Supporting Information

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Methods

Synthesis of MitoNAP [5-(2-Acetylamino-3-mercapto-3-methyl-butyrylamino)-pentyl]-triphenylphosphonium methanesulfonate]. A solution of 5-aminopentyltriphenylphosphonium bromide hydrogen bromide (1) (467 mg, 0.917 mmol) and triethylamine (136 μl, 0.920 mmol) in dichloromethane (10 mL) was stirred for 5 min after which time a solution of *N*-(2,2-dimethyl-4-oxo-thietan-3-yl)-acetamide (2–4) (157 mg, 0.917 mmol) in dichloromethane (20 mL) was added in 1 aliquot and the reaction mixture was stirred for a further 4 h. After the mixture had been washed with a saturated aqueous solution of sodium methane sulfonate (3 × 20 mL), the organic fraction was collected, dried ($MgSO_4$), filtered and the solvents removed in vacuo to give a colorless oil. The oil was dissolved in minimal dichloromethane (2 mL) and then excess diethyl ether (70 mL) was added. After the resultant precipitate had settled, the solvents were decanted and the residue was dried in vacuo (0.1 mm Hg) for 2 h to give a cream powder (374 mg, 0.607 mmol, 66%). A sample (200 mg) was dissolved in water (10 mL) and freeze-dried; HPLC: Prodigy 5 μ OD53 11A 250 × 4 mm, detection 275 nm, flow rate 1.00 mL/min; mobile phase 50% acetonitrile, 50% water (0.1% trifluoroacetic acid); retention time 4.76 min. HRPI ESMS calculated for $C_{30}H_{38}N_2O_2PS^+$: 521.2386, found: 521.2389. Analysis calculated for $C_{31}H_{41}N_2O_5PS_2$: C 60.4, H 6.7, N 4.5, S 10.4%; found C 59.8, H 6.9, N 4.8, S 10.2%. UV ethanol ε_{268} 2,740 $M^{-1}cm^{-1}$, ε_{275} 2,292 $M^{-1}cm^{-1}$. ^{1}H NMR ($CDCl_3$) δ 7.94 (1H, t, J = 5 Hz, CH_2-N**H**), 7.82–7.62 (16H, m, Ar**H**, CH-N**H**), 4.67 (1H, d, J = 10 Hz, C**H**), 3.58–3.47 (2H, m, C**H**$_2$-P$^+$), 3.42–3.27 (1H, m, CH**H**-NH), 3.27–3.14 (1H, m, C**H**H-NH), 2.76 (3H, s, SO_3-C**H**$_3$), 2.69 (1H, s, S**H**), 2.13 (3H, s, CO-C**H**$_3$), 1.55 (3H, s, C**H**$_3$), 1.43 (3H, s, C**H**$_3$), 1.80–1.52 (6H, m, C**H**$_2$) ppm. ^{13}C NMR ($CDCl_3$) δ 170.7, (CO), 170.3, (CO), 135.1 (d, J = 3 Hz, *para*-Ph), 133.6 (d, J = 10 Hz, metaPh), 130.6 (d, J = 13 Hz, ortho-Ph), 118.5 (d, J = 86 Hz, ipso-Ph), 62.3 (**C**H), 46.5 (**C**), 39.8 (**C**H$_3$-SO$_3^-$), 38.2 (**C**H$_2$-NH), 30.7 (**C**H$_3$), 30.6, (**C**H$_3$), 27.6 (**C**H$_2$-CH$_2$-NH), 27.1 (d, J = 17 Hz, **C**H$_2$-CH$_2$-P$^+$), 23.4 (**C**H$_3$), 22.0 (d, J = 53 Hz, **C**H$_2$-P$^+$), and 21.9 (d, J = 5 Hz, **C**H$_2$-CH$_2$-CH$_2$-P$^+$) ppm. ^{31}P NMR ($CDCl_3$) δ 25.4 ppm. For the chemical synthesis, all NMR spectra were obtained on a Varian Unity 300 instrument and mass spectra were acquired on a Bruker QToF instrument.

Synthesis of MitoSNO1 [5-(2-Acetylamino-3-methyl-3-nitrosothio-butyrylamino)-pentyl]-triphenylphosphonium methanesulfonate]. MitoNAP methanesulfonate (50 mg, 0.081 mmol) was dissolved in 1:1 ethanol/water (2 mL) in a sample tube (5 mL). The solution was flushed with argon and held in the dark and then 0.576 mL of a methane sulfonic acid solution (620 mM methane sulfonic acid, 2 mM EDTA, 0.2 mM DTPA, and 0.2 mM neocuproine) was added. The reaction vessel was swirled for 1 min, then 0.420 mL of a sodium nitrite solution (769 mM sodium nitrite, 2 mM EDTA, 0.2 mM DTPA, and 0.2 mM neocuproine) was added and the reaction vessel was swirled again. The reaction mixture, which turned grass green after 15 min, was held in the dark for 2 h at room temperature. Dichloromethane (2 mL) was added and then water (2 mL). The lower dichloromethane layer was removed using an argon-flushed Pasteur pipette, and dried ($MgSO_4$) resulting in a clear green organic solution. The dichloromethane solution was evaporated in vacuo (30 °C, 2 mm Hg) in the dark. The green glassy residue was dissolved in minimal dichloromethane (0.5 mL) and excess diethyl ether added (40 mL). The resulting pale green precipitate coagulated and the solvents were decanted. The residue was dried under vacuum (2 mm Hg) in the dark to yield MitoSNO1 (40 mg, 78%) as the methanesulfonate salt. The green solid was lyophilized from water to give a pale green solid which was stable under inert atmosphere in the dark at room temperature for several days. Methanolic or aqueous solutions of MitoSNO1 were stable for several days under argon and in the dark. However prolonged storage (3 h) in chlorinated solvents resulted in substantial decomposition. MitoSNO1 was shown to be stable for long periods in 0.1% aqueous TFA based on its absorbance at 340 nm. HPLC: Prodigy 5μ OD 53 11A 250 × 4 mm, detection 275 nm, flow rate 1.00 mL/min; Mobile phase 50% acetonitrile, 50% water (0.1% trifluoroacetic acid); retention time 8.17 min. UV ethanol ε_{268} 5,000 $M^{-1}cm^{-1}$ ε_{275} 4,150 $M^{-1}cm^{-1}$ ε_{340} 950 $M^{-1}cm^{-1}$. HRPI ESMS (collisional energy 2 V, quadrupole ionisation energy 2 V) calculated for $C_{30}H_{37}N_3O_3PS$: 550.2288, found: 550.2280. The parent ion (M^+) m/z 550.2280 showed strong fragmentation peaks for $(M\text{-}NO_2)^+$at m/z 520.2290 (expect 520.2308) and $(M\text{-}NO)_2^{2+}$ at m/z 520.2290, 520.7305 (expect 520.2308, 520.7304). Correlation of calculated and experimental isotope patterns confirmed the elemental composition.^{1}H NMR δ (CD_3OD): 8.38 (1H, bt, CH_2-N**H**), 7.73–7.92 (15H, m, Ar-**H**), 5.30 (1H, d, J = 10 Hz, C**H**), 3.47–3.35 (2H, m, P$^+$-C**H**$_2$), 3.22–3.07 (2H, m, C**H**$_2$-NH), 2.68 (3H, s, C**H**$_3$-SO$_3^-$), 2.03 (3H, s, C**H**$_3$), 1.99 (3H, s, C**H**$_3$), 1.96 (3H, s, C**H**$_3$), 1.88–1.40 (6H, m, C**H**$_2$) ppm. ^{13}C NMR δ (CD_3OD): 173.1 (**C**O), 170.8 (**C**O), 136.3 (d, J = 3 Hz, *para*-Ph), 134.8 (d, J = 10 Hz, *meta*-Ph), 131.5 (d, J = 13 Hz, *ortho*-Ph), 119.9 (d, J = 86 Hz, *ipso*-Ph), 61.7 (**C**H), 59.2 (**C**), 39.8 (**C**H$_2$-NH), 39.5 (**C**H$_3$-SO$_3^-$), 29.3 (**C**H$_2$-CH$_2$-NH), 28.7 (d, J = 17 Hz, **C**H$_2$-CH$_2$-P$^+$), 27.1, (**C**H$_3$), 25.9, (**C**H$_3$), 23.0 (d, J = 9 Hz, **C**H$_2$-CH$_2$-CH$_2$-P$^+$), 22.7 (d, J = 56 Hz, **C**H$_2$-P$^+$), and 22.4 (**C**H$_3$) ppm. ^{31}P NMR δ (CD_3OD) 24.9 ppm.

Synthesis of MitoSNO2. In addition, to MitoSNO1, another MitoSNO, MitoSNO2 was made. While MitoSNO2 could be made easily and gave results that were similar to those obtained using MitoSNO1, the compound was generally less stable, and so only data on the more tractable MitoSNO1 are reported. The synthesis of MitoSNO2 is reported below. The precursor for MitoSNO2 was thiobutyltriphenylphosphonium (TBTP) which was synthesised from 4-(acetylthio)butyltriphenylphosphonium bromide as described (5). A typical preparation of MitoSNO2 for use in biological experiments was as follows: acetyl TBTP bromide (40.2 mg; 85.2 μmol) was dissolved in absolute ethanol (100 μL) in a 1.5-mL plastic tube, to give a 0.85 M solution. This was de-acetylated by incubation on ice under argon with 1 M NaOH (100 μL) for 5 min, which gave a pale green solution. Two hundred microliters of 1 M HBr was added to stabilize the phosphonium ion as a Br^- counter ion, then the TBTP was extracted into dichloromethane (4 × 200 μL). The combined dichloromethane extracts were washed with 200 μL of 1% $NaHCO_3$ and with H_2O, and then evaporated under argon. The remaining oily residue was dissolved in absolute ethanol (300 μL) and the extinction coefficient of TBTP (ε_{268} = 3,000 $M^{-1}cm^{-1}$; ref 5) was used to calculate the molarity as 178 mM in 300 μL (63% yield). To S-nitrosate this thiol, HCl (150 μL of 0.62 M HCl, to give a 206 mM final concentration) was added followed by $NaNO_2$ (60 μmol, 150 μL of 400 mM) in the dark, on ice and under argon. The color changed instantaneously from clear to bright pink/red. After 3 min, 1 M NaBr (500 μL) was added, and the MitoSNO2 was extracted into dichloromethane (4 × 350 μL). The pooled dichloromethane fractions were

washed with 1% $NaHCO_3$, followed by H_2O and then the dichloromethane was evaporated under argon. The pink oily residue was redissolved in dichloromethane and precipitated with ether and the supernatant aspirated. This process was repeated to remove remaining impurities, and the final bright pink oily residue was dissolved in absolute ethanol (100 μL) and the TPP concentration was approximately 320 mM, as determined by its UV absorbance spectrum (65% recovery from TBTP). The thiol contamination, determined by reaction with dithionitrobenzoic acid, was less than 0.6% of the TPP concentration. The S-nitrosothiol concentration was approximately 330 mM, as determined by degrading the S-nitrosothiol to NO_2^-, followed by the Griess assay, which suggests that most of the TBTP has been S-nitrosated. The solution absorbed strongly at approximately 330 nm, a characteristic local maximum for absorption by SNO bonds. The A_{268}:A_{330} ratio was approximately 3.78 for this preparation and varied between 3.5 and 4 for fresh preparations of MitoSNO2. The extinction coefficient of the S-nitrosothiol at A_{330} for this MitoSNO preparation was approximately 787 $M^{-1}cm^{-1}$. Different preparations gave ε_{330} approximately 787–800 $M^{-1}cm^{-1}$. To estimate the stability of crude MitoSNO2 in the ethanol stock solution on storage, NO_2^- production from spontaneous S-nitrosothiol degradation was assessed by the Griess assay, which was performed at regular intervals and was compared with the total NO_2^- concentration after treatment with $HgCl_2$ which completely degraded MitoSNO2. Storage at −20 °C for 2 weeks led to negligible decay of MitoSNO2 as judged by the lack of change in nitrite concentration, however after 1 month, an approximate 10% decrease in MitoSNO2 concentration was observed. The decay of MitoSNO2 was also measured from the ratio of the TPP absorbance peak (268 nm) to that of the SNO function (330 nm). This increased from 3.7 ± 0.17 to 3.9 ± 0.04 over 25 days at −20 °C, consistent with the loss of the S-nitrosothiol. NMR analysis of MitoSNO2 was not possible due to the significant solvent signal overlap and instability in the usual NMR solvents.

Nitrite and Nitric Oxide Measurements. Nitrite was analyzed by the Griess assay (Molecular Probes). Nitric oxide was measured using an NO• electrode (World Precision Instruments) connected to an Apollo 4000 Free Radical Analyser. The electrode was inserted into a stirred, sealed 3-mL chamber with a Clark-type O_2 electrode (Rank Brothers) built into its base and was thermostated at 37 °C. The NO• electrode was calibrated by adding SNAP to argon-purged, saturated CuCl. OxyHb was prepared by reduction of bovine methemoglobin with sodium dithionite.

To measure the stability of MitoSNO1 and SNAP in aqueous buffer by nitrite accumulation, samples (50 μM) were incubated at 37 °C in the dark in KCl buffer, then snap frozen on dry ice/ethanol and later assayed for nitrite. Data are means of triplicate determinations. Half-lives were estimated from semi-log plots of concentration against time generated by subtracting the accumulated nitrite from the starting SNO concentration. $HgCl_2$ degraded all MitoSNO1 or SNAP to nitrite, and when ambient light was not excluded nitrite formation from MitoSNO1 increased by approximately 40–50%.

To measure the release of NO• from MitoSNO1 on reaction with GSH, MitoSNO1 (5 μM) or SNAP (5 μM) was incubated in 3 mL KCl buffer at 37 °C in the dark, and NO• was measured using an NO• electrode. Control experiments showed that addition of GSSG (333 μM) to 5 μM MitoSNAP had no effect on NO• production, and that GSH (200 μM) alone did not affect the NO• electrode. To measure the concentrations of O_2 and NO• simultaneously, mitochondria were incubated in KCl medium supplemented with rotenone in a stirred 3-mL chamber. There was no effect of 5 μM MitoNAP on respiration or NO• production.

Measurement of S-nitrosothiols. After incubation the mitochondrial, or mitochondrial membrane, suspensions were supplemented with 10 mM NEM and 1 min later were pelleted by centrifugation (10,000 × *g* for 5 min) and resuspended 1 ml KCl buffer supplemented with 10 mM NEM and pelleted once more. The pellet was then resuspended in 1 mL of 25 mM Hepes pH 7.2, 100 μM DTPA, 10 μM neocuproine, and 10 mM NEM, snap frozen on dry ice/ethanol, freeze/thawed (3 times), and stored at -20 °C until analysis. For measurements on cells, the medium was removed then the cell layer was washed in PBS/10 mM NEM, then scraped into 1 mL PBS/NEM, and snap frozen on dry ice/ethanol, freeze/thawed (3 times) and stored at -20 °C until analysis. Samples (225–450 μL) were thawed rapidly just before analysis and made up to a final volume of 250–500 μL containing 0.1 M HCl and 0.5% sulfanilamide ± 0.2% $HgCl_2$ and incubated in the dark for 30 min. Then duplicate 100–500 μL samples were injected into 20 mL of an acidic I^-/I_2 solution [33 mM KI, 14 mM I_2 in 38% (vol/vol) acetic acid] through which bubbled helium that carried the NO produced through a 1 M NaOH trap to an EcoMedics CLD 88 Exhalyzer (Annex), where it is mixed with ozone and the chemiluminescence measured. The peak area is compared to a standard curve generated from $NaNO_2$ standards. As $HgCl_2$ selectively degrades SNOs, the SNO content was the difference between the samples with and without $HgCl_2$. A control experiment was carried out in which mitochondria were incubated with 5 μM MitoSNO1 for 5 min, then the mitochondrial suspension was supplemented with 10 mM NEM and 1 min later was pelleted by centrifugation (10,000 × *g* for 5 min) as above. The pellet was then resuspended in 1 mL of 5% sulfosalicylic acid to fully lyse mitochondria and precipitate protein which was pelleted and washed again in 1 mL of 5% sulfosalicylic acid, where the protein pellet was resuspended in 1 mL of 25 mM Hepes, pH 7.2, 100 μM DTPA, 10 μM neocuproine, and 10 mM NEM KCl buffer supplemented with 10 mM NEM and assessed for SNOs as above. This gave a significant amount of S-nitrosated proteins (359 ± 89 pmol SNO/mg protein, mean ± SEM of 4), confirming that the SNOs measured in mitochondria are due to S-nitrosated proteins and not to carry over of MitoSNO1 trapped within mitochondria into the SNO assay.

Measurement of Exposed Protein Thiols. To measure exposed mitochondrial protein thiols, mitochondria (1 mg protein/mL) were incubated in 1 mL KCl buffer for 5 min, pelleted by centrifugation and the pellet resuspended in 75 μL of 250 mM sucrose, 5 mM Hepes, and 1 mM EGTA, pH 7.4, supplemented with 0.1% dodecylmaltoside. Small molecules were removed by centrifugation through MicroBioSpin Columns (cut off = 6 kDa), the eluate was clarified by centrifugation (2 min at 10,000 × *g*) and protein thiols measured in triplicate by mixing 10 μL sample or GSH standard with 160 μL of 200 μM dithionitrobenzoic acid (DTNB) in 80 mM NaP_i and 1 mM EDTA, pH 8, in a 96-well plate, and absorbance at 412 nm was measured after 30 min at room temperature and the thiol content determined from the GSH standard curve. The protein concentration was measured in parallel by the bicinchonininc assay using BSA as a standard.

Cell Culture. Cells were grown in medium supplemented with 10% FCS (FCS), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Jurkat cells were cultured in Roswell park memorial institute (RPMI) 1640 medium supplemented with 2 mM Glutamax and 25 mM Hepes. HeLa cells were grown in minimum essential eagle medium (MEM) containing 2 mM glutamine and non-essential amino acids and were grown to 100% confluence before use. C2C12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) and were seeded at approximately 25,000–30,000 cells/cm^2 and grown overnight before experiments.

Preparation of a Mitochondria-enriched Fraction from C2C12 Cells. C2C12 cells were seeded ($\approx 2 \times 10^6$ cells per 14-cm^2 dish) and grown with 25 mL medium/dish for 46 h giving approximately 7×10^6 cells per 14-cm^2 dish. The medium was removed and replaced with 10 mL medium. To this, DMSO or 20 μM FCCP was added and incubated for 5 min at 37 °C in the dark. After this, 5 μM MitoSNO1 or SNAP or ethanol was added and incubated for a further 5 min in the dark. The supernatant was aspirated and the cell sheet was washed first in PBS/10 mM NEM then in buffer A (100 mM sucrose, 1 mM EGTA, 20 mM Mops, pH 7.4, 10 mM NEM, 100 μM DTPA, and 10 μM neocuproine) while on ice. Cells were scraped into 1 mL SEM buffer and the plate washed in 4 mL of the same buffer. The cell suspension was centrifuged ($200 \times g$ for 5 min at 4 °C) and the pellet resuspended in 500 μL buffer B (= buffer A supplemented with 0.1 mg/mL digitonin and 10 mM triethanolamine) and incubated on ice for 3 min then 2.5 volumes of buffer A was added. This was homogenized 20 x using a tight fitting Dounce, then spun 1 min at $1{,}000 \times g$ at RT. The supernatant was kept, and the pellet was washed again in buffer A again the supernatant was retained. These were combined and spun at $10{,}000 \times g$ for 5 min at 4 °C. The supernatant (cytosolic fraction) was snap frozen and the pellets combined in 500 μL of 25 mM Hepes pH 7.2, 100 μM DTPA, 10 μM neocuproine, and 10 mM NEM and then snap frozen. Samples were freeze thawed 3 times and aliquots prepared for S-nitrosothiol analysis.

Electrophoresis and Visualization of S-nitrosated Proteins. Isolated rat liver or rat heart mitochondria (4 mg protein/mL) were suspended in KCl buffer supplemented with 10 mM succinate and 8 μg/mL rotenone and were incubated with no additions, 10 μM MitoSNO1 or 500 μM diamide at 37 °C for 5 min with occasional mixing. Mitochondria were then pelleted by centrifugation and resuspended in a blocking buffer containing 250 mM Hepes, pH 7.7, 1 mM EDTA, 1 mM DTPA, 10 μM neocuproine, 1% SDS, and 50 mM NEM. This blocking reaction was carried out for 5 min at 40 °C. The reaction mixture was then passed 3 times through MicroBioSpin Columns (cut off = 6 kDa: Bio-Rad) to remove NEM. Cy3 maleimide (200 μM; Amersham), 1 mM ascorbate, and 10 μM $CuSO_4$ were added, and the mixture gently vortexed and then incubated for 30 min at 37 °C. The protein (10 μg) was then separated on a 12.5% SDS PAGE gel. After electrophoresis, the gel image was acquired with a Typhoon 9410 variable mode imager.

To assess S-nitrosation, bovine heart mitochondrial membranes (250 μg/mL) were incubated in KCl buffer $\pm$ 75 μM MitoSNO1 at 37 °C for 5 min with occasional mixing. Then 10 mM NEM was added and incubated for 5 min at 40 °C. The membranes were then pelleted by centrifugation and washed 3 times in 1 mL PBS buffer. The pellet was then resuspended in 100 μL PBS supplemented with 200 μM Cy3 maleimide, 1 mM ascorbate, and 10 μM $CuSO_4$ and incubated for 30 min at 37 °C. When testing the reversibility of MitoSNO1 S-nitrosation with GSH, membranes were treated with 1 mM GSH for 15 min before the blocking step. BN-PAGE samples were prepared as described [10] and separated on a 5–12% gradient acrylamide gel. Following electrophoresis, the gel image was acquired with a Typhoon 9410 variable mode imager scanning for Cy3 fluorescence at 532 nm. Proteins were then transferred to a nitrocellulose membrane and probed with rabbit antisera against the bovine 24-kDa Complex I subunit (a gift from Prof John E. Walker), a mouse monoclonal against bovine complex III, core subunit 2 (from Molecular Probes/Invitrogen) or a mouse monoclonal against bovine Complex V β subunit (from Molecular Probes/Invitrogen). These were then probed with secondary antibody-horseradish peroxidase conjugates and visualised by enhanced chemiluminescence (Amersham Biosciences).

RP-HPLC Analysis. MitoSNO1, MitoNAP and their derivatives were separated by reverse phase HPLC (RP-HPLC) on a C18 column (Jupiter 300 Å, Phenomenex) with a Widepore C18 guard column (Phenomenex), using a Gilson 321 pump. Samples were injected manually through a 0.22-μm PVDF filter (Millipore) into a 2-mL sample loop and then a gradient of A (0.1% TFA) and B (100% ACN, 0.1% TFA) was run at 1 mL/min (time in min, %B): 0–5, 5%; 5–10, 5–40%; 10–25, 40–80%; 25–27.5, 80–100%; 27.5–30, 100%; 30–32.5, 100–5%. Peaks were detected at 220 nm using a Gilson UV/VIS 151 spectrophotometer. MitoNAP and MitoSNO1 standards were used to identify peak elution times. To measure the stability of MitoSNO1 in aqueous buffer by RP-HPLC, aliquots of MitoSNO1 (5 μM) in 700 μL KCl buffer without DTPA or neocuproine were incubated at 37 °C in the dark, then mixed with 700 μL of 0.1% trifluoracetic acid (TFA) and analyzed by RP-HPLC. The identities of the RP-HPLC peaks for MitoNAP and the disulfide dimer of MitoNAP obtained after a 21-h incubation were confirmed by electrospray mass spectrometry (ESMS) and the *m/z* values obtained were 521.2 for MitoNAP (expected 521.238) and 520.2 for the MitoNAP disulfide linked dimer (expected 520.3). The amount of MitoSNO1 and MitoNAP were calculated as the percentage of their peak areas relative to the sum of both peak areas.

Mass Spectrometry. MitoSNO1 decay products were identified by electrospray mass spectrometry (ESMS) using a Quattro triple quadrupole mass sepctrometer. RP-HPLC peaks were collected in 50–60% ACN, 0.1% TFA, transferred to sample vials, and compared with 1 μM MitoNAP or MitoSNO1 standards in 50% ACN and 0.1% TFA. An isocratic solvent (50% ACN) was infused continuously at 20 μL/min into a Quattro triple quad mass spectrometer and an automated sample injector was used to infuse samples (30 μL) into the solvent flow. Spectra over 400–700 *m/z* were accumulated continuously over 5 min. A MitoNAP standard used for internal callibration.

Cell Hypoxia Experiments. HeLa cells were seeded and grown to 100% confluence with 15 mL medium/dish on 165-cm^2 dishes. The medium was aspirated and replaced with 15 mL fresh medium. The dishes were then placed in a humidified hypoxia workstation (Coy Laboratories) at 1% O_2 concentration with 5% CO_2 and the balance N_2 for 60 min. The indicated concentration of MitoNAP, MitoSNO1, and myxothiazol was added to individual dishes which were incubated in the dark for a further 30 min. Extracellular pO_2 measurements were taken by fluorescence quenching oximetry (Oxylite-2000; Oxford Optronix). Statistical analysis was performed using SPSS 12.0.1.

Aorta Relaxation Measurements. Sprague-Dawley rats (male 250–350 g) were anesthetized by i.p. injection of ketamine and xylazine (100 and 16 mg/kg, respectively) and thoracic aortas were excized and placed in Krebs-Henseleit buffer (pH 7.3) at 37 °C. Aortic segments were cut into 7 to 8 approximately 3-mm–long rings, mounted in baths containing 15 mL Krebs-Henseleit buffer equilibrated with 21% O_2 and 5% CO_2 at 37 °C, and isometric vessel tension was established using a vessel bioassay system (Radnoti). Indomethacin (5 μM) treated aorta was precontracted with phenylephrine (100 nM) and L-N^G-monomethylarginine (100 μM) and then treated with test compounds. For decomposition, MitoSNO1 and SNAP were exposed to light for 2 h, resulting in >90% SNO degradation. All vasodilatation studies were performed under subdued light settings. The concentrations of MitoSNO1 and SNAP were calculated using ε = 950 $M^{-1}cm^{-1}$ and 1168 $M^{-1}cm^{-1}$ at 340–344 nm respectively in PBS supplemented with 10 μM DTPA. The effects of intact or decomposed MitoSNO1 and SNAP on precontracted rat thoracic aorta mounted in a myo-

graph were determined. Data represent means ± SEM. For MitoSNO1, $n = 8$ from experiments on 2 independent rat aorta preparations. For decomposed MitoSNO1 and SNAP experiments the data ($n = 4$) are from 1 aorta preparation.

Respiration Measurments. Respiration rates were measured in a Clark-type O_2 electrode (Rank Brothers). Heart mitochondria (1 mg protein/mL) were incubated in 1 mL KCl medium supplemented with 1 mM phosphate in an oxygen electrode with 5 mM glutamate and 5 mM malate, or succinate, and incubated with 10 μM MitoSNO1, 10 μM MitoNAP or carrier for 2 min (succinate) or 3 min (glutamate/malate), then 250 μM adenosine diphosphate (ADP) was added and the rate of respiration was measured. Respiration in the presence of MitoSNO1 is expressed as a percentage of that with MitoNAP, and are means ± SEM from 3 separate mitochondrial preparations, each determined in triplicate. Respiration rates of control mitochondria on succinate and glutamate/malate were 140 ± 8 and 92 ± 5 nmol O_2/min/mg protein (means ± SEM, $n = 3$), respectively. MitoNAP decreased control respiration on either substrate by about 3–4%. Bovine heart mitochondrial membranes (0.25 mg protein/mL) were incubated in 1 mL KCl medium in an O_2 electrode at 37 °C, and incubated with 75 μM MitoSNO1 or MitoNAP, or ethanol carrier for 5 min in the presence or absence of rotenone, then 1 mM NADH or 10 mM succinate was added and the rate of respiration was measured. Data are expressed as respiration in the presence of MitoSNO1 or MitoNAP as a percentage of the appropriate controls and are means ± range of 2 separate experiments, each determined in triplicate. Respiration rates of control mitochondrial membranes on NADH or succinate were 198 ± 33 and 56 ± 3 nmol O_2/min/mg protein (means ± range, $n = 2$), respectively. To measure cell respiration, Jurkat cells (55×10^6 cells) were incubated in PBS supplemented with 25 mM Hepes pH 7.2 (NaOH), 1 mM sodium pyruvate, 10 μM neocuproine, and 100 μM DTPA in the stirred 2.5-mL chamber of an oxygen electrode at 37 °C.

Other Assays. An electrode selective for the TPP moiety of MitoSNO1 and MitoNAP was made and used as described previously (6). To measure GSH and GSSG, mitochondria (1 mg protein/mL) were incubated with 0–10 μM MitoSNO1 or MitoNAP for 10 min, pelleted by centrifugation, and the GSH and GSSG contents assessed by the recycling assay (7).

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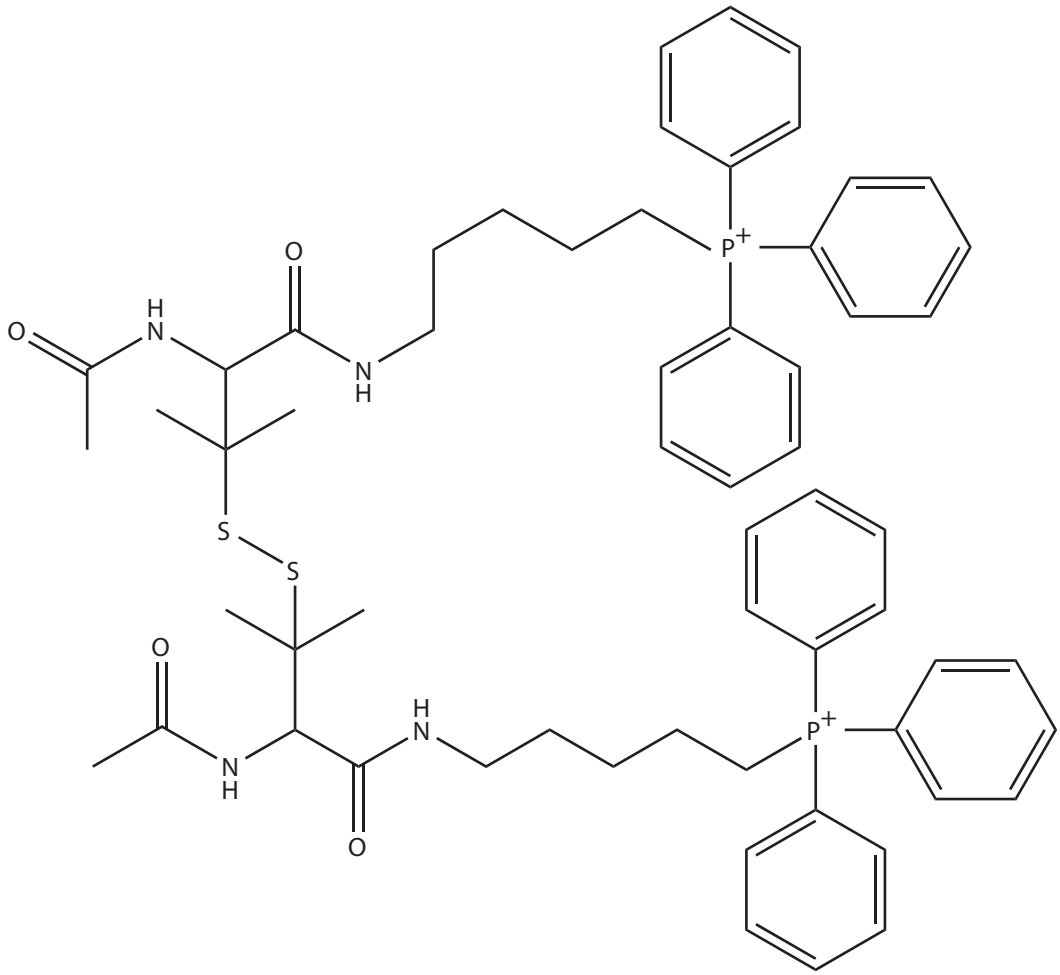

Fig. S1. Structure of the disulfide dimer of MitoNAP.

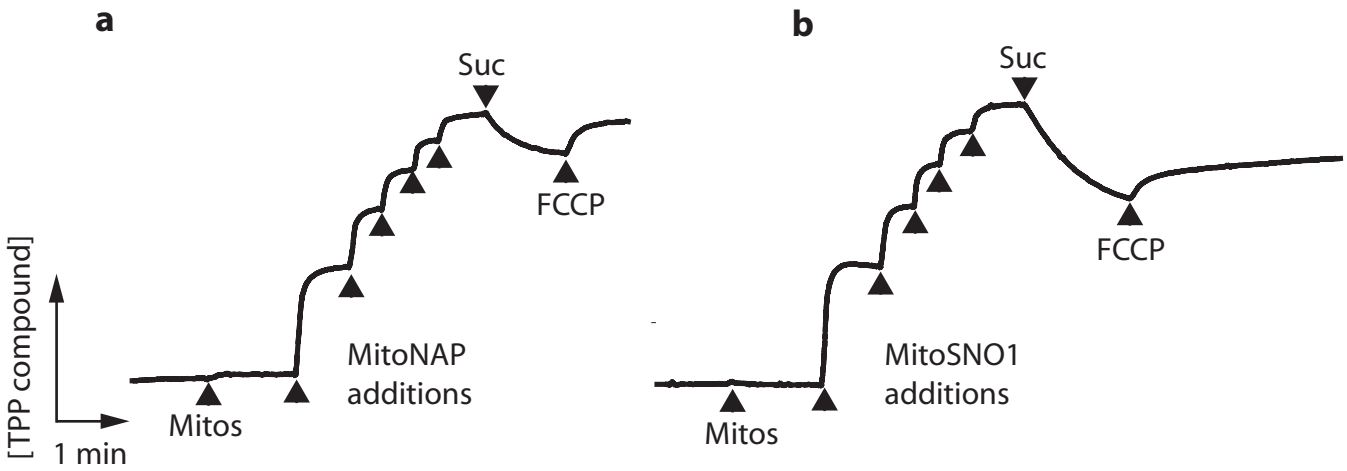

Fig. S2. Uptake of MitoNAP and MitoSNO1 by energized mitochondria measured using an ion-selective electrode. (*a* and *b*) Mitochondria (2 mg protein/mL) were incubated in KCl buffer supplemented with rotenone in a stirred 3-mL chamber thermostated at 37 °C and shielded from ambient light. An electrode selective for the TPP cation was inserted and calibrated by 5 sequential 1 μM additions of MitoNAP (*a*) or MitoSNO1 (*b*). Succinate (10 mM) was then added to energize the mitochondria, followed by the uncoupler FCCP (0.5 μM). Rapid uptake of MitoNAP by energized mitochondria was observed and this was reversed by abolishing the membrane potential with the uncoupler FCCP (*a*). Energized mitochondria also rapidly accumulated MitoSNO1 (*b*). However, in contrast to MitoNAP, uncoupling apparently led to only partial release of the accumulated MitoSNO1 (*b*). This apparent incomplete release of MitoSNO1 on uncoupling is presumably because MitoSNO1 is converted to MitoNAP within mitochondria and this generates a different response from the electrode.

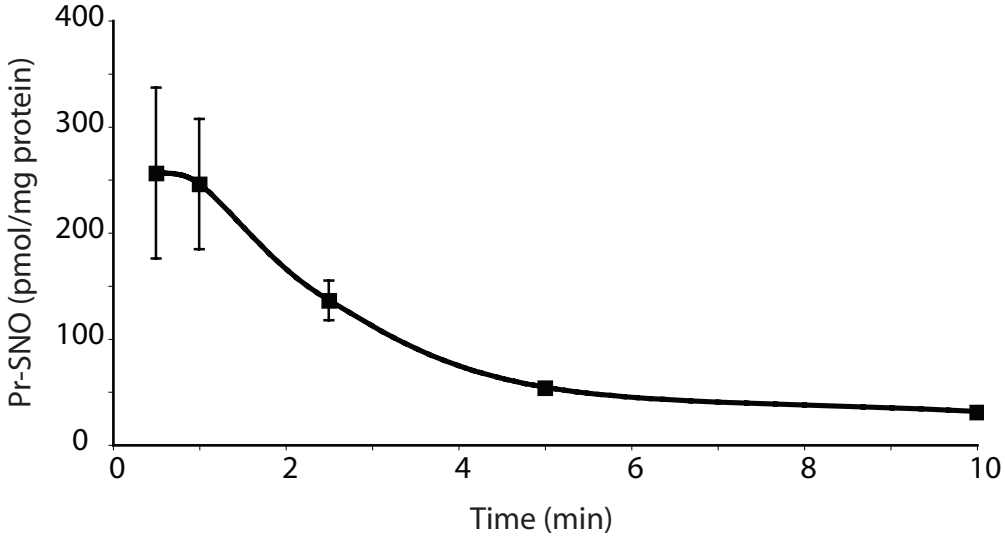

Fig. S3. Effect of OxyHb on *S*-nitrosation of mitochondrial thiols by MitoSNO1. Time course of *S*-nitrosation of mitochondrial proteins by MitoSNO1. Aliquots of liver mitochondria (1 mg protein/mL) were incubated at 37 °C in 1 mL KCl buffer supplemented with rotenone and succinate in the presence of 5 μM MitoSNO1 and 5 μM OxyHb, and at various times mitochondria were treated with 10 mM NEM and isolated by centrifugation and the *S*-nitrosothiol content assessed. Data are of a typical experiment showing means $\pm$ range of duplicate samples for each time point, each measured in duplicate. The experiment was repeated twice with the same result.

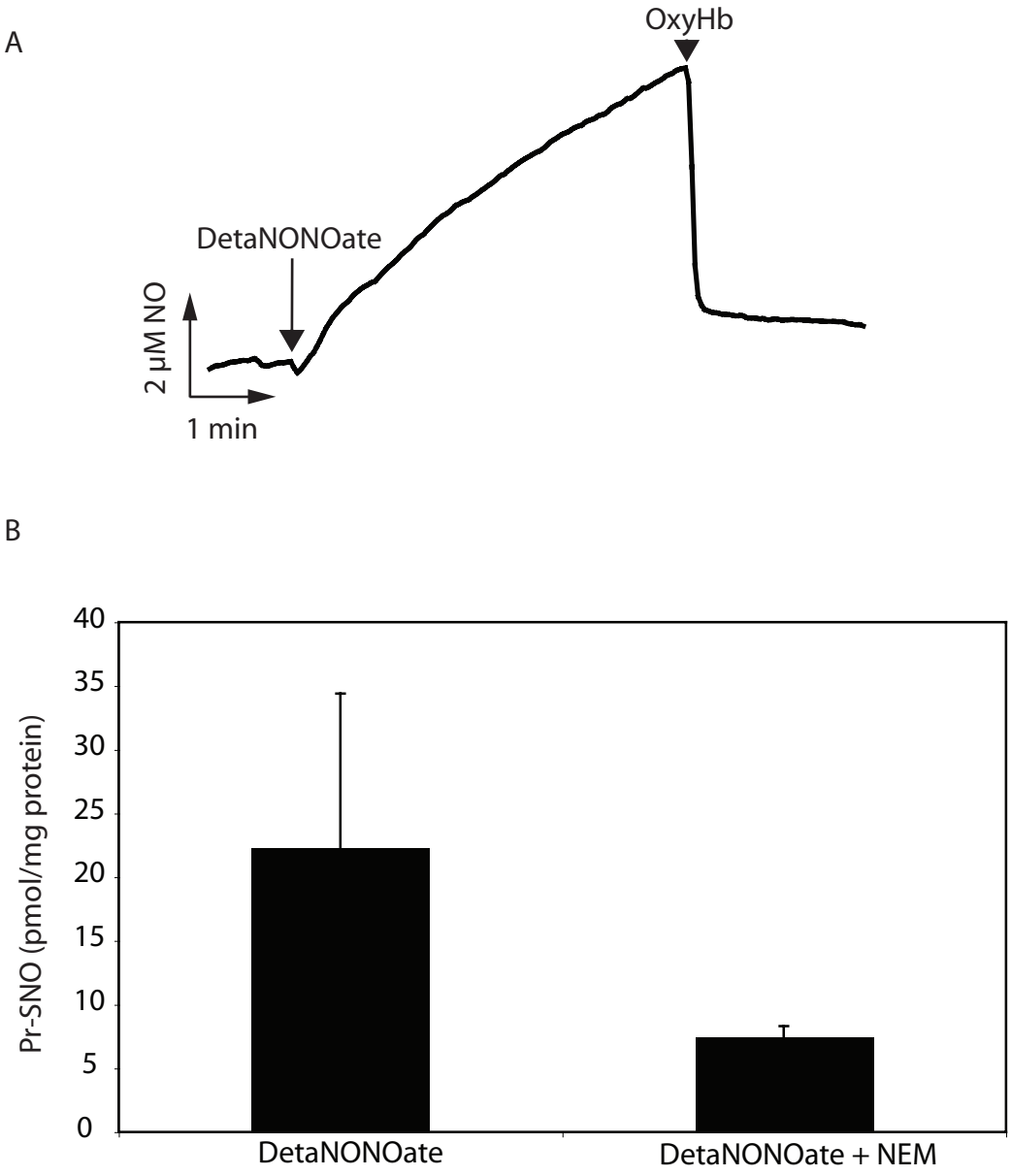

Fig. S4. Limited *S*-nitrosation of mitochondria by DetaNONOate. (*A*) Rat liver mitochondria (1 mg protein/mL) were incubated in KCl medium supplemented with rotenone and succinate at 37 °C in the 3-mL stirred chamber of an NO• electrode. Where indicated 500 μM DetaNONOate was added and the concentration of NO• was measured over time, before 5 μM OxyHb was added. (*B*) Rat liver mitochondria (1 mg protein/mL) were incubated in 1 mL KCl medium supplemented with rotenone and succinate at 37 °C with 500 μM DetaNONOate for 5 min. Then protein pellets were resuspended and processed for analysis of protein *S*-nitrosothiols. Control incubations in the presence of 10 mM NEM were also carried out. Data are means ± range of 2 independent experiments each being the average of 4 mitochondrial incubations.

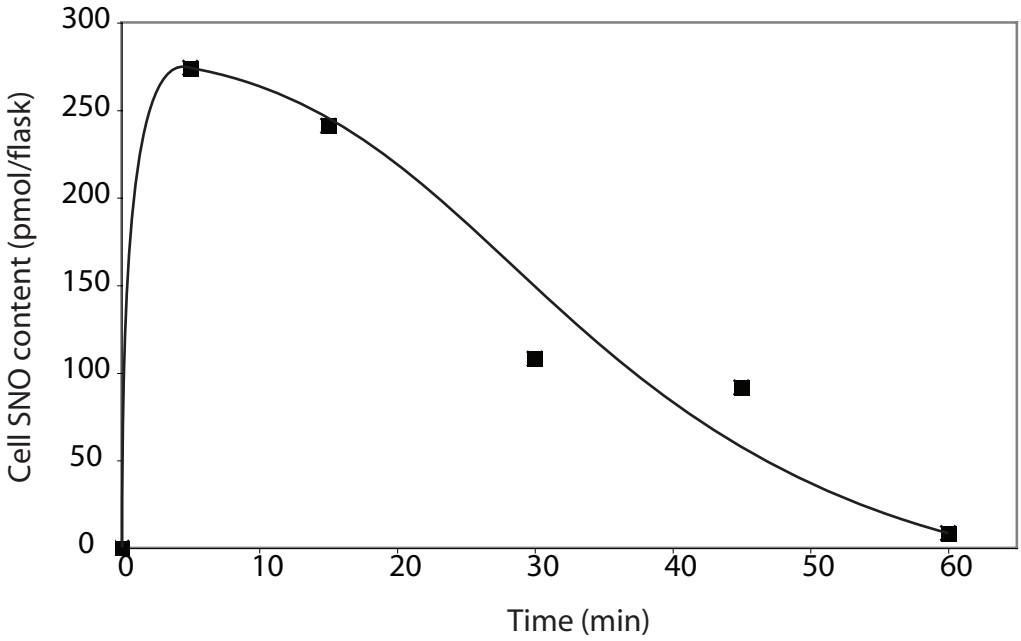

Fig. S5. Time course of *S*-nitrosation of cell proteins by MitoSNO1. Six 25-cm^2 flasks were seeded with C2C12 cells in 2 mL cell culture medium and incubated overnight. Then 5 μM MitoSNO1 was added to each flask and they were incubated for the indicated times at 37 °C in the dark. The cells were then processed to measure *S*-nitrosated protein thiols. The experiment was repeated twice with similar results.

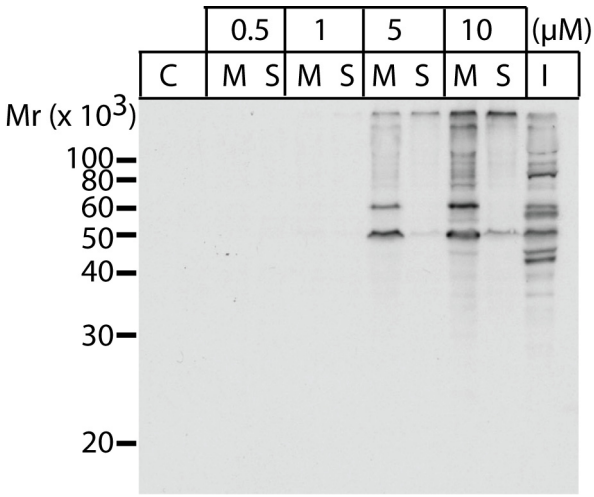

Fig. S6. Binding of MitoSNO1 to mitochondrial thiol proteins. Mitochondria (0.5 mg protein/mL) were incubated in 500 μL KCl medium supplemented with rotenone and succinate at 37 °C in the dark with no further additions (C) or with the indicated concentrations of MitoSNO1 (S), MitoNAP (M), or 10 μM IBTP (I) for 10 min. Then 500 μL of 100 mM NEM in KCl buffer was added and the mitochondria were pelleted by centrifugation (14,000 $\times$ *g* for 2 min) and the pellets resuspended in Laemmli loading buffer, modified by omitting a thiol reductant and addition of 50 mM NEM, and 50 μg protein was separated on a 12.5% acrylamide non-reducing SDS/PAGE gel, blotted to nitrocellulose, and probed with rabbit antiserum against the TPP moiety (8), followed by anti rabbit IgG conjugated to horseradish peroxidase and visualized using enhanced chemiluminescence. Mitochondrial samples treated with 10 μM MitoSNO1, but which were not treated with NEM and were instead incubated with 50 mM DTT, lost all of the MitoSNO1 binding, although 1 band at about 55 kDa was difficult to eliminate by DTT.

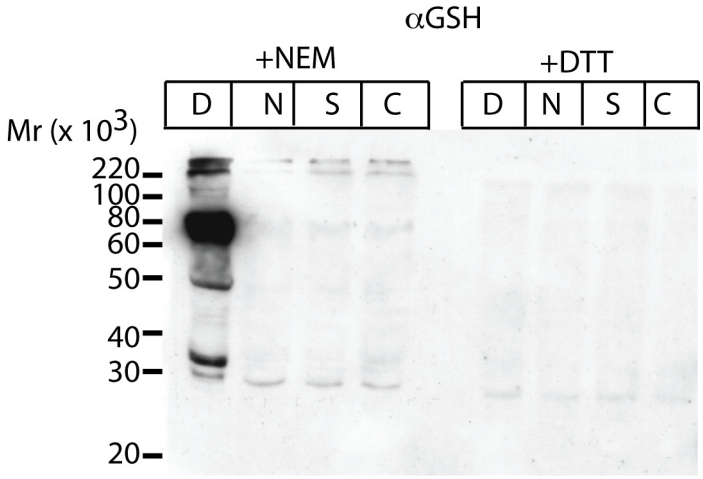

Fig. S7. Lack of extensive glutathionylation of mitochondrial thiol proteins by MitoSNO1. Mitochondria (1 mg protein/mL) were incubated in 1 mL KCl supplemented with rotenone and succinate at 37 °C in the dark for 10 min with no additions (C), 5 μM MitoSNO1 (S), 5 μM MitoNAP (N), or 1 mM diamide (D). Then the samples were split into 2 $\times$ 500 μL and pelleted by centrifugation (1 min at 14,000 $\times$ *g*) and the pellets resuspended in 100 μL Laemmli loading buffer either containing 50 mM NEM and no thiol reductant or with 100 mM DTT. Then 50 μg protein was separated on a 12.5% SDS/PAGE gel, blotted to nitrocellulose, and probed with a mouse monoclonal antibody against glutathionylated proteins (Virogen) then probed with a secondary antimouse IgG conjugated to horseradish peroxidase and visualized using enhanced chemiluminescence.

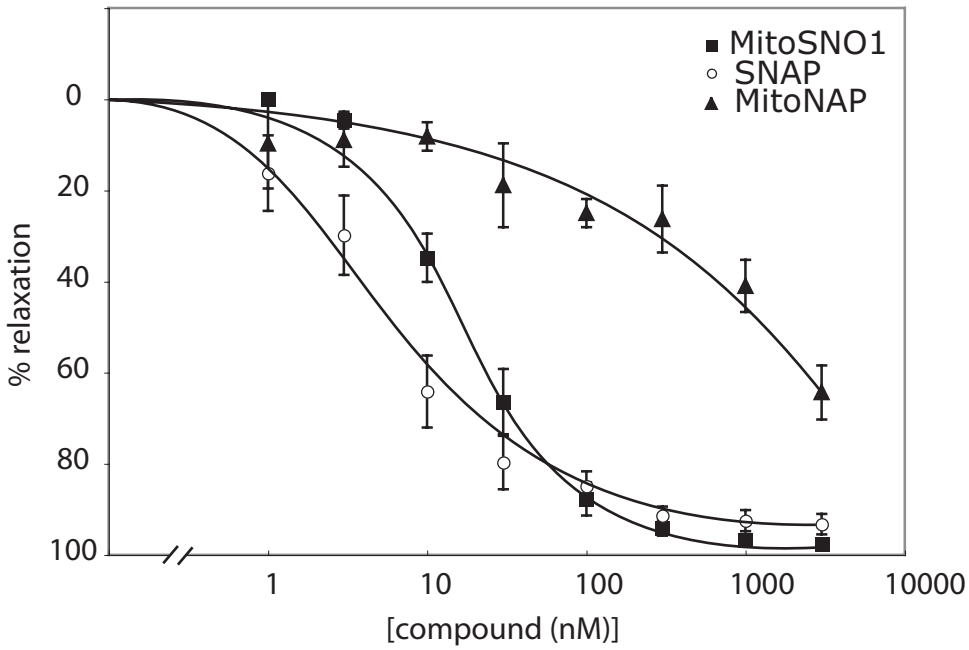

Fig. S8. Vasorelaxation of endothelium-denuded rat small mesenteric artery preparations by MitoSNO1 and SNAP. To measure vessel relaxation in endothelium-denuded rat mesenteric artery male Wistar rats (250–350 g) were anesthetized with sodium pentobarbitone (60 mg/kg i.p. Sagatal, Rhone Merieux). The mesentery was removed and placed in ice-cold, gassed (95% O_2/5% CO_2) Krebs-Henseleit buffer. Segments (2 mm in length, 250–350 μm in diameter) of third order branches of the superior mesenteric artery were removed, endothelium was removed by rubbing the intima with a human forearm hair and mounted in a Mulvany-Halpern myograph (Model 500A, JP Trading) as described (9). Vessels were maintained at 37 °C in Krebs-Henseleit solution containing indomethacin (10 μM) and bubbled with 95%O_2/5% CO_2 and were allowed to equilibrate under zero tension for 60 min. After equilibration vessels were normalized to a tension equivalent that generated at 90% of the diameter of the vessel at 100 mm Hg (10). Vessels were precontracted with methoxamine and then sequential cumulative additions of test compounds were made to relax the vessels shielded from direct light. Relaxation of muscle tone are expressed as a percentage of relaxation of the initial tone. Data are means of 4 determination of MitoNAP and MitoSNO1 and of 6 for SNAP. Data from the dose-response curves were fitted to a logistic curve, with the following parameters: MitoSNO1, EC_{50} = 16.5 ± 1.1 nM, max. relaxation of induced tone = 96 ± 1% and slope function (Hill slope) = 1.3 ± 0.1; SNAP, EC_{50} = 5.2 ± 0.5 nM, max. relaxation of induced tone = 92 ± 2% and slope function (Hill slope) = 1.0 ± 0.1. The data for MitoNAP did not fit to a logistic curve.

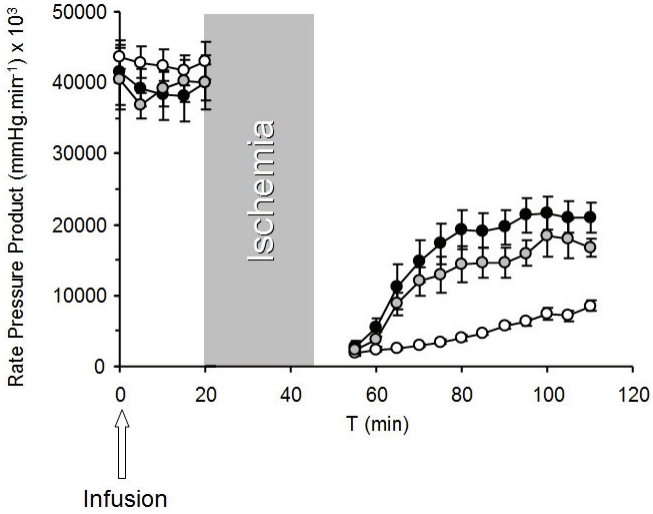

Fig. S9. Effect of preischemic administration of MitoSNO1 or MitoNAP on recovery of heart function (rate pressure product) from I/R injury. Experiments were carried out exactly as described in Fig. 7C and *D* of the main manuscript, except that MitoSNO1 or MitoNAP were infused into the heart for 20 min, followed by a 2-min wash-out period, before the onset of ischemia. White symbols, vehicle control; black symbols, MitoSNO1; gray symbols, MitoNAP. Data are means ± SEM, $n \geq 6$.