

# Nitroalkenes Confer Acute Cardioprotection via Adenine Nucleotide Translocase 1<sup>\*S</sup>

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Sergiy M. Nadtochiy<sup>‡</sup>, Qiuyu Zhu<sup>§</sup>, William Urciuoli<sup>‡</sup>, Ruslan Rafikov<sup>¶1</sup>, Stephen M. Black<sup>¶</sup>, and Paul S. Brookes<sup>‡2</sup>

From the <sup>‡</sup>Department of Anesthesiology and <sup>§</sup>Aab Cardiovascular Research Institute, University of Rochester Medical Center, Rochester, New York 14642 and the <sup>¶</sup>Vascular Biology Center, Georgia Health Sciences University, Augusta, Georgia 30912

**Background:** Nitroalkenes are cardioprotective. We investigated the role of ANT1 in this process.

**Results:** Nitro-linoleate modifies Cys<sup>57</sup> on ANT1. Knockdown of ANT1 inhibits cytoprotection by nitro-linoleate.

**Conclusion:** ANT1 is required for nitro-linoleate cytoprotection and possibly for its cardioprotection.

**Significance:** This is the first evidence for modification of a specific cysteine in a mitochondrial protein by a physiologically (and clinically) relevant electrophile.

Electrophilic nitrated lipids (nitroalkenes) are emerging as an important class of protective cardiovascular signaling molecules. Although species such as nitro-linoleate (LNO<sub>2</sub>) and nitro-oleate can confer acute protection against cardiac ischemic injury, their mechanism of action is unclear. Mild uncoupling of mitochondria is known to be cardioprotective, and adenine nucleotide translocase 1 (ANT1) is a key mediator of mitochondrial uncoupling. ANT1 also contains redox-sensitive cysteines that may be targets for modification by nitroalkenes. Therefore, in this study we tested the hypothesis that nitroalkenes directly modify ANT1 and that nitroalkene-mediated cardioprotection requires ANT1. Using biotin-tagged LNO<sub>2</sub> infused into intact perfused hearts, we obtained mass spectrometric (MALDI-TOF-TOF) evidence for direct modification (nitroalkylation) of ANT1 on cysteine 57. Furthermore, in a cell model of ischemia-reperfusion injury, siRNA knockdown of ANT1 inhibited the cardioprotective effect of LNO<sub>2</sub>. Although the molecular mechanism linking ANT1-Cys<sup>57</sup> nitroalkylation and uncoupling is not yet known, these data suggest that ANT1-mediated uncoupling may be a mechanism for nitroalkene-induced cardioprotection.

Mitochondria play a central role in cardiac ischemia-reperfusion (IR)<sup>3</sup> injury (1). IR severely impairs mitochondrial function, causing inhibition of Ox-Phos (2), enhanced ROS generation, and Ca<sup>2+</sup> dysregulation (3), which in combination with high inorganic phosphate and low ATP promote opening of the mitochondrial permeability transition (PT) pore (4). The latter

is a key factor in IR-induced cell death (5). The composition of the PT pore is still a matter of debate, and whereas a role for cyclophilin D is well defined (6), opposing data exist regarding involvement of the adenine nucleotide translocase (ANT) (7, 8). In addition to its potential role in the PT pore and cell death regulation (7, 9, 10), ANT plays essential roles in mitochondrial bioenergetics (10) and ANT1 (the major cardiac isoform (11)) is known to mediate a basal proton leak in mitochondria (12, 13). Mild increases in mitochondrial proton leak (uncoupling) can confer protection against IR injury (14–16), and this raises the possibility that ANT may play a role in cardioprotection.

Nitroalkenes, such as nitro-linoleic acid (LNO<sub>2</sub>) and nitro-oleic acid (OANO<sub>2</sub>), are electrophiles that trigger a variety of intracellular signaling events (17, 18). The mechanisms of endogenous nitroalkene formation are poorly understood, but several factors present in mitochondria are thought to be important (19). This includes a large mitochondrial pool of unsaturated membrane lipids (20), a ready availability of nitrite (21), and elevated levels of reactive oxygen/nitrogen species (22). The conditions encountered during ischemia, including acidosis, may promote endogenous nitroalkene formation (23, 24). However, far from being pathologic in nature, nitroalkenes are thought to play an important role in the endogenous cardioprotective paradigm of ischemic preconditioning (IPC) (15). Formation of LNO<sub>2</sub> has been shown in mitochondria isolated from perfused hearts subjected to IPC (15). Furthermore, prior administration of synthetic nitroalkenes prevented IR injury in both *in vivo* hearts and isolated cardiomyocytes (15, 24).

One mechanism of nitroalkene signaling is the nitroalkylation of nucleophilic protein residues such as cysteines (25). We previously obtained immunoblot data strongly supportive of ANT1 nitroalkylation in isolated mitochondria and cardiomyocytes. This was accompanied by an increase in mitochondrial proton leak and by potent cytoprotection against simulated IR injury (15). ANT1 is an attractive candidate for cardioprotective nitroalkene signaling, as it contains a number of redox active cysteines (26) that have been shown to play critical roles in adenine nucleotide binding (27) and opening of the PT pore (28, 29). Notably, however, the role of these cysteines in regulating proton conductance by ANT1 is unknown. Furthermore, a direct role for ANT1 in the acute cardioprotective effect of

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<sup>S</sup> This article contains supplemental Table S1 and Figs. S1–S4.

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<sup>2</sup> To whom correspondence should be addressed: Dept. of Anesthesiology, Box 604, University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642. Tel.: 585-273-1626; E-mail: paul\_brookes@urmc.rochester.edu.

<sup>3</sup> The abbreviations used are: IR, ischemia-reperfusion; LA, linoleic acid; OA, oleic acid; LNO<sub>2</sub>, nitro-linoleate; Bt-LNO<sub>2</sub>, biotinylated LNO<sub>2</sub>; OANO<sub>2</sub>, nitro-oleate; ANT1, adenine nucleotide translocase 1; IPC, ischemic preconditioning; PT pore, permeability transition pore; TMRE, tetramethylrhodamine ethyl; Ctrl, control; CHCA,  $\alpha$ -cyano-4-hydroxycinnamic acid.

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nitroalkenes has not been established. Therefore, the goal of this study was to determine the requirement for ANT1 in nitroalkene-mediated cardioprotection.

### EXPERIMENTAL PROCEDURES

**Animals and Reagents**—Full experimental details are in the supplemental material. Two-month-old male C57/BL6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained under the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were housed under a 12 h light/dark cycle with food and water available *ad libitum*. All chemicals were purchased from Sigma unless otherwise specified.

**Nitroalkene Synthesis, Purification, and Quantification**—Nitroalkenes (OANO<sub>2</sub>, LNO<sub>2</sub>) were synthesized by nitrosylation as previously described (30). Biotin-tagged LNO<sub>2</sub> (Bt-LNO<sub>2</sub>) was synthesized from fresh LNO<sub>2</sub> stock using EZ-Link Biotin Hydrazide (Pierce) followed by purification and quantification as previously published (31). All nitroalkenes and native fatty acids (OA and LA) were stored in pure HPLC grade methanol at  $-80^{\circ}\text{C}$  under argon.

**Heart Preparation**—Isolated heart perfusion was performed as previously described (15, 32). Hearts were divided in several groups: (i) IR alone ( $n = 8$ ); (ii) LA + IR ( $n = 7$ ); (iii) LNO<sub>2</sub> + IR ( $n = 8$ ); (iv) Bt-LNO<sub>2</sub> + IR ( $n = 7$ ); (v) OA + IR ( $n = 7$ ); (vi) OANO<sub>2</sub> + IR ( $n = 7$ ). Nitroalkenes and related agents were infused via a port above the perfusion cannula for 20 min before ischemia at 100 nM. Global ischemia was induced by stopping flow and immersing the heart in deoxygenated buffer at  $37^{\circ}\text{C}$  for 25 min. Reperfusion (1 h) was initiated by raising flow back to the preischemic rate over a period of 1 min. Parameters measured included heart rate, left ventricular developed pressure (LVDP), rate pressure product (heart rate  $\times$  LVDP), and infarct size (2,3,5-triphenyltetrazolium chloride staining).

**Immunoprecipitation, SDS-PAGE, and In-gel Digestion**—In separate experiments hearts from groups (i) and (iv) were harvested before ischemia and subjected to two separate immunoprecipitation protocols, described previously (15, 33). Briefly, whole hearts were homogenized in buffer containing 20 mM Tris, 50 mM NaCl, 1 mM EGTA, 0.5% (v/v) Nonidet P-40, 1% (v/v) Triton X-100, 1% (w/v) *n*-dodecyl- $\beta$ -D-maltoside supplemented with protease inhibitor mixture tablets (Roche Applied Science), pH 7.4, at  $4^{\circ}\text{C}$ . ANT1 was pulled down from 2 mg of whole heart homogenate using anti-ANT1 antibodies (1:200). Immunocomplexes were captured using protein G-agarose (EMD, Gibbstown, NJ) suspension (15  $\mu\text{l}/\mu\text{g}$  antibody). After dissociation of immunocomplexes from the beads by boiling in sample buffer, proteins were separated by 12.5% SDS-PAGE gel and immunoblotted using either anti-ANT1 antibodies (MitoSciences, Eugene, OR) or streptavidin-HRP (GE Healthcare).

In reverse experiments, biotinylated proteins (*i.e.* from hearts treated with biotin-tagged nitroalkenes) were immunoprecipitated from whole heart homogenates using neutravidin-agarose (Pierce) (15). Samples were subjected to 12.5% SDS-PAGE and run in duplicate. Proteins from one gel were transferred to nitrocellulose, and membranes were probed with anti-ANT1 antibodies (1:2000 dilution). Proteins from the second gel were visualized by Coomassie Blue stain, and the prominent bands at

$>250$ , 80, 55, and 32 kDa were excised. Bands were destained and subjected to overnight in-gel digestion with sequencing grade trypsin (25 ng/ $\mu\text{l}$  in 25 mM ammonium bicarbonate buffer, pH 7.8). Peptides were extracted with 0.1% TFA, 75% acetonitrile and evaporated to near dryness.

**MALDI-TOF-TOF Mass Spectrometry**—Peptide calibration standards and matrix CHCA were from Applied Biosystems (Carlsbad, CA). All spectra were taken on an ABSciex 5800 MALDI-TOF-TOF mass spectrometer in positive reflector mode (10 kV) with a matrix of CHCA. At least 1000 laser shots were averaged to obtain each spectrum. Masses were calibrated to known peptide standards. 5- $\mu\text{l}$  aliquots of the ANT1 (band at 32 kDa) tryptic digest were cleaned on a C18 ZipTip (Millipore, Bedford MA) as per manufacturer's instructions. Bound peptides were desalted with two 5- $\mu\text{l}$  washes of 0.1% TFA and then eluted with 2.5  $\mu\text{l}$  of aqueous, acidic acetonitrile (75% CH<sub>3</sub>CN, 0.1% TFA). The eluant was mixed with 2.5  $\mu\text{l}$  of freshly prepared CHCA stock solution (20 mg/ml CHCA in aqueous acetonitrile as above), and 1.5- $\mu\text{l}$  portions of this mixture were spotted onto a MALDI sample plate for air drying. 1.5  $\mu\text{l}$  of crude peptides were additionally mixed with 1.5  $\mu\text{l}$  of CHCA and spotted. MS/MS of the 1438.8  $m/z$  peak was performed in positive reflector mode without collision-induced dissociation. MS and MS/MS spectra were analyzed in Protein Pilot 3.0, Mascot Distiller, and PEAKS software packages.

**Cell Culture and siRNA Transfection**—H9c2 cardiomyocytes were maintained in DMEM with 4 mM glutamine, 1% penicillin/streptomycin, and 10% heat inactivated FBS at  $37^{\circ}\text{C}$  in a 5% CO<sub>2</sub> atmosphere. Cells were used between passages 20 and 40 and plated at 30,000/well on 22-mm 12-well plates (Greiner Bio-One) in antibiotic-free media. After 24 h, half the wells were transfected with 100 nM ANT1 siRNA (Qiagen, Valencia CA; #SI03107496) using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. The remaining wells were transfected with 100 nM concentrations of scrambled (control) siRNA (#1027281). Media were changed after 24 h. Successful knockdown was verified after 72 h using ANT1 immunoblotting.

**In Vitro IR Model**—72 h post-transfection, growth medium was replaced with 1 ml/well normoxic buffer comprising DMEM, 10 mM glucose, 10 mM HEPES, pH 7.4, at  $37^{\circ}\text{C}$ . LNO<sub>2</sub> (100 nM) was added in half of controls and half of ANT1 siRNA wells and incubated for 30 min at  $37^{\circ}\text{C}$ . After treatment, medium in each well was replaced with hypoxic buffer (glucose-free DMEM, 10 mM HEPES, pH 6.5, at  $37^{\circ}\text{C}$ ), which was pre-gassed with N<sub>2</sub>. The plate was then transferred to a hypoxic chamber (Coy Inc., Grass Lake, MI) pre-equilibrated with N<sub>2</sub>, thermostatic to  $37^{\circ}\text{C}$ . Reperfusion comprised removal of the plate from the hypoxic chamber, replacement of media with normoxic buffer, and incubation at  $37^{\circ}\text{C}$  for 1.5 h. Cell death was then assayed by measuring LDH release using a kit (Cytotoxicity Detection, Roche Applied Science) according to the manufacturer's protocol. LDH release was expressed as a percentage of total LDH, the latter obtained by adding 0.1% Triton X-100 to lyse cells. The model of IR injury was validated by testing a known cardioprotective agent, the volatile anesthetic isoflurane, which yielded a  $31 \pm 3\%$  reduction in IR-induced cell death (data not shown).

**Statistics**—Where appropriate, Student's *t* test and multiple way analysis of variance were applied to the data.

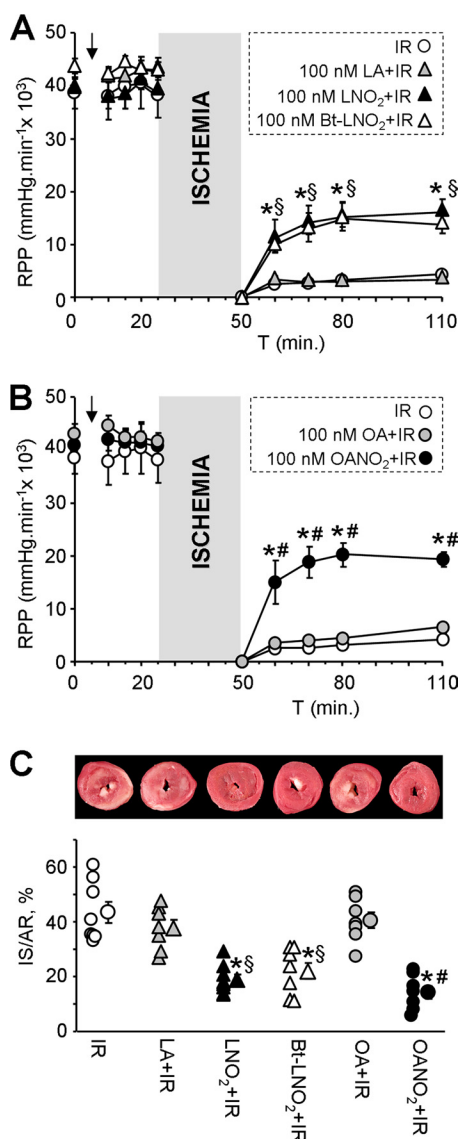
## RESULTS

An acute cardioprotective effect of nitroalkenes was demonstrated in adult cardiomyocytes (15). Furthermore, a long term *in vivo* treatment with nitroalkenes in mice conferred protection against IR injury. However, the ability of nitroalkenes to afford acute protection at the isolated heart level has not been investigated. Fig. 1 demonstrates that nitroalkenes significantly improved cardiac contractile function (rate  $\times$  pressure product) during reperfusion and drastically decreased infarct size compared with vehicle treated controls. Both LNO<sub>2</sub> and OANO<sub>2</sub> provided equal magnitudes of protection, and regular non-nitrated LA and OA were not protective, indicating that the small doses used herein (100 nM) were not sufficient to provide any cardioprotective effect of free fatty acids, as has been observed in other studies (34, 35). Together these data support the cardioprotective efficacy of nitroalkenes against IR injury.

The time-frame for this effect (15 min. infusion) indicates that the mechanism of protection was most likely via protein nitroalkylation rather than at the level of transcriptional activity. To investigate protein modifications by LNO<sub>2</sub> in the perfused heart, Bt-LNO<sub>2</sub> was synthesized. Bt-LNO<sub>2</sub> was administered in the same manner as LNO<sub>2</sub> and exhibited the same degree of protection against IR injury (Figs. 1, A and C). These data indicate that the addition of the biotin group did not interfere with the bioactivity of LNO<sub>2</sub>, suggesting that the active portion of the LNO<sub>2</sub> molecule is not the biotin-blocked carboxyl group.

Based on our previous studies (15), we focused next on the nitroalkene modification of ANT1. After Bt-LNO<sub>2</sub> delivery to perfused hearts, homogenates were subjected to immunoprecipitation with either anti-ANT1 antibodies or neutravidin to capture ANT1 and other biotin-tagged proteins followed by Western blotting. Fig. 2 shows that Bt-LNO<sub>2</sub> adducted several proteins, including ANT1, suggesting that nitroalkenes can cross the vascular and plasma membranes and enter mitochondria. This is in agreement with our previous results (15) where Bt-LNO<sub>2</sub> modified ANT1 in both isolated mitochondria and intact adult cardiomyocytes. Although the modification site was not elucidated, we demonstrated that pretreatment with the ANT inhibitor carboxyatractyloside abrogated Bt-LNO<sub>2</sub> modification (15). Because carboxyatractyloside is known to block the accessibility of a cysteine residue in ANT (Cys<sup>57</sup>), we therefore speculated this may be a site of LNO<sub>2</sub> modification.

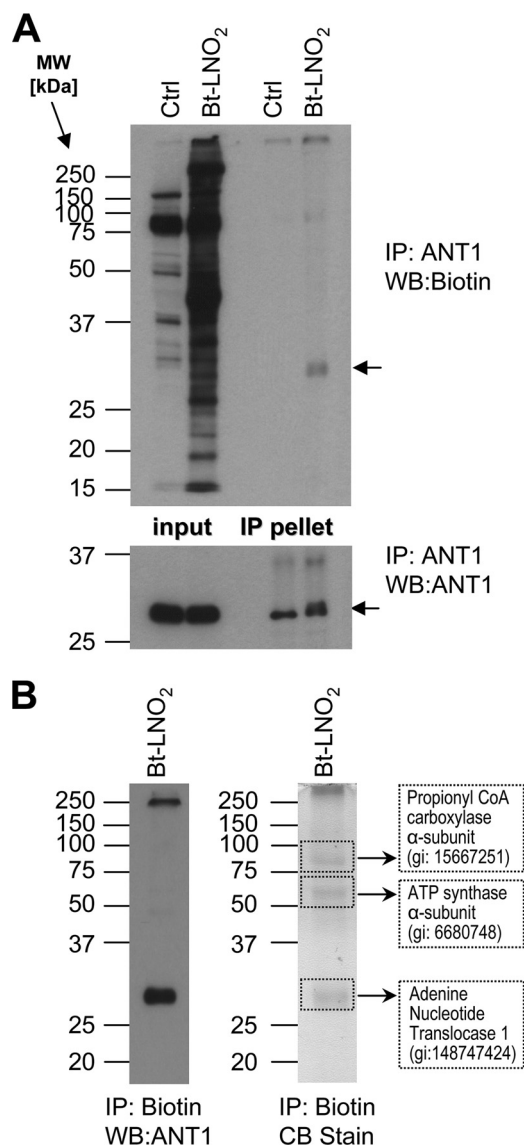
To check if any of the four known cysteines in ANT were modified in perfused heart after Bt-LNO<sub>2</sub> treatment, cardiac homogenates were immunoprecipitated using neutravidin beads. The immunoprecipitate pellet was separated on duplicate SDS-PAGE gels. One gel was transferred and probed for ANT1 to verify successful nitroalkylation and pull-down of the Bt-LNO<sub>2</sub>-ANT1 complex (Fig. 2B, left panel). The second gel was stained (Fig. 2B, right panel), and the prominent bands at >250, 80, 55, and 32 kDa were excised and subjected to mass spectrometric identification. The >250-kDa band could not be identified and is likely a nonspecific protein aggregate. The



**FIGURE 1. Cardioprotective effects of nitroalkenes in perfused mouse hearts.** A and B, perfused hearts were subjected to IR injury with or without infusion of agents (see “Experimental Procedures”). Left-ventricular contractile function was monitored, and graphs show rate  $\times$  pressure product (RPP). For clarity, experimental groups with linoleate-based and oleate-based treatments are shown in two separate panels, although the IR alone group is the same in each panel. Final concentration of nitroalkenes and related reagents was 100 nM. Data are the means  $\pm$  S.E.,  $n \geq 7$ ; \*,  $p < 0.05$  versus IR alone; §,  $p < 0.05$  versus IR + LA; #,  $p < 0.05$  versus IR + OA (analysis of variance). C, following IR protocols, hearts were stained with 2,3,5-triphenyltetrazolium chloride, and infarct size was measured as the % of the left ventricular area. The upper images show representative 2,3,5-triphenyltetrazolium chloride-stained hearts. In the graph, infarct is quantified, with individual data points for each condition shown on the left and the means  $\pm$  S.E. on the right ( $n \geq 7$ ). \*,  $p < 0.05$  versus IR alone; §,  $p < 0.05$  versus IR + LA; #,  $p < 0.05$  versus IR + OA (analysis of variance). IS/AR, infarct/area-at-risk.

band at 80 kDa was the  $\alpha$ -subunit of propionyl CoA-carboxylase, a biotin-containing enzyme that often contaminates biotin detection systems. The 55-kDa band was the  $\alpha$ -subunit of ATP synthase (see “Discussion”).

The 32-kDa band was identified as ANT1, with 80% sequence coverage, including all four cysteine residues (supplemental Fig. S1). The confidence of identification was above >98 for at least 5 peptides. Further MS/MS analysis (Fig. 3) revealed that a peptide at  $m/z$  1438.8 containing Cys<sup>57</sup> was modified by Bt-



**FIGURE 2. ANT1 modification by Bt-LNO<sub>2</sub>.** A, ANT1 was immunoprecipitated from whole heart homogenates (Ctrl or treated with 100 nM Bt-LNO<sub>2</sub>) using anti-ANT1 antibodies (see “Experimental Procedures”). The first two lanes are inputs (*i.e.* intact homogenate), and the next two lanes are immunoprecipitated (IP) pellets. Upper and lower panels correspond to immunoblots (WB) for biotin and ANT1, respectively. At least two independent experiments were performed for each condition. B, biotinylated proteins were immunoprecipitated from whole heart homogenates using neutravidin. The left panel represents immunoblot for ANT1, and the right panel is the corresponding Coomassie Blue (CB)-stained gel. Four bands at >250, 80, 55, and 32 kDa (highlighted by dotted lines) were analyzed by mass spectrometry (MALDI-TOF) and are identified as labeled in the figure. Mass spectrometry of the gel band at 32 kDa corresponding to ANT1 is shown in supplemental Fig. S1B.

LNO<sub>2</sub>, as indicated by a mass shift of 564 Da corresponding to the molecular mass of Bt-LNO<sub>2</sub> (supplemental Fig. S2). These data are the first identification of a nitroalkylation site in ANT1.

Although supraphysiological doses of electrophiles have previously been shown to modify mitochondrial proteins (36, 37) and modification of ANT cysteines has been shown using various thiol reagents (38), to the best of our knowledge this is the first ever *in situ* identification of a cysteine modification in ANT by a low dose (100 nM) of a physiologically relevant (15, 39) electrophile, delivered to an intact tissue. Notably, the levels of LNO<sub>2</sub> found in mitochondria in preconditioned hearts are in

the micromolar range, suggesting they may be capable of nitroalkylating ANT *in situ*, although preliminary studies in this area<sup>4</sup> have so far failed to detect nitroalkylation of ANT in hearts exposed to IPC.

To investigate if ANT1 was required for LNO<sub>2</sub>-mediated protection, we employed an *in vitro* model of IR injury. ANT1 was knocked down by siRNA in the H9c2 cardiomyocyte cell line. Fig. 4A demonstrates that 72 h post-transfection, ANT1 levels were 35% of control. The levels of another mitochondrial protein (70-kDa subunit of respiratory complex II) did not change, and a scrambled siRNA control was without effect.

To verify the metabolic effects of ANT1 knockdown in cells, we employed extracellular flux analysis (40) and TMRE spectrofluorimetry (15). ANT1 knockdown caused an ~30% decrease in basal respiration rate (124 ± 20 pmol/min in ANT1 siRNA versus 177 ± 26 pmol/min in Ctrl siRNA, *n* = 7, *p* = 0.001), likely due to the known bioenergetic role of ANT1. To assess mitochondrial uncoupling (proton leak), respiration in the presence of oligomycin (enforced state 4) was used as a surrogate marker, as previously described (15, 41, 42). ANT1 knockdown caused a significant decrease in state 4 respiration (37 ± 5 pmol/min in ANT1 siRNA versus 51 ± 6 pmol/min in Ctrl siRNA, *n* = 7, *p* = 0.001), consistent with a role for ANT1 in basal proton leak (12). Furthermore, although the addition of LNO<sub>2</sub> caused a small but significant increase in state 4 respiration in control cells (51 ± 6 pmol/min → 61 ± 9 pmol/min, *n* = 7, *p* = 0.02), LNO<sub>2</sub> was without effect in ANT1 siRNA cells (37 ± 5 pmol/min → 41 ± 6 pmol/min, *n* = 7, *p* = 0.185) (supplemental Fig. S3). To further characterize proton leak, mitochondrial membrane potential was also measured in H9c2 cells using TMRE, normalizing the signal to MitoTracker Green. Consistent with the respiration data, in oligomycin-enforced state 4, LNO<sub>2</sub> addition caused a significant decrease in Δψ in control cells (TMRE/MitoTracker Green signal 1439 ± 124 → 900 ± 80, *p* = 0.021) but not in ANT1 knock-down cells (TMRE/MitoTracker Green signal 1456 ± 371 → 1017 ± 216, *p* = 0.365). Together, these data indicate that the ability of LNO<sub>2</sub> to induce mitochondrial proton leak is partly dependent on ANT1.

Finally, because a mild induction of mitochondrial proton leak can elicit cardioprotection (14, 43) and we previously suggested this may be a mechanism of LNO<sub>2</sub>-induced protection (15), we sought to investigate whether knocking down ANT1 would also block LNO<sub>2</sub> protection. Fig. 4B shows that pretreatment with LNO<sub>2</sub> resulted in a 26 ± 3% decrease in IR-induced cell death, consistent with our previous finding in adult cardiomyocytes (15) and consistent with the effects of another cardioprotective agent, isoflurane (see “Experimental Procedures”). Most notably, administration of LNO<sub>2</sub> to ANT1 knockdown cells did not afford protection. This suggests that ANT1 is required for LNO<sub>2</sub>-mediated acute protection against IR injury.

## DISCUSSION

The present study establishes a role for ANT1 in LNO<sub>2</sub>-mediated acute protection against IR injury. At least two possible

<sup>4</sup> S. M. Nadtochiy and P. S. Brookes, unpublished information.

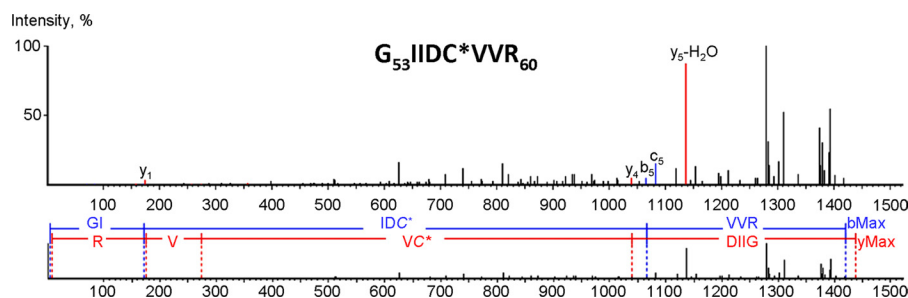


FIGURE 3. **Detection of Bt-LNO<sub>2</sub>-Cys adduct site in ANT1.** The MS/MS spectrum of 1438.8 *m/z* ion was obtained in positive reflector mode fitted with peptide <sup>53</sup>GI IDC\*VVR<sup>60</sup> from a ANT1 mouse sequence (gi:148747424) modified at Cys<sup>57</sup> by Bt-LNO<sub>2</sub>. Colored numbers represent masses found in MS/MS with mass error less than 0.3 ppm. Collision of biotinylated nitro-lipid-modified peptides during MS/MS produces multiple products of nitrolipid side-chain breakage, which PEAKS software cannot take into account. We performed manual assignment of the four major peaks on the MS/MS; 1136.931 corresponds to the y<sub>5</sub>-H<sub>2</sub>O ion, 1393.184 corresponds to decarboxylated metastable ion, 1279.124 corresponds to metastable ion with water and biotin ring loss, 1311.005 corresponds to y<sub>7</sub> ion with C<sub>5</sub>H<sub>10</sub> neutral loss from nitro-lipid side chain. Supplemental Table S1 shows the full list of b and y ions.

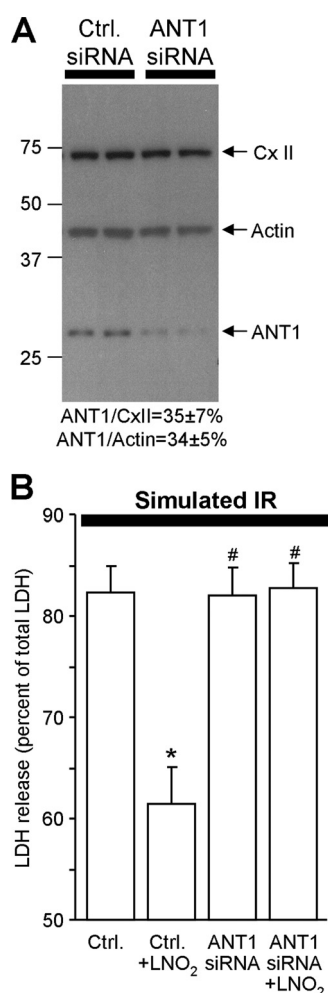


FIGURE 4. **ANT1 knock down abolishes effects of LNO<sub>2</sub> in H9c2 cells.** A, knockdown of ANT1 in H9c2 cells is shown. 72 h post-transfection cells (Ctrl and ANT1 siRNA) were harvested for immunoblotting analysis using anti-ANT1, anti-70 kDa complex II, and anti-actin antibodies. A blot image representative of four independent experiments is shown. Densitometry of ANT1, 70-kDa complex II (CXII), and actin bands was performed using Scion Image™ software. Relative ANT1 expression (in siRNA cells versus controls) is shown below the blot (means ± S.E., *n* = 4). B, is shown LDH release upon simulated IR injury. Transfected cells were subjected to simulated IR (see the “Experimental Procedures”). LDH release (cell death) is expressed as a percentage of total LDH (upon lysis with Triton X-100). Data are the means ± S.E., *n* = 7. \*, *p* < 0.05 versus Ctrl siRNA; #, *p* < 0.05 versus Ctrl siRNA + LNO<sub>2</sub>.

mechanisms of protection have been revealed; (i) modification of Cys<sup>57</sup> on ANT1 and (ii) ANT1-mediated mild uncoupling. Currently, the mechanistic details linking these two phenomena are not known. There are 4 cysteines in ANT1 at positions 57, 129, 159, and 256 in the mouse protein. Cys<sup>57</sup> is localized on the matrix side of ANT1 in the hydrophobic pocket Gly<sup>53</sup>-Val<sup>59</sup> (44, 45) and is accessible for modification by thiol reactive agents (46). Furthermore, this thiol faces the central pore of ANT1 (supplemental Fig. S4), and the electronegative nitro-group of nitroalkenes is retained upon covalent adduction of the lipid to cysteine. As such, a nitroalkene on Cys<sup>57</sup> would be expected to introduce an electronegative moiety into the central pore of ANT, and this may play a role in the mechanism of proton leak.

Modification of Cys<sup>57</sup> by Bt-LNO<sub>2</sub> is in agreement with this cysteine being inaccessible in the *c*-conformation of ANT, which is enforced by the inhibitor carboxyatractyloside. We previously showed that carboxyatractyloside prevented ANT adduction by Bt-LNO<sub>2</sub>, whereas the alternative inhibitor bongkrekate, which enforces the *m*-conformation in which Cys<sup>57</sup> is still accessible, did not (15).

There could be several mechanisms by which modification of ANT1 at Cys<sup>57</sup> could protect the heart from IR injury. This includes effects on the PT pore, ADP/ATP transport, and proton leak. From the perspective of PT pore opening, oxidative modification of ANT1 Cys<sup>57</sup> has been implicated in PT pore formation (46), potentially via regulation of cyclophilin D binding to ANT1 (29, 47, 48). Although we did show that high concentrations of LNO<sub>2</sub> can induce PT pore opening (similar to other electrophiles such as 4-HNE and 15d-PGJ<sub>2</sub> (49, 50)), low levels of LNO<sub>2</sub> were without effect on the PT pore (15). Given the known importance of ROS in regulating PT pore opening in the post-ischemic heart (5), it is possible that nitroalkylation of Cys<sup>57</sup> may serve to prevent further oxidative modification of this residue and thus prevent PT pore opening. Notably, such a “cap and protect” phenomenon has been proposed as one mechanism by which *S*-nitrosation of thiols can protect them against oxidative damage (51). Clearly, further work is needed to clarify the interplay between nitroalkylation versus oxidative modification at Cys<sup>57</sup>.

Regarding bioenergetic effects, it is known that irreversible modification of Cys<sup>57</sup> drastically reduces ADP/ATP transport via ANT (45) and may impair intracellular ATP levels upon

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reperfusion regardless of PT pore opening. Thus, reversible nitroalkylation of Cys<sup>57</sup> before reperfusion may prevent irreversible oxidative modification of this critical residue and thus permit rescue of ADP/ATP transport in the post-ischemic state.

With respect to proton leak, it is known that LNO<sub>2</sub> stimulates mild mitochondrial uncoupling and that mild uncoupling itself is protective against IR injury (14, 16, 33). The mechanism linking mild uncoupling to protection may include inhibition of ROS generation and/or Ca<sup>2+</sup> overload (for review, see Ref. 52). Furthermore, it has recently been shown that mild uncoupling of mitochondria can stimulate autophagy (53), and there is substantial evidence for a protective role of autophagy against IR injury (54, 55). It is not yet known if nitroalkenes can stimulate autophagy, but the recent finding that autophagy is required for IPC (56) coupled with our previous discovery of endogenous nitroalkene formation in IPC (15) suggests that nitroalkenes may be an upstream signal-regulating autophagy.

Although we were able to detect significant changes in mitochondrial proton leak by combining measurements of both membrane potential (TMRE) and respiration (extracellular flux (XF)-24), these methods are not high resolution. Likewise, it was previously shown that a cardioprotective concentration of FCCP (100 nM) stimulated oxygen consumption in cardiomyocytes but did not cause a detectable drop in membrane potential (57). Furthermore, the requirement to measure proton leak in state 4 typically necessitates the use of oligomycin, which depletes cellular ATP and induces mitochondrial fragmentation (58). Thus, development of more reliable methods for measuring proton leak in intact cells is desirable.

An important discrepancy between our cell and intact heart data is that LNO<sub>2</sub> reduced infarct size in the heart by 56% but only reduced death in the cell model of IR by 26%. One reason for this may be that LNO<sub>2</sub> modifies different proteins in hearts *versus* cells. Indeed, in addition to ANT1, several other proteins were modified by Bt-LNO<sub>2</sub> in intact hearts. One such protein identified herein was the  $\alpha$ -subunit of ATP synthase (Fig. 2B). This protein contains a redox-sensitive cysteine (Cys<sup>294</sup>) that was recently shown to be modified by reactive nitrogen species in the context of heart failure (59). Thus, it is possible that nitroalkylation of Cys<sup>294</sup> may modulate ATP synthase activity, with important implications for cardiac dysfunction.

It is also possible that other mitochondrial redox-sensitive proteins not detected in this study may react with nitroalkenes to bring about cardioprotection. Two examples are UCP-2 and p66<sup>shc</sup>. Previously we obtained evidence suggestive of UCP-2 modification by LNO<sub>2</sub> (15), and studies are ongoing to confirm this at the molecular level. Regarding p66<sup>shc</sup>, it is known to regulate energy metabolism (60) and H<sub>2</sub>O<sub>2</sub> generation during cardiac reperfusion (61). The redox active cysteine (Cys<sup>59</sup>) in p66<sup>shc</sup> plays an important role in its activation (62) and may be a target for nitroalkylation. Clearly, as further nitroalkene target proteins continue to be identified, their role in cardioprotection by nitroalkenes can be determined.

Regarding the physiological relevance of the ANT nitroalkylation event in IPC, we have so far failed to detect nitroalkylation of ANT by endogenously generated LNO<sub>2</sub> in hearts exposed to IPC. This is likely due to uncertainty regarding the

exact molecular nature of the nitroalkenes generated in IPC, thus precluding the ability to use single-reaction monitoring to detect ANT modifications by mass spectrometry. Furthermore, because ANT is such a large part of the mitochondrial protein complement, immuno-precipitating all of the ANT (modified and unmodified) from a sample is technically challenging. In addition, the stability of endogenous nitroalkylation adducts during mitochondrial isolation from tissue is unclear.

Finally, although it is unlikely that the well documented ability of nitroalkenes to regulate transcription factors (24, 63, 64) contributes to the acute timescale protection observed in this study, such long term gene regulation effects might nevertheless be important for nitroalkene-mediated cardioprotection in a larger disease context. It is notable that nitroalkenes are currently being pursued in the context of type 2 diabetes (Complexa, Inc.), raising the possibility that nitroalkene administration in humans may be beneficial both in short term and long term therapeutic windows.

In summary, herein we show that acute protection from IR injury by LNO<sub>2</sub> requires ANT1 and that LNO<sub>2</sub> modifies a specific cysteine (Cys<sup>57</sup>) on the protein. The mechanistic links between this post-translational modification event and cardioprotection remain to be explored.

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