Online Supplemental Information

Nitroalkenes confer acute cardioprotection via adenine nucleotide translocase 1

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SUPPLEMENTAL METHODS

Reagents

Both ANT1 and mitochondrial complex II 70 kDa subunit antibodies were from MitoScience-Abcam Cambridge, MA), anti-actin was from Calbiochem/EMD (Gibbstown NJ). Tetramethylrhodamine ethyl ester (TMRE; 555nm ex – 615nm em) and MitoTracker Green FM (MTG; 470nm ex - 535nm em) were purchased from (Molecular Probes, Eugene , OR). All other reagents were from Sigma (St. Louis MO) unless listed otherwise in the methods text.

Verification of ANT1 knocks down in H9c2 cells

72 hrs. post-transfection cells were washed twice with 1xPBS and lysed in 2x sample loading buffer comprised of 12mM Tris, 4% SDS, 10% glycerol, pH 6.8. Proteins were separated on 12.5% reducing SDS-PAGE, and transferred on nitrocellulose membrane followed by probing with antibodies against ANT1 (1:2000), 70 kDa complex II (1:10000) and actin (1:10000). Densitometry of ANT1, 70 kDa complex II and actin bands was performed using Scion Image software.

Respiration rate measurements

Respiration rates were measured in transfected cells in 24-well V7-PET plates using Seahorse XF24 Extracellular Flux analyzer (Seahorse Bioscience, Billerica MA) (1). Cells were plated at 12,000/well on Seahorse 24-well V7-PET plates in media containing 15% FBS. After 5 hrs. medium was replaced with antibiotic-free media. Cells were transfected with control or ANT1 siRNA (main methods). After 72 hrs. original media was removed and replaced with un-buffered media 30 min. before the measurements. After 8 min. of equilibration LNO_2 (1 μM) was injected in half of controls and half of ANT1 siRNA wells, then 10 min. later oligomicin (1 μg/ml) was injected into each well to subject cells to state 4 respiration rate.

TMRE/MTG fluorescence

For measurements of TMRE/MTG fluorescence, cells were plated at 20,000/plate on 35mm petri dishes with a coverglass (MatTek Corp., Ashland, MA). Cells were transfected with control or ANT1

siRNA as described in the main methods. 72 hrs. post-transfection medium was replaced with un-buffered media (in order to replicate the same conditions as used to determine respiration, in the XF-24), and cells were loaded with 100 nM MTG and incubated for 30 min. at +37°C. Cells were then washed twice and loaded with 20 nM TMRE for 30 min. at +37°C. Fluorescence microscopy was performed using an Eclipse TE2000-S (Nikon, Avon MA). Images were taken using 40x oil objectives. TMRE/MTG ratios were measured at three different time-points: (i) before addition of agents; (ii) 15 min. oligomicin (1μg/ml); and (iii) 15 min. LNO_2 (1μM) in the presence of oligomicin. To ensure valid TMRE labeling, FCCP (1μM) was added at the end of each experimental run to collapse mitochondrial membrane potential and fully dissipate TMRE fluorescence. Data were analyzed using TILL Photonics Imaging System Software.

LITERATURE CITED

1. Gerencser, A. A., Neilson, A., Choi, S. W., Edman, U., Yadava, N., Oh, R. J., Ferrick, D. A., Nicholls, D. G., and Brand, M. D. (2009) *Anal. Chem.* **81,** 6868-6878

SUPPLEMENTAL RESULTS

Table S1. MS/MS fragmentation of $G_{53}IIDC^{*}VVR_{60}$ peptide of ANT1 with biotinylated Cys. Colored numbers represent masses found in MS/MS (Figure 3) with mass error less than 0.3 ppm.

#	I	b	b-H_2O	a	c	Seq	y	y-H_2O	z	z'	y(2+)	#
53	86	58	40	30	75	G	1381	1363	1364	1365	691	60
54	86	171	153	143	188	I	1268	1250	1251	1252	635	59
55	86	284	266	256	301	I	1155	1137	1138	1139	578	58
56	88	399	381	371	416	D	1040	1022	1023	1024	520	57
57	639	1065	1048	1038	1083	***C****	373	355	356	357	187	56
58	72	1165	1147	1136	1182	V	274	256	257	258	138	55
59	72	1264	1246	1236	1281	V	175	157	158	159	88	54
60	129					R						53

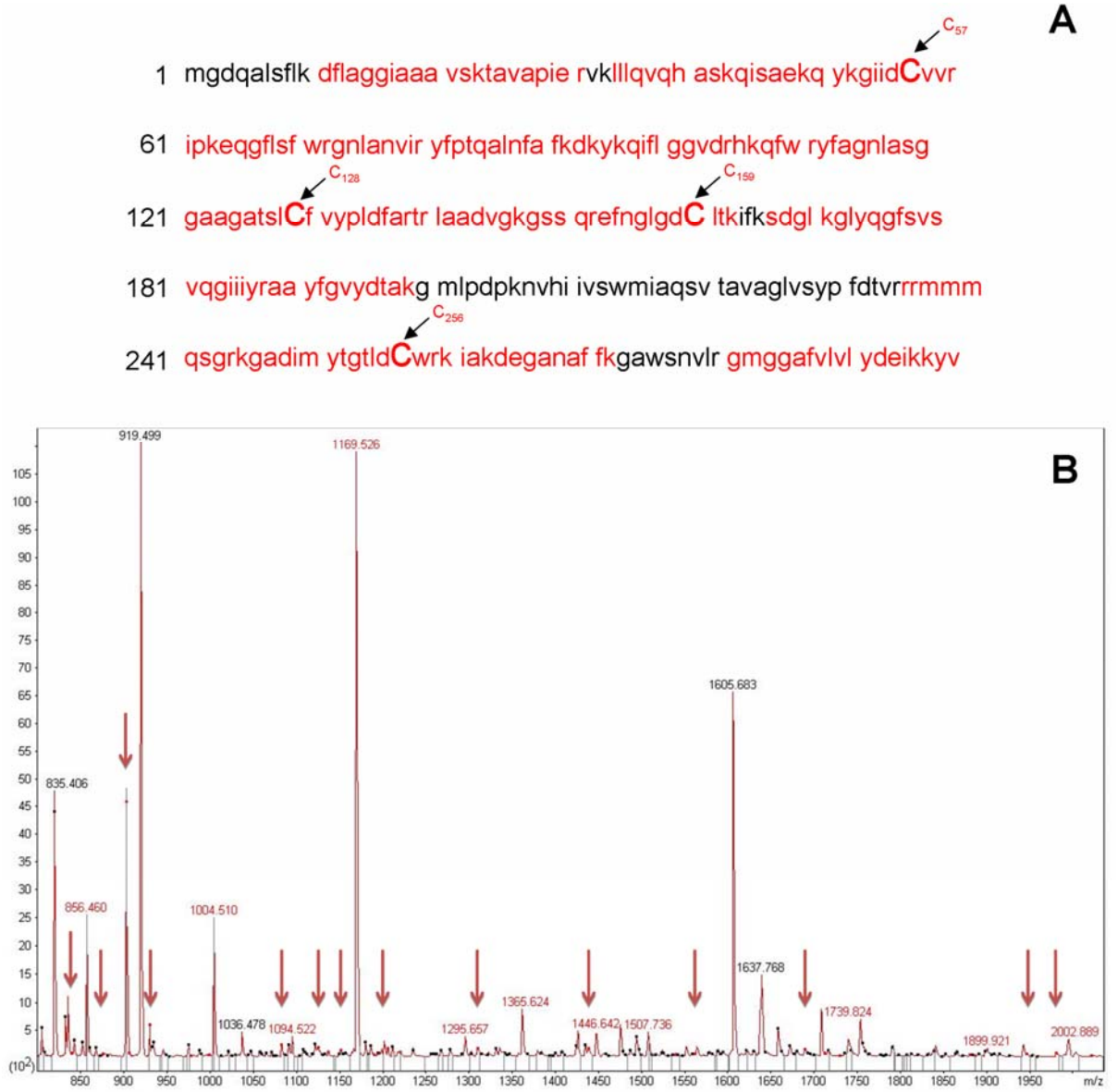

Figure S1. ANT1 protein coverage map and mass spectrometry. (A): Red colored amino acids indicate peptides found in MALDI-TOF MS analysis of in-gel tryptic digest from band corresponding to ANT1 (~32kDa). The ANT1 sequence is that for mouse (gi: 148747424**). (B):** Mass spectrometry of the gel band from biotinylated protein pull-down experiment (Fig. 2B). Tryptic digest peptide masses originating from ANT1 protein are indicated as brown-red (values for highly abundant peptides, and arrows for low abundance peptides).

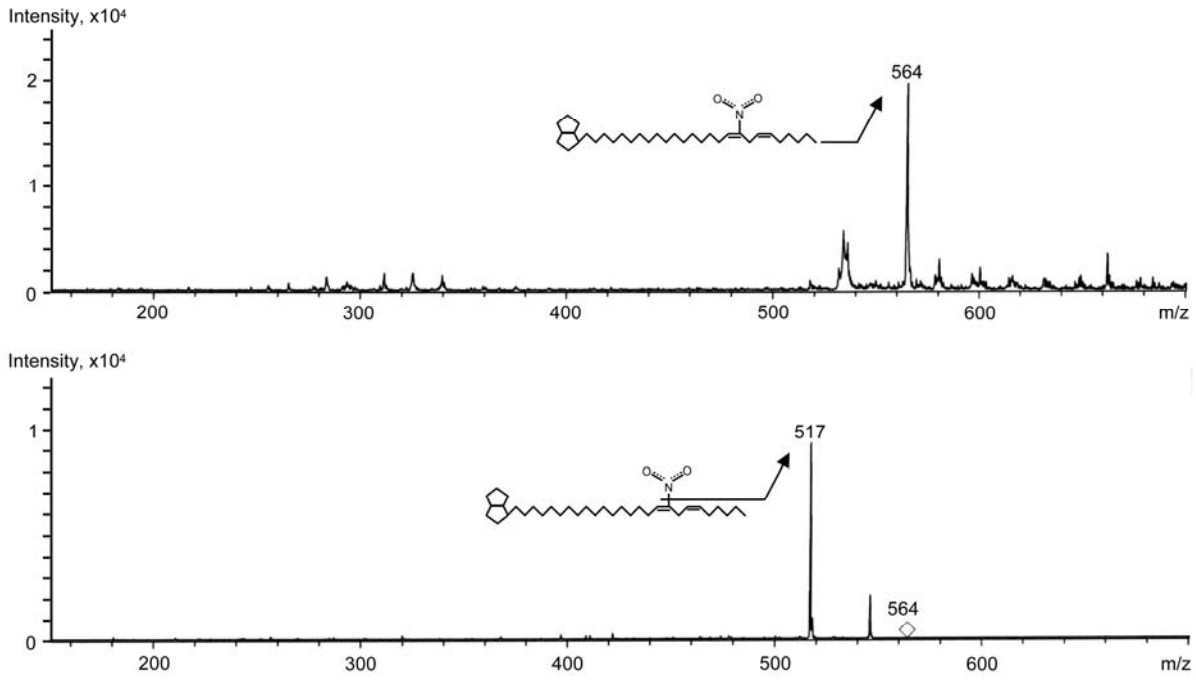

Figure S2. MS analysis of Bt-LNO$_2$. Synthetic Bt-LNO$_2$ was analyzed by direct infusion on a Bruker Daltonics MS/MS system, in negative ion mode. Single reaction monitoring was used to detect species undergoing loss of m/z 46 (NO_2 group). Parent spectrum (Bt-LNO$_2$, m/z 564) is shown in upper panel, and daughter spectrum (Bt-LA, m/z 517) in the lower panel.

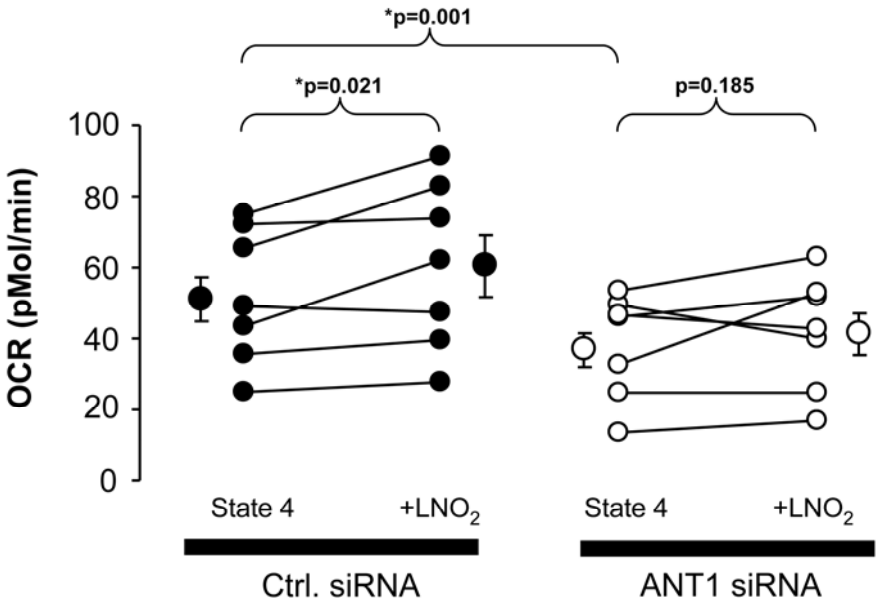

Figure S3. State 4 respiration rate in H9c2 cells. Oxygen consumption rate (OCR) was measured using a Seahorse Biosciences XF-24 analyzer, as detailed in the methods. All data are for enforced state 4 respiration, in the presence of oligomyicin. LNO_2 (1 μM) was present where indicated). Individual data points (n=7), connected by lines show the effect of LNO_2 addition in each case, with average values (±SEM) shown adjacent to these points. Statistical significance between groups (paired *t*-test) is shown above the data.

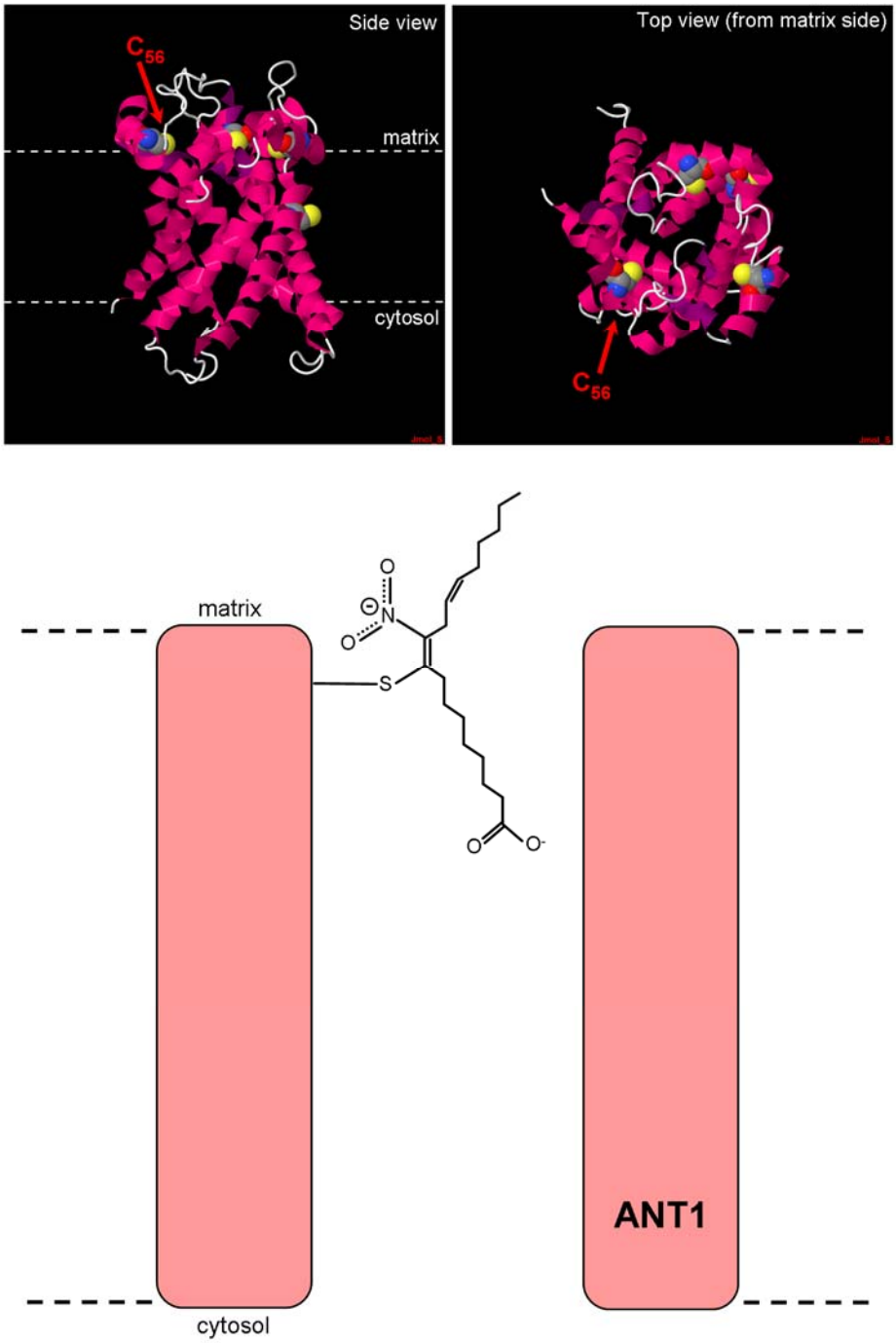

Figure S4. ANT1 structure and localization of modified cysteine. Crystal structure of bovine ANT1 at 2.8 Å resolution was obtained from the protein database (dx.doi.org/10.2210/pdb2c3e/pdb). Images were rendered in Jmol (jmol.sourceforge.net/). Backbone of the structure is shown as ribbons (pink), with the four cysteine residues highlighted in ball-and-stick formation. The position of C_{56} is indicated by the arrows (Note, C_{56} in the bovine protein corresponds to C_{57} in mouse ANT1). Dotted lines denote position of the membrane, with the matrix and cytosol indicated for orientation. Left panel shows side view, right panel shows top view from the matrix side, indicating the position of C_{56} with the thiol facing the central membrane-spanning pore of the protein. Lower panel shows a theoretical location of nitro-linoleate adduct in the ANT structure, indicating the presence of the electronegative nitro group.