoxic conditions (Figure 16b).

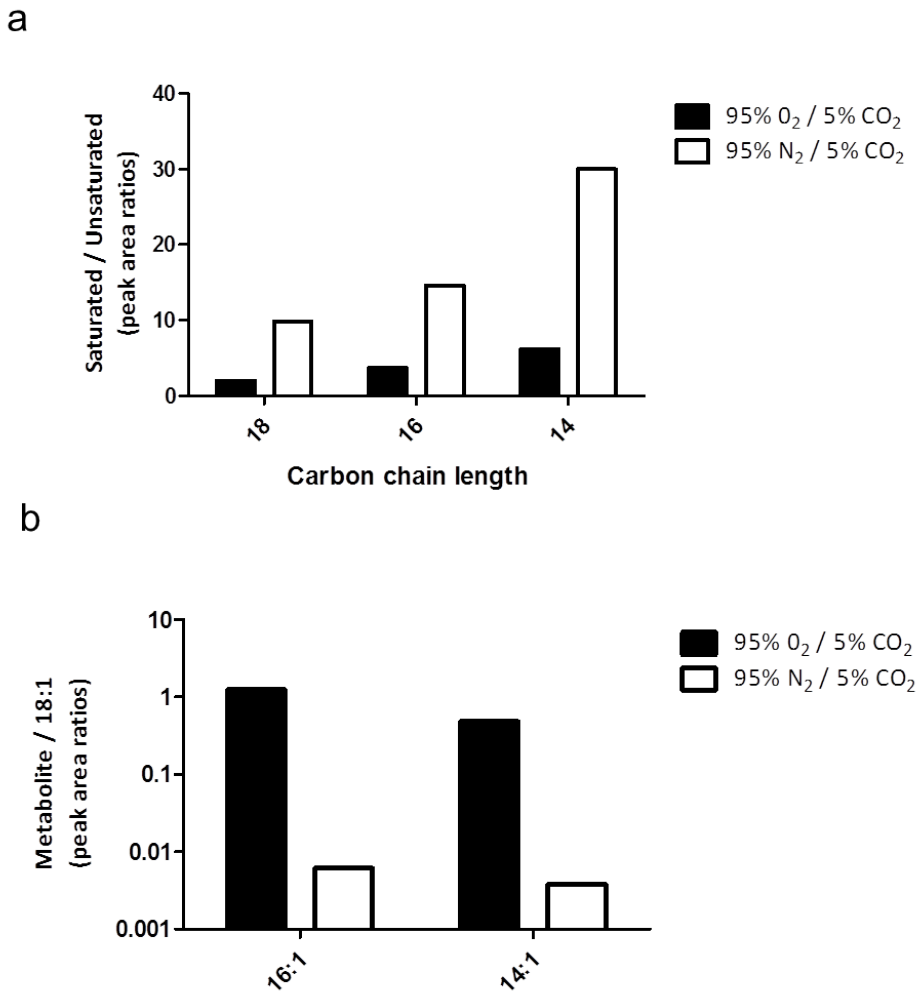

Figure 16. Hypoxia effects on $OANO_2$ metabolism by the isolated heart. Isolated perfused hearts were perfused with KH buffer equilibrated with 95% O_2 / 5% CO_2 or 95% N_2 / 5% CO_2 for 30 min, followed by infusion of 5 µM $OANO_2$ for 15 min followed by 30 min washout. Hearts were flash-frozen, lipids extracted from homogenates and metabolites determined by LC/MS. **a)** Peak area ratios of reduced nitroalkanes to nitroalkenes at 18, 16, and 14 carbon chain lengths in hypoxic and normoxic hearts. **b)** 16- and 14-carbon nitroalkene peak areas expressed as fractions of $OANO_2$ peak area.

3.5 DISCUSSION

Inhibition of the electrophile-inactivating enzyme PGR1 with indomethacin increased nitroalkene to nitroalkane ratios in isolated hearts treated with $OANO_2$. Electrophilic nitroalkene β-oxidation products of $OANO_2$ were also evident at enhanced levels in indomethacin-treated hearts relative to controls. This likely reflected the impact of PGR1 inhibition on the increased availability of $OANO_2$ for mitochondrial metabolism. This was consistent with indomethacin-induced inhibition of metabolism of $OANO_2$, lower levels of saturated $OANO_2$, metabolites over the first 90 min and increased in 16 and 14 carbon electrophilic nitroalkenes metabolites 90 min and 24 hr after $OANO_2$ addition to cells. In cardiomyocyte-based observations, the appearance of 18:0 was blunted less dramatically by indomethacin than the disappearance of 18:1, which may also suggest the involvement of another indomethacin target in NO_2-FA metabolism.

The relevance of decreased PGR1 metabolism in the indomethacin-treated isolated heart to NO_2-FA signaling was implied by enhanced protein adduction by $OANO_2$ and its electrophilic 16 and 14 carbon β-oxidation metabolites. While this analysis defines net electrophilic fatty acid-protein adduction reactions, it is presumed that specific signaling targets would also be modified to a greater extent by indomethacin. Indeed, in H9C2 cardiomyocytes, indomethacin enhanced the magnitude of $OANO_2$-induced expression of the Nrf2 target gene HO-1. Indomethacin itself is not protective in cardiac IR, and in some studies blunts the effects of cardioprotective agents whose mechanisms rely on the production of vasodilatory prostaglandins (100). Because proposed mechanisms of $OANO_2$-mediated cardioprotection do not involve prostaglandin generation, indomethacin pretreatment or co-administration may enable tissue preservation at lower $OANO_2$ concentrations. Given the broad tissue expression of PGR1, this principle may apply *in vivo* to NO_2-FA effects in a variety of inflammatory processes. Further, as PGR1 “deactivates” a multitude of electrophilic substrates, including α,β-unsaturated ketones and nitroalkenes (95,96), the inhibition of this enzyme by indomethacin other clinically-prescribed COX inhibitors may prevent the metabolism of these agents, increasing the half-life of electrophilic compounds.

Inhibition of β-oxidation with etomoxir significantly reduced levels of shorter chain nitroalkene $OANO_2$ metabolites in H9C2 cells, while concomitantly enhancing the formation of non-electrophilic nitroalkanes having 18, 16, and 14 carbon chain lengths. While protein adduction was not assessed in this cell model, it would be anticipated that the reduction in electrophile levels would limit target protein thiol adduction and downstream signaling reactions.

In aggregate, these analyses in the presence of indomethacin and etomoxir suggest that PGR1-mediated reduction is an important inactivation pathway than β-oxidation for NO_2-FA signaling.

Another NO_2-FA clearance pathway not examined in this study is conjugation to glutathione (GSH), which occurs via both non-enzymatic Michael addition and enzymatic catalysis by glutathione-S-transferases (GSTs), followed by export through multidrug resistance proteins (MRPs). Whether GSTs or Michael addition are responsible for the formation of GSH-NO_2-FA adducts *in vivo* is unclear. While a previous study suggest that fatty acid nitroalkenes are poor substrates for and good inhibitors of several GST isoforms *in vitro*, overexpression of the GSTP1a isoform potently inhibited PPARγ-mediated signaling by LNO_2. As GSH-LNO_2 adducts were not measured following GSTP1a overexpression, this may have been due to enzymatic GSH conjugation or sequestration of LNO_2 by reactive thiols on GST (101). Because GSTs are often expressed at elevated levels in inflamed tissue, including heart following reperfusion, this mode of NO_2-FA metabolism may be particularly relevant in diseased target organs (102). In addition, electrophile activation of the Nrf2/Keap1 pathway upregulates GSTs, so this pathway may play a greater role in NO_2-FA clearance with repeated administration (103). GSTs are overexpressed in many cancers and contributes to chemotherapy resistance, and several GST inhibitors are in development for use as adjuvant cancer therapies (104). These compounds will aid our understanding of the contributions of GSTs to electrophile inactivation, and may have a role in enhancing the efficacy of electrophilic therapeutics as well. Finally, regardless of GST involvement in the formation of GSH-nitroalkene conjugates, MRP1 export is an important pathway in clearance of GSH adducts, and its expression decreases NO_2-FA signaling (105).

The effect of hypoxia on NO_2-FA metabolism in the intact heart was also examined. Despite reports that the PGR1 cofactor NADPH does not accumulate in hypoxia like NADH, we observed an increase in nitroalkane metabolites of $OANO_2$ under low O_2 conditions. As anticipated, β-oxidation of $OANO_2$ was reduced in hypoxia. Though the effects on thiol binding are yet to be determined; one might anticipate increased binding based on other published work. In one previous study a biotinylated derivative of the electrophile 15-deoxy-prostaglandin J2 (15d-PGJ_2) displayed enhanced protein binding when incubated with thoracic aorta under hypoxic conditions versus normoxia (106). This was attributed to the reduction of partially oxidized sulfenic acid (-SOH)-containing cysteine residues under hypoxic conditions, as previously shown (107). Other thiol modifications have been shown to increase under hypoxic conditions as well, including S-nitrosation and glutathionylation (108,109).

The implications of this study are that PGR1-mediated reduction of NO_2-FA represents a critical pathway for electrophile removal in myocardium, and that pharmacological inhibition of this pathway provides a mechanism for enhancing NO_2-FA signaling. In contrast, pharmacological inhibition of β-oxidation appears to shift metabolism away from the generation of shorter chain electrophiles and toward reduced, non-electrophilic nitroalkanes. Finally, while hypoxia appears to enhance PGR1-mediated inactivation of NO_2-FA (possibly by increasing NADPH levels), the increase in reduced protein thiol availability at low O_2 may compensate for this, allowing increased protein binding and signaling.

4.0 DISCUSSION

Nitric oxide (NO) is a diffusible and lipophilic mediator which facilitates physiological processes in nearly all tissues studied, from vasodilation in arterioles to neurotransmission in the CNS. Inflammatory conditions lead to the generation of secondary oxides of nitrogen from NO which modify macromolecules to influence cellular function. Of relevance to the current work, unsaturated fatty acids undergo nitration by the free radical nitrogen dioxide ($^{\cdot}NO_2$) to generate electrophilic NO_2-FA. *In vivo*, $^{\cdot}NO_2$ is produced by the homolytic scission of the peroxynitrous acid (ONOOH) or nitrosoperoxycarbonate ($ONOOCO_2$), the one-electron oxidation of nitrite (NO_2^-) by heme peroxidases, or from the decomposition of nitrous acid (HNO_2) formed from NO_2^- in acidic environments (110). Once produced, NO_2-FA adduct nucleophilic amino acid residues, modulating protein function and affecting gene expression in a pattern that is overall anti-inflammatory (111).

Recent data have shown that the primary lipid target of $^{\cdot}NO_2$-mediated nitration is conjugated linoleic acid (CLA), a group of linoleic acid isomers (18 carbons, 2 double bonds, most commonly *cis*-9,*trans*-11 CLA (*c*9,*t*11-CLA), and *trans*-10,*cis*-12 CLA (*t*10,*c*12-CLA)) in which the double bonds form a conjugated diene (112). This moiety is ~20-fold more susceptible to nitration than the methylene-interrupted double bond system in linoleic acid, or the single double bond in oleic acid (18:1) (57). The nitro-cLA ($cLNO_2$) generated by this mechanism has two potential electrophilic carbons and reduced stability compared to the nitroalkene compounds used pharmacologically in the present studies (LNO_2 and $OANO_2$), and may exhibit cell signaling differences from these better-characterized species. Notably, the susceptibility of $cLNO_2$ to reduction by PGR1, which the above data show is a critical mediator of NO_2-FA metabolism, is markedly increased relative to that of LNO_2 or $OANO_2$ (95). Synthetic chemistry strategies for the production of $cLNO_2$ are still in development; future work will seek to compare its signaling activity and therapeutic potential to other fatty acid nitroalkenes.

CLA is produced from linoleic acid during its biohydrogenation to stearic acid (18:0) by bacterial enzymes in the gut of ruminants, and consumed in beef and dairy products (113). Like

n-3 fatty acids, CLA is noted for its biological activities and commercially available as a dietary supplement. Among the beneficial properties attributed to CLA in disease models are anti-atherosclerotic effects, cancer chemoprevention, and attenuation of obesity (113-115). Intriguingly, these same effects are reported or under investigation for NO_2-FA and/or other electrophiles (53,91,93), raising the possibility that some of the observed anti-inflammatory signaling actions of CLA are due to its "activation" to an electrophilic nitroalkene. To probe this hypothesis, LC/MS analysis of tissues from CLA-treated animals can be used to demonstrate formation of $cLNO_2$ and its metabolites, coupled with proteomic analysis to determine adduction of critical proteins by $cLNO_2$, or with immunoblotting to demonstrate upregulation of Nrf2 target genes such as HO-1. In a recent study validating gastric nitration of CLA by nitrite, the latter approach was used to show that HO-1 upregulation occurs alongside cLNO2 generation in the GI tract when nitrite and CLA are administered together, but not with either treatment alone (57). Another possibility would be testing the effect of nitric oxide synthase (NOS) knockdown or inhibition on the protective effects of CLA.

The nitration of CLA to $cLNO_2$ may occur in any compartment where CLA and $^{\cdot}NO_2$ coexist. These include the stomach, where acidified dietary nitrite may facilitate nitration of CLA in the lumen, inflammatory cells such as activated macrophages where iNOS induction provides abundant oxides of nitrogen, or in mitochondria, where high lipid content, low pH and reactive species generation may all coincide, particularly under inflammatory conditions (e.g. ischemia). Indeed, the addition of nitrite alone to isolated rat heart mitochondria under mildly acidic conditions promotes the nitration of endogenous CLA (57). Mitochondria are also a noted source of endogenous fatty acid nitroalkene production in ischemic preconditioning (31), and it is now known that the endogenous LNO_2 reported in these studies was most likely the $cLNO_2$ isomer. The observation that conjugated double bonds are readily nitrated *in vivo* has interesting implications. For instance, nitration of the amino acid tyrosine by $^{\cdot}NO_2$, often used as a biomarker of oxidative stress, is a much less thermodynamically favorable process than CLA nitration, as addition of tyrosine in 500 molar excess fails to impair the latter reaction (57). This suggests that $cLNO_2$ or its protein adducts may be a more sensitive biomarker of oxidative stress and nitrating reactions than nitrotyrosine, and with the detection of $cLNO_2$ and its metabolites in human urine this may prove clinically relevant (57). In addition, other compounds containing conjugated double bond systems (including polyene pigments such as carotenoids) may be subject to biological nitration (107). Research is underway to identify additional nitro-containing electrophiles in post-ischemic tissue.

The mitochondrial origin of endogenous fatty acid nitroalkenes and the role of mitochondria in adaptive redox signal transduction motivated the study of their mitochondrial effects. While all respiratory complexes are inhibited by various thiol-reactive compounds, complex II was most sensitive to inhibition by NO_2-FA. Complex II consists of four polypeptide subunits and five prosthetic groups (flavin adenine dinucleotide, three iron-sulfur centers, heme *b*) and occupies a unique niche as a direct link between electron transport and the TCA cycle (as succinate dehydrogenase), catalyzing the reduction of ubiquinone coupled to the oxidation of succinate to fumarate (108). While other dehydrogenases can couple ubiquinone reduction to the oxidation of organic compounds, these enzymes are structurally less intricate, leading to speculation that complex II has additional functions (109). For example, the small subunit of cytochrome *b* (subunit D) in complex II has been proposed to function as an O_2 sensor, as mutations in this subunit have been linked to hereditary paragangliomas, benign tumors of the carotid body phenotypically similar to the sporadic form caused by chronic hypoxia (92). In addition, complex II inhibition is linked to the opening of a mitochondrial K_{ATP} channel, though the molecular identity of this channel is unknown and the relationship of complex II proteins to its activity unclear (112). Further, interference with complex II activity influences superoxide production at complexes I and III (80). Combined with the role of complex II in cellular respiration, one or more of these functions could provide a mechanistic link between inhibition of complex II and cardioprotection in IR. Because reactive species generation and mito-K_{ATP} channel activity were not measured here, future efforts may be directed toward understanding the effects of fatty acid nitroalkenes on these parameters.

Redox modifications of complex II are myriad and often discussed in the context of irreversible damage to electron transport and loss of mitochondrial function. Rat heart mitochondria show reduced complex II activity and 4-hydroxynonenal (HNE) modification of the 70-kDa Fp subunit (by Western blot) following induction of diabetes by an intraperitoneal injection of streptozotocin (112). During reperfusion injury, complex II undergoes tyrosine nitration concomitant with a loss of electron transport activity (113). In isolated mitochondria, treatment with Angeli's salt (HNO) leads to oxidation of a thiol near the active site and loss of activity (71). There are also data suggesting a more positive role of post-translational thiol modifications in complex II: a loss of glutathionylation from the 70-kDa subunit accompanies reperfusion injury, and *in vitro* data show that a loss of GSH adducts correlate with decreased electron transfer efficiency and increased $O_2^{\cdot-}$ formation (90). Additional work is required to reconcile the presence of oxidative "damage" to complex II in injured or inflamed tissue with the apparent benefits of its inhibition in IR, though the reversible binding of many cardioprotective

complex II inhibitors (including NO_2-FA), in contrast with the relative irreversibility of thiol oxidation to sulfinic/sulfonic acid or HNE adduction provides a partial explanation (114,115). Timing of compound administration may also play an important role in determining the effect of complex II inhibition. For example, HNO has protective effects when used as a pretreatment in IR, but exacerbates injury when added during reperfusion (116,117).

In aggregate the data presented herein suggest a cardioprotective mechanism for NO_2-FA involving a shift from respiratory to glycolytic metabolism. This approach to protecting ischemic heart tissue has its historical origins in the 1960s, when glucose, insulin, and potassium (GIK) treatment of acute MI was first conceptualized (51). Insulin was used to prevent myocardial uptake of free fatty acids, which are systemically released during MI secondary to elevated stress-induced catecholamine levels, with glucose provided as an alternative energy source (potassium was used primarily to aid in recovery of sinus rhythm) (118,119). While GIK therapy never became standard practice and its efficacy is still debated, the reasoning underlying its use (i.e. that glucose oxidation is a more efficient use of O_2 and should be promoted under hypoxic conditions) remains appealing. In stable severe angina pectoris, where stress-induced myocardial O_2 demand outstrips supply causing chest pain, β-oxidation inhibitors such as perhexiline (a highly efficacious but somewhat toxic inhibitor which acts on carnitine palmitoyl transferase 1 (CPT1)), trimetazidine, and ranolazine (both long-chain 3-ketoacyl-CoA thiolase inhibitors with moderate anti-angina activity) have been successfully employed in the clinic, though none are first-line therapies (120). Etomoxir, used in the present work to inhibit β-oxidation of fatty acid nitroalkenes at the level of CPT1, was used as a congestive heart failure therapy in the ERGO (etomoxir for the recovery of glucose oxidation) trial. This study was halted due to elevations of liver transaminases in plasma, though trends toward improved exercise time were observed in the treatment group (121). Together these examples confirm that shifting metabolism away from fatty acid oxidation and toward glycolysis is clinically effective in ischemic heart disease, though there are concerns about systemic toxicity with certain molecular targets or chronic use. Thus, developing metabolic agents with improved safety profiles is a priority, and NO_2-FA may meet this criterion. A more direct approach to this issue can be seen in a recent study in which a cell-based screen of FDA-approved medications identified the anti-nausea medication meclizine (a histamine receptor antagonist) as a mediator of the metabolic transition from respiration to glycolysis, and followed this with a demonstration of its protective ability in cardiac and cerebral IR (62). Clearly a medication with a known safety profile would be an ideal candidate for clinical use.

The realization that superoxide and its byproducts play a deleterious role in inflammatory disease has led to decades of research on free radical and oxidant scavengers, both naturally occurring (ascorbate, tocopherols, N-acetylcysteine, catalase and superoxide dismutase (SOD)) and synthetic (manganese porphyrin SOD mimetics). While in certain contexts these approaches have proven safe and effective (N-acetylcysteine in acetaminophen-induced liver injury, for instance (122)), overall drugs directly targeting free radicals have failed to live up to their promise. A large, randomized controlled trial of d-α-tocopherol (vitamin E) supplementation in a population at high cardiovascular risk (the HOPE study) showed no benefit and modest trends toward adverse cardiovascular events in the supplement group (123). Clinical trials with ascorbate (vitamin C) have also failed to show positive impacts on cardiovascular morbidity and mortality, though slight decreases in blood pressure are frequently observed (124,125). While several studies have shown the benefits of native SOD and catalase administration in IR (126,127), the short half-life, antigenicity and cost of protein-based therapies makes them impractical options. SOD mimetics, such as the manganese metalloporphyrins, have been developed to improve on these parameters, with success in experimental models (128). These compounds vary in their selectivity for superoxide versus other reactive species, catalytic efficiency and cell permeability, and clinical trials are ongoing for multiple agents (129). More recently, agents have been developed with the goal of scavenging ROS in specific compartments. The addition of a triphenylphosphonium moiety is a popular method of targeting redox active compounds to mitochondria, as its delocalized positive charge and lipophilicity promote matrix accumulation. These mitochondrially-targeted antioxidants are also effective in cardiovascular disease models (130). Regardless of the efficacy of these approaches in inflammation, there is emerging evidence that suppression of “adaptive” free radical generation is harmful, which may be problematic when indiscriminate radical scavengers are used. For example, the insulin sensitivity-promoting effects of exercise are suppressed by supplementation of vitamins C and E (131).

The therapeutic benefits of oxidant-derived nitro-fatty acids (and perhaps by extension other electrophiles produced under inflammatory conditions) suggest that *in vivo* generation and signaling of these compounds represents one such adaptive response to oxidative stress. In addition to insulin sensitization as mentioned above, current evidence indicates that free radicals are involved in ischemic preconditioning (116) and adaptive vascular responses to stress (117). If mild oxidant stress can be viewed as beneficial, this provides a rationale for the pharmacological use of oxidant-generated signaling compounds to enhance native defense mechanisms. Endogenous free radical and electrophile generation are typically under tight

control, and various genomic pathways have evolved to sense reactive species and upregulate enzymes which promote their detoxification. Administration of reactive species at sub-toxic doses represents one pharmacological approach for increasing expression of antioxidant and electrophile-detoxifying genes and preventing tissue injury in inflammation. The Nrf2/Keap1 and heat shock response pathways are two well-characterized example of this. Keap1 is a cysteine-rich E3 ubiquitin ligase that binds Nrf2 in the cytosol and tags it for degradation. Oxidation or electrophilic Michael addition of Keap1 thiols frees Nrf2 which then migrates to the nucleus, binding to gene promoters containing an antioxidant response element (ARE) and amplifying their expression. Proteins such as NAD(P)H:quinone oxidoreductase-1 NQO1, enzymes of the GSH synthetic pathway, glutathione-S-transferase (GST), and heme oxygenase-1 (HO-1) are among the Nrf2 target genes which protect the cell against oxidative damage (25). Analogously, heat shock factor-1 (HSF1), basally inactive in the cytosol and bound to heat shock proteins (HSPs) 40, 70 and 90, is released for nuclear translocation by a variety of stresses that induce protein damage and misfolding including including high temperature, oxidative stress, and electrophile adduction, and following hyperphosphorylation in the nucleus binds to gene promoters containing heat shock elements (HSE). HSP chaperone expression is increased through this pathway, promoting proper protein conformation and preventing aggregation (27). Electrophilic mediators currently under study as potential pharmacological agents, including triterpenoids, sulforaphane, and fatty acid nitroalkenes, activate the antioxidant and heat shock response pathways as part of their mechanism of action. Further, in the present work it is suggested that mitochondrial responses to electrophiles may constitute an acute stress-response pathway, altering metabolism to mediate protection in IR. Whether this approach is clinically viable, and in which pathologies it will be most effective, is yet to be determined.

Another important pharmacological issue for NO_2-FA addressed here was the metabolism of the active parent compound. Fatty acid nitroalkenes are subject to the same metabolic pathways as other fatty acids: they undergo β-oxidation in mitochondria, in the process forming shorter chain nitroalkenes and CoA and carnitine derivatives, and are likely esterified to phospholipids and triglycerides (detection of nitroalkene-containing complex lipids is an ongoing project in the laboratory) (86). By virtue of their electrophilic functionality, they are also metabolized by protein thiol adduction, the nitroalkene reductase activity of prostaglandin reductase 1 (PGR1) and adduction by GSH followed by export through MRP1. While decreases in MRP1 and PGR1 activity enhance NO_2-FA signaling, it is less clear whether blocking fatty acid metabolism yields similar results. Likely this is because short chain nitroalkenes, CoA and carnitine derivatives, and esterified NO_2-FA retain electrophilicity. Further, it is reasonable to

speculate that fatty acid trafficking alters NO_2-FA signaling relative to that of other electrophiles: under conditions where cells rely on β-oxidation to meet their energetic needs, local concentrations of fatty acid nitroalkenes in mitochondria would be expected to increase, enhancing adduction of mitochondrial versus cytosolic protein targets. As myocardial reperfusion is one such situation, NO_2-FA may represent mitochondrially targeted metabolic modulators for IR.

In conclusion, this work shows that fatty acid nitroalkenes mediate acute cardioprotection from IR in the isolated heart, and inhibit respiration in cultured cardiomyoblasts, likely via reversible adduction of complex II, promoting glycolytic flux. In addition, PGR1-mediated saturation of NO_2-FA in heart tissue and cardiomyoblasts was identified as a critical means of inactivation for this electrophile relative to β-oxidation, and pharmacological inhibition of the former pathway emerged as a potential means of enhancing nitroalkene signaling in myocardium. Preliminary results from studies conducted at low pO_2 suggest that NO_2-FA metabolism and signaling may also be altered in the hypoxic myocardium. Together these studies suggest a novel mechanism of action for an emerging class of cardioprotective mediators, and reveal methods of analyzing and optimizing their metabolism and signaling by the heart.

5.0 APPENDIX A: SUPPLEMENTAL INFORMATION

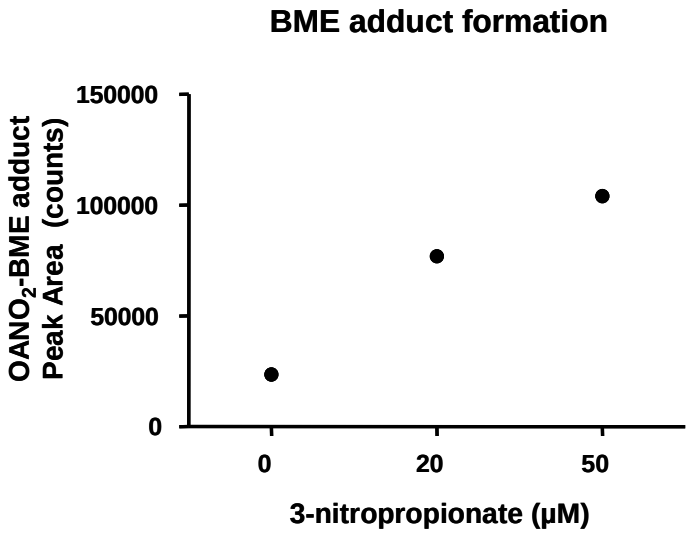

Figure 17. 3-NP does not inhibit $OANO_2$ binding to the Fp subunit of complex II. 100 μg of mitochondrial lysate was incubated with 5 μM $OANO_2$ following pre-incubation with varying concentrations of 3-nitropropionate (0, 20 or 50 μM) followed by SDS-PAGE and protein labeling by Coomassie stain. The band corresponding to the 70 kDa subunit of complex II was removed, dried with ACN and rehydrated in the presence of 500 mM BME. Peak areas for BME-$OANO_2$ adducts are shown for 0, 20, and 50 μM $OANO_2$.

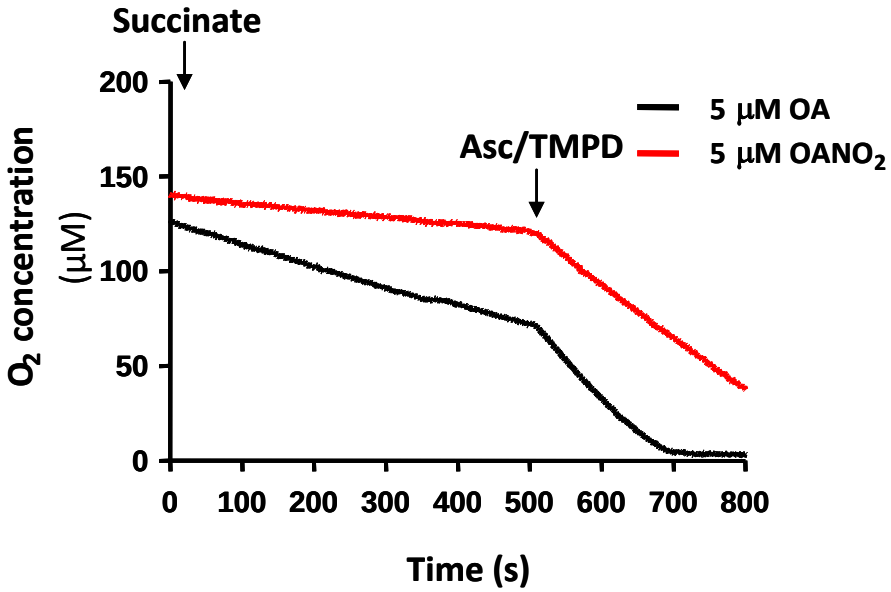

Figure 18. Ascorbate/TMPD rescues respiratory inhibition by $OANO_2$. Freshly isolated rat heart mitochondria (0.5 mg, all RCR > 4) were incubated in respiration buffer (pH 7.2) for 5 min with OA or $OANO_2$ (5 µM) prior to substrate addition. Representative O_2 concentration traces following addition of the complex II-linked substrate succinate (10 mM) are show. After 500 s (to establish inhibition by $OANO_2$), 20 mM ascorbate and 20 µM TMPD were added to both chambers.

ABBREVIATIONS

NO_2-FA, nitro-fatty acids; $OANO_2$, nitro-oleic acid; OA, oleic acid; TMPD, tetramethylphenylenediamine; FCCP, BME, β-mercaptoethanol. HNE, hydroxynonenal. OCR, oxygen consumption rate. ECAR, extracellular acidification rate. RCR, respiratory control ratio. 3-NP, 3-nitropropionic acid, XF, extracellular flux. UCP, uncoupling protein; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; RNS, reactive nitrogen species; FAO, fatty acid oxidation; PTP, permeability transition pore; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; SERCA, sarco/endoplasmic reticulum calcium ATPase; T2DM, type 2 diabetes mellitus; 4-HNE, 4-hydroxynonenal; 4-ONE, 4-oxononenal; 15-$dPGJ_2$, 15-deoxy-prostaglandin J2; COX, cyclooxygenase; LOX, lipoxygenase; oxo-ETE, oxo-eicosatetraenoic acid; $ONOO^-$, peroxynitrite; $^{\cdot}NO_2$, nitrogen dioxide; NO_2^-, nitrite; $L^{\cdot}$, lipid alkoxyl radical; $LOO^{\cdot}$, lipid peroxyl radical; TZD, thiazoladinedione; NF-κB, nuclear factor κB; PPARγ, peroxisome proliferator-activated receptor g; Nrf2, nuclear erythroid 2-related factor 2; Keap1, kelch ECH associating protein; GSH, glutathione; TNF-α, tumor necrosis factor α; NADH, nicotinamide adenine dinucleotide (reduced); NAD^+, nicotinamide adenine dinucleotide (oxidized).

BIBLIOGRAPHY

1. Lloyd-Jones, D., Adams, R., Carnethon, M., De Simone, G., Ferguson, T. B., Flegal, K., Ford, E., Furie, K., Go, A., Greenlund, K., Haase, N., Hailpern, S., Ho, M., Howard, V., Kissela, B., Kittner, S., Lackland, D., Lisabeth, L., Marelli, A., McDermott, M., Meigs, J., Mozaffarian, D., Nichol, G., O'Donnell, C., Roger, V., Rosamond, W., Sacco, R., Sorlie, P., Stafford, R., Steinberger, J., Thom, T., Wasserthiel-Smoller, S., Wong, N., Wylie-Rosett, J., and Hong, Y. (2009) Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* **119**, 480-486
2. Wang, Q. D., Pernow, J., Sjoquist, P. O., and Ryden, L. (2002) Pharmacological possibilities for protection against myocardial reperfusion injury. *Cardiovasc Res* **55**, 25-37
3. Rudolph, V., Rudolph, T. K., Schopfer, F. J., Bonacci, G., Woodcock, S. R., Cole, M. P., Baker, P. R., Ramani, R., and Freeman, B. A. (2009) Endogenous generation and protective effects of nitro-fatty acids in a murine model of focal cardiac ischaemia and reperfusion. *Cardiovasc Res*
4. Forman, H. J., Fukuto, J. M., Miller, T., Zhang, H., Rinna, A., and Levy, S. (2008) The chemistry of cell signaling by reactive oxygen and nitrogen species and 4-hydroxynonenal. *Arch Biochem Biophys* **477**, 183-195
5. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* **87**, 1620-1624
6. Denicola, A., Freeman, B. A., Trujillo, M., and Radi, R. (1996) Peroxynitrite reaction with carbon dioxide/bicarbonate: kinetics and influence on peroxynitrite-mediated oxidations. *Arch Biochem Biophys* **333**, 49-58
7. Greenacre, S. A., and Ischiropoulos, H. (2001) Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. *Free Radic Res* **34**, 541-581
8. Wink, D. A., Darbyshire, J. F., Nims, R. W., Saavedra, J. E., and Ford, P. C. (1993) Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O2 reaction. *Chem Res Toxicol* **6**, 23-27
9. Gutierrez, H. H., Nieves, B., Chumley, P., Rivera, A., and Freeman, B. A. (1996) Nitric oxide regulation of superoxide-dependent lung injury: oxidant-protective actions of endogenously produced and exogenously administered nitric oxide. *Free Radic Biol Med* **21**, 43-52

10. Ceaser, E. K., Moellering, D. R., Shiva, S., Ramachandran, A., Landar, A., Venkartraman, A., Crawford, J., Patel, R., Dickinson, D. A., Ulasova, E., Ji, S., and Darley-Usmar, V. M. (2004) Mechanisms of signal transduction mediated by oxidized lipids: the role of the electrophile-responsive proteome. *Biochem Soc Trans* **32**, 151-155
11. Halliwell, B., and Chirico, S. (1993) Lipid peroxidation: its mechanism, measurement, and significance. *Am J Clin Nutr* **57**, 715S-724S; discussion 724S-725S
12. O'Donnell, V. B., and Freeman, B. A. (2001) Interactions between nitric oxide and lipid oxidation pathways: implications for vascular disease. *Circ Res* **88**, 12-21
13. Freeman, B. A., Baker, P. R., Schopfer, F. J., Woodcock, S. R., Napolitano, A., and d'Ischia, M. (2008) Nitro-fatty acid formation and signaling. *J Biol Chem* **283**, 15515-15519
14. Rudolph, T. K., and Freeman, B. A. (2009) Transduction of redox signaling by electrophile-protein reactions. *Sci Signal* **2**, re7
15. Schneider, C., Tallman, K. A., Porter, N. A., and Brash, A. R. (2001) Two distinct pathways of formation of 4-hydroxynonenal. Mechanisms of nonenzymatic transformation of the 9- and 13-hydroperoxides of linoleic acid to 4-hydroxyalkenals. *J Biol Chem* **276**, 20831-20838
16. Stevens, J. F., and Maier, C. S. (2008) Acrolein: sources, metabolism, and biomolecular interactions relevant to human health and disease. *Mol Nutr Food Res* **52**, 7-25
17. Fam, S. S., Murphey, L. J., Terry, E. S., Zackert, W. E., Chen, Y., Gao, L., Pandalai, S., Milne, G. L., Roberts, L. J., Porter, N. A., Montine, T. J., and Morrow, J. D. (2002) Formation of highly reactive A-ring and J-ring isoprostane-like compounds (A4/J4-neuroprostanes) in vivo from docosahexaenoic acid. *J Biol Chem* **277**, 36076-36084
18. Hall, L. M., and Murphy, R. C. (1998) Electrospray mass spectrometric analysis of 5-hydroperoxy and 5-hydroxyeicosatetraenoic acids generated by lipid peroxidation of red blood cell ghost phospholipids. *J Am Soc Mass Spectrom* **9**, 527-532
19. Milne, G. L., and Morrow, J. D. (2006) Isoprostanes and related compounds: update 2006. *Antioxid Redox Signal* **8**, 1379-1384
20. Liu, X., Miller, M. J., Joshi, M. S., Thomas, D. D., and Lancaster, J. R., Jr. (1998) Accelerated reaction of nitric oxide with O2 within the hydrophobic interior of biological membranes. *Proc Natl Acad Sci U S A* **95**, 2175-2179
21. O'Donnell, V. B., Eiserich, J. P., Chumley, P. H., Jablonsky, M. J., Krishna, N. R., Kirk, M., Barnes, S., Darley-Usmar, V. M., and Freeman, B. A. (1999) Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion. *Chem Res Toxicol* **12**, 83-92
22. Parola, M., Bellomo, G., Robino, G., Barrera, G., and Dianzani, M. U. (1999) 4-Hydroxynonenal as a biological signal: molecular basis and pathophysiological implications. *Antioxid Redox Signal* **1**, 255-284
23. Isom, A. L., Barnes, S., Wilson, L., Kirk, M., Coward, L., and Darley-Usmar, V. (2004) Modification of Cytochrome c by 4-hydroxy- 2-nonenal: evidence for histidine, lysine, and arginine-aldehyde adducts. *J Am Soc Mass Spectrom* **15**, 1136-1147
24. Wong, H. L., and Liebler, D. C. (2008) Mitochondrial protein targets of thiol-reactive electrophiles. *Chem Res Toxicol* **21**, 796-804
25. Osburn, W. O., and Kensler, T. W. (2008) Nrf2 signaling: an adaptive response pathway for protection against environmental toxic insults. *Mutat Res* **659**, 31-39

26. Itoh, T., Fairall, L., Amin, K., Inaba, Y., Szanto, A., Balint, B. L., Nagy, L., Yamamoto, K., and Schwabe, J. W. (2008) Structural basis for the activation of PPARgamma by oxidized fatty acids. *Nat Struct Mol Biol* **15**, 924-931
27. Jacobs, A. T., and Marnett, L. J. (2007) Heat shock factor 1 attenuates 4-Hydroxynonenal-mediated apoptosis: critical role for heat shock protein 70 induction and stabilization of Bcl-XL. *J Biol Chem* **282**, 33412-33420
28. Gutierrez, J., Ballinger, S. W., Darley-Usmar, V. M., and Landar, A. (2006) Free radicals, mitochondria, and oxidized lipids: the emerging role in signal transduction in vascular cells. *Circ Res* **99**, 924-932
29. Kagan, V. E., Bayir, H. A., Belikova, N. A., Kapralov, O., Tyurina, Y. Y., Tyurin, V. A., Jiang, J., Stoyanovsky, D. A., Wipf, P., Kochanek, P. M., Greenberger, J. S., Pitt, B., Shvedova, A. A., and Borisenko, G. (2009) Cytochrome c/cardiolipin relations in mitochondria: a kiss of death. *Free Radic Biol Med* **46**, 1439-1453
30. Madesh, M., and Balasubramanian, K. A. (1997) Activation of liver mitochondrial phospholipase A2 by superoxide. *Arch Biochem Biophys* **346**, 187-192
31. Nadtochiy, S. M., Baker, P. R., Freeman, B. A., and Brookes, P. S. (2009) Mitochondrial nitroalkene formation and mild uncoupling in ischaemic preconditioning: implications for cardioprotection. *Cardiovasc Res* **82**, 333-340
32. Bonacci, G., Schopfer, F. J., and Freeman, B. A. (2009) Unpublished Observations.
33. Kaplan, P., Tatarkova, Z., Racay, P., Lehotsky, J., Pavlikova, M., and Dobrota, D. (2007) Oxidative modifications of cardiac mitochondria and inhibition of cytochrome c oxidase activity by 4-hydroxynonenal. *Redox Rep* **12**, 211-218
34. Humphries, K. M., Yoo, Y., and Szweda, L. I. (1998) Inhibition of NADH-linked mitochondrial respiration by 4-hydroxy-2-nonenal. *Biochemistry* **37**, 552-557
35. Hill, B. G., Dranka, B. P., Zou, L., Chatham, J. C., and Darley-Usmar, V. (2009) Importance of the bioenergetic reserve capacity in response to cardiomyocyte stress induced by 4-hydroxynonenal. *Biochem J*
36. Ceaser, E. K., Ramachandran, A., Levonen, A. L., and Darley-Usmar, V. M. (2003) Oxidized low-density lipoprotein and 15-deoxy-delta 12,14-PGJ2 increase mitochondrial complex I activity in endothelial cells. *Am J Physiol Heart Circ Physiol* **285**, H2298-2308
37. Landar, A., Zmijewski, J. W., Dickinson, D. A., Le Goffe, C., Johnson, M. S., Milne, G. L., Zanoni, G., Vidari, G., Morrow, J. D., and Darley-Usmar, V. M. (2006) Interaction of electrophilic lipid oxidation products with mitochondria in endothelial cells and formation of reactive oxygen species. *Am J Physiol Heart Circ Physiol* **290**, H1777-1787
38. Nakamura, K., Miura, D., Kusano, K. F., Fujimoto, Y., Sumita-Yoshikawa, W., Fuke, S., Nishii, N., Nagase, S., Hata, Y., Morita, H., Matsubara, H., Ohe, T., and Ito, H. (2009) 4-Hydroxy-2-nonenal induces calcium overload via the generation of reactive oxygen species in isolated rat cardiac myocytes. *J Card Fail* **15**, 709-716
39. Lee, J. Y., Jung, G. Y., Heo, H. J., Yun, M. R., Park, J. Y., Bae, S. S., Hong, K. W., Lee, W. S., and Kim, C. D. (2006) 4-Hydroxynonenal induces vascular smooth muscle cell apoptosis through mitochondrial generation of reactive oxygen species. *Toxicol Lett* **166**, 212-221
40. Uchida, K. (2003) 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Prog Lipid Res* **42**, 318-343

41. Manoli, I., Alesci, S., Blackman, M. R., Su, Y. A., Rennert, O. M., and Chrousos, G. P. (2007) Mitochondria as key components of the stress response. *Trends Endocrinol Metab* **18**, 190-198
42. Bugger, H., and Abel, E. D. (2008) Molecular mechanisms for myocardial mitochondrial dysfunction in the metabolic syndrome. *Clin Sci (Lond)* **114**, 195-210
43. Kelley, D. E., He, J., Menshikova, E. V., and Ritov, V. B. (2002) Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* **51**, 2944-2950
44. Finck, B. N., Lehman, J. J., Leone, T. C., Welch, M. J., Bennett, M. J., Kovacs, A., Han, X., Gross, R. W., Kozak, R., Lopaschuk, G. D., and Kelly, D. P. (2002) The cardiac phenotype induced by PPARalpha overexpression mimics that caused by diabetes mellitus. *J Clin Invest* **109**, 121-130
45. Boudina, S., and Abel, E. D. (2006) Mitochondrial uncoupling: a key contributor to reduced cardiac efficiency in diabetes. *Physiology (Bethesda)* **21**, 250-258
46. Karmazyn, M. (1998) The myocardial sodium-hydrogen exchanger (NHE) and its role in mediating ischemic and reperfusion injury. *Keio J Med* **47**, 65-72
47. Griffiths, E. J., and Halestrap, A. P. (1995) Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion. *Biochem J* **307 (Pt 1)**, 93-98
48. Zweier, J. L., and Talukder, M. A. (2006) The role of oxidants and free radicals in reperfusion injury. *Cardiovasc Res* **70**, 181-190
49. Burwell, L. S., Nadtochiy, S. M., and Brookes, P. S. (2009) Cardioprotection by metabolic shut-down and gradual wake-up. *J Mol Cell Cardiol* **46**, 804-810
50. Fragasso, G., Spoladore, R., Cuko, A., and Palloshi, A. (2007) Modulation of fatty acids oxidation in heart failure by selective pharmacological inhibition of 3-ketoacyl coenzyme-A thiolase. *Curr Clin Pharmacol* **2**, 190-196
51. Lopaschuk, G. D., Ussher, J. R., Folmes, C. D., Jaswal, J. S., and Stanley, W. C. (2010) Myocardial fatty acid metabolism in health and disease. *Physiological reviews* **90**, 207-258
52. Zhang, J., Villacorta, L., Chang, L., Fan, Z., Hamblin, M., Zhu, T., Chen, C. S., Cole, M. P., Schopfer, F. J., Deng, C. X., Garcia-Barrio, M. T., Feng, Y. H., Freeman, B. A., and Chen, Y. E. (2010) Nitro-oleic acid inhibits angiotensin II-induced hypertension. *Circ Res* **107**, 540-548
53. Rudolph, T. K., Rudolph, V., Edreira, M. M., Cole, M. P., Bonacci, G., Schopfer, F. J., Woodcock, S. R., Franek, A., Pekarova, M., Khoo, N. K., Hasty, A. H., Baldus, S., and Freeman, B. A. (2010) Nitro-fatty acids reduce atherosclerosis in apolipoprotein E-deficient mice. *Arteriosclerosis, thrombosis, and vascular biology* **30**, 938-945
54. Batthyany, C., Schopfer, F. J., Baker, P. R., Duran, R., Baker, L. M., Huang, Y., Cervenansky, C., Branchaud, B. P., and Freeman, B. A. (2006) Reversible post-translational modification of proteins by nitrated fatty acids in vivo. *J Biol Chem* **281**, 20450-20463
55. Bonacci, G., Schopfer, F. J., Batthyany, C. I., Rudolph, T. K., Rudolph, V., Khoo, N. K., Kelley, E. E., and Freeman, B. A. (2011) Electrophilic fatty acids regulate matrix metalloproteinase activity and expression. *J Biol Chem* **286**, 16074-16081
56. Nadtochiy, S. M., Zhu, Q., Urciuoli, W., Rafikov, R., Black, S. M., and Brookes, P. S. (2012) Nitroalkenes confer acute cardioprotection via adenine nucleotide translocase 1. *J Biol Chem* **287**, 3573-3580

57. Bonacci, G., Salvatore, S. R., Vitturi, D. A., Woodcock, S. R., Rudolph, T. K., Rudolph, V., Koenitzer, J. R., Schopfer, F. J., and Freeman, B. A. (2012) Unpublished Observations.
58. Queliconi, B. B., Wojtovich, A. P., Nadtochiy, S. M., Kowaltowski, A. J., and Brookes, P. S. (2011) Redox regulation of the mitochondrial K(ATP) channel in cardioprotection. *Biochimica et biophysica acta* **1813**, 1309-1315
59. Honda, H. M., Korge, P., and Weiss, J. N. (2005) Mitochondria and ischemia/reperfusion injury. *Annals of the New York Academy of Sciences* **1047**, 248-258
60. Halestrap, A. P. (2010) A pore way to die: the role of mitochondria in reperfusion injury and cardioprotection. *Biochem Soc Trans* **38**, 841-860
61. Adlam, V. J., Harrison, J. C., Porteous, C. M., James, A. M., Smith, R. A., Murphy, M. P., and Sammut, I. A. (2005) Targeting an antioxidant to mitochondria decreases cardiac ischemia-reperfusion injury. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **19**, 1088-1095
62. Gohil, V. M., Sheth, S. A., Nilsson, R., Wojtovich, A. P., Lee, J. H., Perocchi, F., Chen, W., Clish, C. B., Ayata, C., Brookes, P. S., and Mootha, V. K. (2010) Nutrient-sensitized screening for drugs that shift energy metabolism from mitochondrial respiration to glycolysis. *Nature biotechnology* **28**, 249-255
63. Woodcock, S. R., Marwitz, A. J., Bruno, P., and Branchaud, B. P. (2006) Synthesis of nitrolipids. All four possible diastereomers of nitrooleic acids: (E)- and (Z)-, 9- and 10-nitro-octadec-9-enoic acids. *Organic letters* **8**, 3931-3934
64. Wittig, I., Braun, H. P., and Schagger, H. (2006) Blue native PAGE. *Nature protocols* **1**, 418-428
65. Schopfer, F. J., Batthyany, C., Baker, P. R., Bonacci, G., Cole, M. P., Rudolph, V., Groeger, A. L., Rudolph, T. K., Nadtochiy, S., Brookes, P. S., and Freeman, B. A. (2009) Detection and quantification of protein adduction by electrophilic fatty acids: mitochondrial generation of fatty acid nitroalkene derivatives. *Free Radic Biol Med* **46**, 1250-1259
66. Ferrick, D. A., Neilson, A., and Beeson, C. (2008) Advances in measuring cellular bioenergetics using extracellular flux. *Drug discovery today* **13**, 268-274
67. Bell, R. M., Mocanu, M. M., and Yellon, D. M. (2011) Retrograde heart perfusion: the Langendorff technique of isolated heart perfusion. *J Mol Cell Cardiol* **50**, 940-950
68. Picklo, M. J., Amarnath, V., McIntyre, J. O., Graham, D. G., and Montine, T. J. (1999) 4-Hydroxy-2(E)-nonenal inhibits CNS mitochondrial respiration at multiple sites. *Journal of neurochemistry* **72**, 1617-1624
69. Lin, D., Saleh, S., and Liebler, D. C. (2008) Reversibility of covalent electrophile-protein adducts and chemical toxicity. *Chem Res Toxicol* **21**, 2361-2369
70. Zhang, L., Chen, C. L., Kang, P. T., Garg, V., Hu, K., Green-Church, K. B., and Chen, Y. R. (2010) Peroxynitrite-mediated oxidative modifications of complex II: relevance in myocardial infarction. *Biochemistry* **49**, 2529-2539
71. Shiva, S., Crawford, J. H., Ramachandran, A., Ceaser, E. K., Hillson, T., Brookes, P. S., Patel, R. P., and Darley-Usmar, V. M. (2004) Mechanisms of the interaction of nitroxyl with mitochondria. *Biochem J* **379**, 359-366
72. Lashin, O. M., Szweda, P. A., Szweda, L. I., and Romani, A. M. (2006) Decreased complex II respiration and HNE-modified SDH subunit in diabetic heart. *Free Radic Biol Med* **40**, 886-896

73. Dranka, B. P., Hill, B. G., and Darley-Usmar, V. M. (2010) Mitochondrial reserve capacity in endothelial cells: The impact of nitric oxide and reactive oxygen species. *Free Radic Biol Med* **48**, 905-914
74. Rudolph, V., Rudolph, T. K., Schopfer, F. J., Bonacci, G., Woodcock, S. R., Cole, M. P., Baker, P. R., Ramani, R., and Freeman, B. A. (2010) Endogenous generation and protective effects of nitro-fatty acids in a murine model of focal cardiac ischaemia and reperfusion. *Cardiovasc Res* **85**, 155-166
75. Wojtovich, A. P., and Brookes, P. S. (2008) The endogenous mitochondrial complex II inhibitor malonate regulates mitochondrial ATP-sensitive potassium channels: implications for ischemic preconditioning. *Biochimica et biophysica acta* **1777**, 882-889
76. Wojtovich, A. P., and Brookes, P. S. (2009) The complex II inhibitor atpenin A5 protects against cardiac ischemia-reperfusion injury via activation of mitochondrial KATP channels. *Basic research in cardiology* **104**, 121-129
77. Garlid, K. D., Paucek, P., Yarov-Yarovoy, V., Murray, H. N., Darbenzio, R. B., D'Alonzo, A. J., Lodge, N. J., Smith, M. A., and Grover, G. J. (1997) Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K+ channels. Possible mechanism of cardioprotection. *Circ Res* **81**, 1072-1082
78. Ockaili, R. A., Bhargava, P., and Kukreja, R. C. (2001) Chemical preconditioning with 3-nitropropionic acid in hearts: role of mitochondrial K(ATP) channel. *Am J Physiol Heart Circ Physiol* **280**, H2406-2411
79. Ma, X. L., Gao, F., Liu, G. L., Lopez, B. L., Christopher, T. A., Fukuto, J. M., Wink, D. A., and Feelisch, M. (1999) Opposite effects of nitric oxide and nitroxyl on postischemic myocardial injury. *Proc Natl Acad Sci U S A* **96**, 14617-14622
80. Drose, S., Bleier, L., and Brandt, U. (2011) A common mechanism links differently acting complex II inhibitors to cardioprotection: modulation of mitochondrial reactive oxygen species production. *Molecular pharmacology* **79**, 814-822
81. Vanden Hoek, T. L., Becker, L. B., Shao, Z., Li, C., and Schumacker, P. T. (1998) Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J Biol Chem* **273**, 18092-18098
82. Coles, C. J., Edmondson, D. E., and Singer, T. P. (1979) Inactivation of succinate dehydrogenase by 3-nitropropionate. *J Biol Chem* **254**, 5161-5167
83. Tomasiak, T. M., Archuleta, T. L., Andrell, J., Luna-Chavez, C., Davis, T. A., Sarwar, M., Ham, A. J., McDonald, W. H., Yankovskaya, V., Stern, H. A., Johnston, J. N., Maklashina, E., Cecchini, G., and Iverson, T. M. (2011) Geometric restraint drives on- and off-pathway catalysis by the Escherichia coli menaquinol:fumarate reductase. *J Biol Chem* **286**, 3047-3056
84. Huang, L. S., Sun, G., Cobessi, D., Wang, A. C., Shen, J. T., Tung, E. Y., Anderson, V. E., and Berry, E. A. (2006) 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**, 5965-5972
85. Beal, M. F., Brouillet, E., Jenkins, B. G., Ferrante, R. J., Kowall, N. W., Miller, J. M., Storey, E., Srivastava, R., Rosen, B. R., and Hyman, B. T. (1993) Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **13**, 4181-4192

86. Rudolph, V., Schopfer, F. J., Khoo, N. K., Rudolph, T. K., Cole, M. P., Woodcock, S. R., Bonacci, G., Groeger, A. L., Golin-Bisello, F., Chen, C. S., Baker, P. R., and Freeman, B. A. (2009) Nitro-fatty acid metabolome: saturation, desaturation, beta-oxidation, and protein adduction. *J Biol Chem* **284**, 1461-1473
87. Baker, P. R., Schopfer, F. J., Sweeney, S., and Freeman, B. A. (2004) Red cell membrane and plasma linoleic acid nitration products: synthesis, clinical identification, and quantitation. *Proc Natl Acad Sci U S A* **101**, 11577-11582
88. Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G., and Tsien, R. Y. (1998) Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc Natl Acad Sci U S A* **95**, 6803-6808
89. Shiva, S., and Gladwin, M. T. (2009) Nitrite mediates cytoprotection after ischemia/reperfusion by modulating mitochondrial function. *Basic research in cardiology* **104**, 113-119
90. Chen, Y. R., Chen, C. L., Pfeiffer, D. R., and Zweier, J. L. (2007) Mitochondrial complex II in the post-ischemic heart: oxidative injury and the role of protein S-glutathionylation. *J Biol Chem* **282**, 32640-32654
91. Koenitzer, J. R., and Freeman, B. A. Redox signaling in inflammation: interactions of endogenous electrophiles and mitochondria in cardiovascular disease. *Ann N Y Acad Sci* **1203**, 45-52
92. Koenitzer, J. R., Bonacci, G., Woodcock, S. R., J., W., Malik, T. M., Freeman, B. A., and Schopfer, F. J. (2012) Fatty acid nitroalkenes modulate mitochondrial respiration at complex II and induce resistance to ischemic cardiac injury.
93. Deacon, C. F., Hughes, T. E., and Holst, J. J. (1998) Dipeptidyl peptidase IV inhibition potentiates the insulinotropic effect of glucagon-like peptide 1 in the anesthetized pig. *Diabetes* **47**, 764-769
94. Pergola, P. E., Raskin, P., Toto, R. D., Meyer, C. J., Huff, J. W., Grossman, E. B., Krauth, M., Ruiz, S., Audhya, P., Christ-Schmidt, H., Wittes, J., and Warnock, D. G. Bardoxolone methyl and kidney function in CKD with type 2 diabetes. *N Engl J Med* **365**, 327-336
95. Vitturi, D. A., Bonacci, G., Salvatore, S. R., Woodcock, S. R., Schopfer, F. J., and Freeman, B. A. (2012) Unpublished observations.
96. Anggard, E., Larsson, C., and Samuelsson, B. (1971) The distribution of 15-hydroxy prostaglandin dehydrogenase and prostaglandin-delta 13-reductase in tissues of the swine. *Acta Physiol Scand* **81**, 396-404
97. Ceconi, C., Bernocchi, P., Boraso, A., Cargnoni, A., Pepi, P., Curello, S., and Ferrari, R. (2000) New insights on myocardial pyridine nucleotides and thiol redox state in ischemia and reperfusion damage. *Cardiovasc Res* **47**, 586-594
98. Gupte, S. A., and Wolin, M. S. (2006) Hypoxia promotes relaxation of bovine coronary arteries through lowering cytosolic NADPH. *Am J Physiol Heart Circ Physiol* **290**, H2228-2238
99. Morgan, A. H., Hammond, V. J., Morgan, L., Thomas, C. P., Tallman, K. A., Garcia-Diaz, Y. R., McGuigan, C., Serpi, M., Porter, N. A., Murphy, R. C., and O'Donnell, V. B. Quantitative assays for esterified oxylipins generated by immune cells. *Nature protocols* **5**, 1919-1931

100. Penna, C., Mancardi, D., Tullio, F., and Pagliaro, P. (2008) Postconditioning and intermittent bradykinin induced cardioprotection require cyclooxygenase activation and prostacyclin release during reperfusion. *Basic research in cardiology* **103**, 368-377
101. Bates, D. J. P., Lively, M. O., Gorczynski, M. J., King, S. B., Townsend, A. J., and Morrow, C. S. (2009) Noncatalytic Interactions between Glutathione S-Transferases and Nitroalkene Fatty Acids Modulate Nitroalkene-Mediated Activation of Peroxisomal Proliferator-Activated Receptor gamma. *Biochemistry* **48**, 4159-4169
102. Porreca, E., Del Boccio, G., Lapenna, D., Di Febbo, C., Pennelli, A., Cipollone, F., Di Ilio, C., and Cuccurullo, F. (1994) Myocardial antioxidant defense mechanisms: time related changes after reperfusion of the ischemic rat heart. *Free Radic Res* **20**, 171-179
103. Kensler, T. W., Wakabayashi, N., and Biswal, S. (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* **47**, 89-116
104. Laborde, E. Glutathione transferases as mediators of signaling pathways involved in cell proliferation and cell death. *Cell Death Differ* **17**, 1373-1380
105. Alexander, R. L., Bates, D. J. P., Wright, M. W., King, S. B., and Morrow, C. S. (2006) Modulation of nitrated lipid signaling by multidrug resistance protein 1 (MRP1): Glutathione conjugation and MRP1-mediated efflux inhibit nitrolinoleic acid-induced, PPAR gamma-dependent transcription activation. *Biochemistry* **45**, 7889-7896
106. Charles, R. L., Burgoyne, J. R., Mayr, M., Weldon, S. M., Hubner, N., Dong, H., Morisseau, C., Hammock, B. D., Landar, A., and Eaton, P. Redox regulation of soluble epoxide hydrolase by 15-deoxy-delta-prostaglandin J2 controls coronary hypoxic vasodilation. *Circ Res* **108**, 324-334
107. Charles, R. L., Schroder, E., May, G., Free, P., Gaffney, P. R. J., Wait, R., Begum, S., Heads, R. J., and Eaton, P. (2007) Protein sulfenation as a redox sensor - Proteomics studies using a novel biotinylated dimedone analogue. *Molecular & Cellular Proteomics* **6**, 1473-1484
108. Takahashi, H., Shin, Y., Cho, S. J., Zago, W. M., Nakamura, T., Gu, Z. Z., Ma, Y. L., Furukawa, H., Liddington, R., Zhang, D. X., Tong, G., Chen, H. S. V., and Lipton, S. A. (2007) Hypoxia enhances S-nitrosylation-mediated NMDA receptor inhibition via a thiol oxygen sensor motif. *Neuron* **53**, 53-64
109. Qanungo, S., Starke, D. W., Pai, H. V., Mieyal, J. J., and Nieminen, A. L. (2007) Glutathione supplementation potentiates hypoxic apoptosis by S-glutathionylation of p65-NF kappa B. *Journal of Biological Chemistry* **282**, 18427-18436
110. Schopfer, F. J., Cipollina, C., and Freeman, B. A. (2011) Formation and signaling actions of electrophilic lipids. *Chemical reviews* **111**, 5997-6021
111. Koenitzer, J. R., and Freeman, B. A. (2010) Redox signaling in inflammation: interactions of endogenous electrophiles and mitochondria in cardiovascular disease. *Annals of the New York Academy of Sciences* **1203**, 45-52
112. Gebauer, S. K., Destaillats, F., Mouloungui, Z., Candy, L., Bezelgues, J. B., Dionisi, F., and Baer, D. J. (2011) Effect of trans fatty acid isomers from ruminant sources on risk factors of cardiovascular disease: Study design and rationale. *Contemporary Clinical Trials* **32**, 569-576
113. Onakpoya, I. J., Posadzki, P. P., Watson, L. K., Davies, L. A., and Ernst, E. (2012) The efficacy of long-term conjugated linoleic acid (CLA) supplementation on body

composition in overweight and obese individuals: a systematic review and meta-analysis of randomized clinical trials. *European Journal of Nutrition* **51**, 127-134
114. McClelland, S., Cox, C., O'Connor, R., de Gaetano, M., McCarthy, C., Cryan, L., Fitzgerald, D., and Belton, O. (2010) Conjugated linoleic acid suppresses the migratory and inflammatory phenotype of the monocyte/macrophage cell. *Atherosclerosis* **211**, 96-102
115. Lee, K. W., Lee, H. J., Cho, H. Y., and Kim, Y. J. (2005) Role of the conjugated linoleic acid in the prevention of cancer. *Critical Reviews in Food Science and Nutrition* **45**, 135-144
116. Cohen, M. V., Baines, C. P., and Downey, J. M. (2000) Ischemic preconditioning: from adenosine receptor to KATP channel. *Annual review of physiology* **62**, 79-109
117. Wolin, M. S. (2000) Interactions of oxidants with vascular signaling systems. *Arteriosclerosis, thrombosis, and vascular biology* **20**, 1430-1442
118. Horowitz, J. D., Chirkov, Y. Y., Kennedy, J. A., and Sverdlov, A. L. (2010) Modulation of myocardial metabolism: an emerging therapeutic principle. *Current opinion in cardiology* **25**, 329-334
119. Schipke, J. D., Friebe, R., and Gams, E. (2006) Forty years of glucose-insulin-potassium (GIK) in cardiac surgery: a review of randomized, controlled trials. *European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery* **29**, 479-485
120. Abozguia, K., Clarke, K., Lee, L., and Frenneaux, M. (2006) Modification of myocardial substrate use as a therapy for heart failure. *Nature clinical practice. Cardiovascular medicine* **3**, 490-498
121. Holubarsch, C. J., Rohrbach, M., Karrasch, M., Boehm, E., Polonski, L., Ponikowski, P., and Rhein, S. (2007) A double-blind randomized multicentre clinical trial to evaluate the efficacy and safety of two doses of etomoxir in comparison with placebo in patients with moderate congestive heart failure: the ERGO (etomoxir for the recovery of glucose oxidation) study. *Clin Sci (Lond)* **113**, 205-212
122. Prescott, L. F., Park, J., Ballantyne, A., Adriaenssens, P., and Proudfoot, A. T. (1977) Treatment of paracetamol (acetaminophen) poisoning with N-acetylcysteine. *Lancet* **2**, 432-434
123. Lonn, E., Bosch, J., Yusuf, S., Sheridan, P., Pogue, J., Arnold, J. M., Ross, C., Arnold, A., Sleight, P., Probstfield, J., Dagenais, G. R., Hope, and Investigators, H.-T. T. (2005) Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. *JAMA : the journal of the American Medical Association* **293**, 1338-1347
124. Sesso, H. D., Buring, J. E., Christen, W. G., Kurth, T., Belanger, C., MacFadyen, J., Bubes, V., Manson, J. E., Glynn, R. J., and Gaziano, J. M. (2008) Vitamins E and C in the prevention of cardiovascular disease in men: the Physicians' Health Study II randomized controlled trial. *JAMA : the journal of the American Medical Association* **300**, 2123-2133
125. Juraschek, S. P., Guallar, E., Appel, L. J., and Miller, E. R., 3rd. (2012) Effects of vitamin C supplementation on blood pressure: a meta-analysis of randomized controlled trials. *Am J Clin Nutr* **95**, 1079-1088

126. Myers, M. L., Bolli, R., Lekich, R. F., Hartley, C. J., and Roberts, R. (1985) Enhancement of recovery of myocardial function by oxygen free-radical scavengers after reversible regional ischemia. *Circulation* **72**, 915-921
127. Deshmukh, D. R., Mirochnitchenko, O., Ghole, V. S., Agnese, D., Shah, P. C., Reddell, M., Brolin, R. E., and Inouye, M. (1997) Intestinal ischemia and reperfusion injury in transgenic mice overexpressing copper-zinc superoxide dismutase. *The American journal of physiology* **273**, C1130-1135
128. Rabkin, S. W., and Klassen, S. S. (2008) Metalloporphyrins as a therapeutic drug class against peroxynitrite in cardiovascular diseases involving ischemic reperfusion injury. *European journal of pharmacology* **586**, 1-8
129. Batinic-Haberle, I., Reboucas, J. S., and Spasojevic, I. (2010) Superoxide dismutase mimics: chemistry, pharmacology, and therapeutic potential. *Antioxid Redox Signal* **13**, 877-918
130. Graham, D., Huynh, N. N., Hamilton, C. A., Beattie, E., Smith, R. A., Cocheme, H. M., Murphy, M. P., and Dominiczak, A. F. (2009) Mitochondria-targeted antioxidant MitoQ10 improves endothelial function and attenuates cardiac hypertrophy. *Hypertension* **54**, 322-328
131. Ristow, M., Zarse, K., Oberbach, A., Kloting, N., Birringer, M., Kiehntopf, M., Stumvoll, M., Kahn, C. R., and Bluher, M. (2009) Antioxidants prevent health-promoting effects of physical exercise in humans. *Proc Natl Acad Sci U S A* **106**, 8665-8670