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Supporting Online Material for

Regulated Protein Denitrosylation by Cytosolic and Mitochondrial Thioredoxins

Moran Benhar, Michael T. Forrester, Douglas T. Hess, Jonathan S. Stamler*

*To whom correspondence should be addressed. E-mail: staml001@mc.duke.edu

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Supporting Online Material

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Materials and Methods

Materials

Propylamine propylamine NONOate (PAPA-NONOate) and N^G-monomethyl-L-arginine (L-NMMA) were from Cayman Chemical. Stock solutions of PAPA-NONOate were prepared in 10 mM NaOH. *S*-nitroso-cysteine (CysNO) was synthesized from L-cysteine using acidified nitrite. Auranofin was from BioMol. Mouse monoclonal antibodies to human caspase-3, thioredoxin 1 (Trx1) and protein tyrosine phosphatase 1B (PTP1B) were from BD Biosciences. Polyclonal antibody to iNOS and mouse monoclonal antibody to GAPDH were from Chemicon. Polyclonal antibodies to Trx2, Trx1 reductase (TrxR1) and TrxR2 were from Abcam. Rabbit antiserum to human thioredoxin-like protein 14 (TRP14) was generously provided by Dr. S. G. Rhee. Polyclonal antibodies to human caspase-3 and caspase-9 and monoclonal antibody to human caspase-8 were from Cell Signaling Technology. Monoclonal anti-Fas antibody (human activating, clone CH11) was from Upstate Biotechnology. The human pRSC-caspase-3 and pET23b-caspase-3 plasmids were generously provided by Dr. E. S. Alnemri and Dr. G. S. Salvesen, respectively. Human TrxR1 (GenBank accession number BC018122, IMAGE clone ID 3883490) in the vector pCMV-SPORT6 was from American Type Culture Collection (ATCC). pEBG-GST-Trx, pEBG-GST-Trx(C35S) and pET28a-Trx were generously provided by Dr. K. Kwon. Trx(C32S) in vector pET28a was generated by site-directed mutagenesis using the QuickChange Kit (Stratagene). Recombinant rat TrxR1 was from American Diagnostica. Recombinant human Trx1 from *E. coli* or human T-cells, and all other reagents, were from Sigma unless indicated.

Cell culture

HeLa, RAW264.7 and human embryonic kidney (HEK-293) cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C under 5% CO_2. Jurkat (human T leukemia cell line) and 10C9 (human Burkitt's lymphoma B cell line) cells were maintained in RPMI medium 1640 supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C under 5% CO_2.

Overexpression and purification of recombinant proteins.

Human caspase-3 was expressed in *E. coli* strain BL21(DE3). The His_6-tagged protein was purified by affinity chromatography on nickel-nitrilotriacetic acid-agarose (Qiagen) as described (*1*). Briefly, bacterial lysates were prepared in buffer (50 mM Tris, pH 8.0, and 100 mM NaCl) and incubated with nickel-nitrilotriacetic acid-agarose for 1 hour. Caspase-3 was eluted with an imidazole linear gradient from 0-200 mM. Fractions containing caspase-3 were pooled and concentrated by ultrafiltration. Because caspase-3 oxidizes spontaneously during purification, the enzyme was reduced with 100 mM DTT for 30 min at room temperature before use. Excess DTT was removed by Sephadex G-25 (Amersham) chromatography. GST-tagged Trx and His-tagged Trx were expressed in *E. coli* and purified on GSH-agarose or nickel columns, respectively.

***S*-Nitrosylation of caspase-3 and reactivation assay**

Reduced caspase-3 (10 μM in buffer H: 50 mM Hepes, pH 7.5, 100 mM NaCl and 0.1% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) was *S*-nitrosylated (SNO-caspase-3) by exposure to 2 mM PAPA-NONOate (half-life ~ 77 min; 2 moles NO per mole PAPA-NONOate) for 1 hour at room temperature followed by buffer exchange through Sephadex G-25 equilibrated with buffer H. Under these conditions, we observed a stoichiometry of ~ 0.9 mol SNO per mol caspase, as measured by Hg-coupled photolysis chemiluminescence (*2*). The flowthrough, which contains SNO-caspase-3, was then diluted 1:4 with buffer H followed by incubation in 96-well HisGrab plates (Pierce) for 1 hour at room temperature. Unbound material was removed by 3 washes with buffer H, and immobilized SNO-caspase-3 (~ 100 nM) was incubated for 30 min with cell extracts (diluted to 1 mg protein per ml in buffer H that contained 0.1 mM diethylenetriaminepentaacetic acid) or with recombinant proteins. After 3 washes with buffer H, caspase activity was measured with the EnzCheck kit (Molecular Probes) using N-acetyl-(Asp-Glu-Val-Asp)-7-amido-4-methylcoumarin (Z-DEVD-AMC) and evaluated at 340/450 nm (excitation/emission). In selected experiments, caspase was eluted from the plate and SNO content was determined by Hg-coupled photolysis-chemiluminescence (*2*).

Chromatographic purification of cellular denitrosylating activity

Jurkat cells (5 L culture) were collected, washed once in phosphate-buffered saline (PBS), resuspended in isotonic buffer (10 mM Tris pH, 7.6, 210 mM mannitol, 70 mM sucrose) supplemented with 1 x protease inhibitor mixture (Roche), and homogenized with 30-35 strokes of a Dounce homogenizer (Wheaton). Nuclei and unbroken cells were removed by centrifugation at 1000*g* for 10 min. The postnuclear supernatant was centrifuged at 20,000*g* for 30 min, and the resultant supernatant was loaded onto a Mono Q HR 10/10 column pre-equilibrated with 10 mM Tris, pH 7.5, and developed with a linear salt gradient from 0 to 1 M NaCl. Active fractions were pooled,

concentrated and loaded onto a hydroxyapatite column. The column was washed with 25 mM sodium phosphate and developed with a 25 mM – 400 mM sodium phosphate gradient (pH 7.6). After this step, denitrosylating activity (>90%) was present in the unbound material, and was dependent on the addition of eluted material (fraction II). Fraction II had negligible activity, but when added in limiting amounts (~10% of unbound protein) reconstituted full activity of the unbound fraction and of material purified during subsequent steps. The unbound material from the last step was then loaded onto a Phenyl-Sepharose column, which was eluted with a gradient of decreasing ammonium sulphate concentration (0.75-0 M). The concentrated active fractions were then loaded onto a Supredex-200 column pre-equilibrated with 100 mM Tris, pH 7.5, at a flow rate of 0.5 ml/min. One ml fractions were collected, and SNO-caspase-3 denitrosylating activity was assessed using the caspase reactivation assay. Proteins (5 μg) from the most active fraction (designated, fraction I) were separated by SDS-PAGE (8-16% gradient) and visualized using Brilliant Blue-G Colloidal Stain (Sigma). All protein bands were identified using an ABI 4700 matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) tandem mass spectrometer (MS), internally calibrated with trypsin autoproteolysis peaks. The MS spectrum was searched against the National Center for Biotechnology Information (NCBI) database, using the on-line version of Protein Prospector (http://prospector.ucsf.edu/). MS analysis was carried out at Michael Hooker Proteomics and Mass Spectrometry Facility, University of North Carolina.

Detection of protein *S*-nitrosylation with biotin-switch and DAF-2 assays

Detection of endogenously *S*-nitrosylated proteins was performed via the biotin-switch method (*3*) with some modifications (*4*), and as follows. Whole cell lysates were prepared with lysis buffer containing N-ethylmaleimide (50 mM Hepes, pH 7.5, 1% NP-40, 150 mM NaCl, 0.1 mM EDTA, 10 mM NEM (Pierce), 1 mM phenylmethanesulfonyl fluoride (PMSF) and protease inhibitor cocktail (Roche)). Fractions enriched for mitochondria were prepared as described previously (*5*). Lysates were adjusted to 1% SDS and incubated at room temperature for 30 min in the dark to block free thiols. To remove excess NEM, proteins were precipitated with 3 volumes of acetone at –20 °C for 30 min. The proteins were recovered by centrifugation at 5,000g for 5 min, and the pellets were then resuspended in 240 μl of HENS buffer (250 mM Hepes, 1 mM EDTA, 0.1 mM neocuproine, pH 7.7, 1% SDS). After addition of sodium ascorbate (20 mM) and biotin-HPDP (0.5 mM; Pierce), biotinylation was carried out for 1 hour at room temperature. After removal of excess biotin-HPDP by acetone precipitation, biotinylated proteins were pulled down overnight with streptavidin-agarose and analyzed by Western blotting using West Femto substrate (Pierce). SNO-PTP1B was detected similarly, with the blocking step performed at 50°C. Detection of SNO-GAPDH and UV exposures (to validate SNO identification) were performed as previously described (*4*). Typically, for SNO quantitation, ascorbate-dependent signals were measured by densitometry and normalized to total protein.

The DAF-2 assay was adapted from (*6*). In brief, cell extracts (50 µl) were adjusted to 100 µl with PBS containing DAF-2 (50 µM) and $HgCl_2$ (1 mM) and incubated for 1 hour at room temperature, to yield the highly fluorescent triazolofluorescein (DAF-2T). Fluorescence was monitored using excitation and emission wavelengths of 485 and 520 nm, respectively.

Determination of soluble thiols

Acid-soluble (low molecular weight) thiols were measured spectrophotometrically after reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB, Ellman's reagent). An equal volume of 10% metaphosphoric acid was added to cell extracts, the precipitate was removed by centrifugation, and the supernatant was neutralized with triethanolamine. Thiols were then measured by addition of an equal volume of DTNB solution (1 mM final concentration). The absorbance of the Ellman's reagent adduct was measured at 405 nm. Values were derived by comparison with GSH standards and were normalized to protein concentration in the extract.

Cell transfections

HEK and HEK-nNOS cells (seeded on polylysine-coated culture dishes) were transiently transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Briefly, sub-confluent cells grown in 10 cm dishes were transfected with 12 µg total DNA and 30 µl Lipofectamine 2000 in 3 ml of Opti-MEM reduced-serum medium (Gibco). The plasmid DNA ratio of Trx-1:caspase-3 or TrxR1:caspase-3 was 2:1. Approximately 80% of HEK cells were successfully transfected under these conditions as determined by a control transfection using a GFP construct. When TrxR1 was overexpressed, the culture media was supplemented with 1 μM sodium selenite as described (*7*).

RNA interference and immunodepletion

Two small interfering RNAs (siRNAs) (designated oligo 1 and 2) that target human Trx1 were based on nucleotides 258-276 and 149-167 relative to the translation start site; siRNA that targets human TRP14 was based on nucleotides 189-209; siRNA that targets human TrxR2 were based on nucleotides 536-554 (oligo 1) and 1160-1178 (oligo 2); siRNA that targets human TrxR1 was based on the smart pool approach (Dharmacon), and a second, individual oligo was based on nucleotides 303-321; siRNA that targets human iNOS was based on nucleotides 302-320. siRNAs were introduced into HeLa or HEK cells as described (*8*). In brief, the cells were harvested following exposure to trypsin, diluted with fresh antibiotic-free medium and transferred to 6-well plates. After incubation for 24 hours, the cells were transfected with siRNA for 3 days employing Oligofectamine (Invitrogen). The siCONTROL non-targeting siRNA #1 (Dharmacon) served as a negative control for siRNA activity. A mixture of 12 µl of Opti-MEM reduced-serum medium and 3 µl of Oligofectamine was incubated for 10 min at

room temperature and was then combined with 10 µl of siRNA (20 µM) in 175 µl of Opti-MEM. The resulting mixture (200 µl) was incubated for 20 min at room temperature to allow complex formation and then overlaid onto each well of cells to yield a final volume of 1.5 ml/well. siRNA oligonucleotides were introduced into 10C9 cells by square wave electroporation. Briefly, annealed siRNA duplexes (5 µM) were mixed with cells resuspended in Opti-MEM medium, and the mixture was pulsed once (2.5 kV/cm; 100 µsec) using the Gene Pulser Xcell system (Bio-Rad). Three days after electroporation, protein knockdown was determined by Western blotting.

For immunodepletion, antibodies (4 µg) were bound to protein A-agarose beads (Amersham; 20 µl bead volume) in lysis buffer (50 mM Hepes, pH 7.5, 1% NP-40, 150 mM NaCl, 0.1 mM EDTA, 1 mM PMSF and protease inhibitor cocktail (Roche)), brought to a total volume of 250 µl and incubated at 4 °C for 1 hour. Antibody-bound beads were washed 3 times with buffer H to remove unbound antibodies. Freshly prepared HeLa cell extract (50 µl) was added to the beads and incubated with constant rotation overnight at 4 °C. Beads were pelleted at 1000*g* for 2 min. Supernatants were removed and used for *in vitro* assays and Western blotting.

GST pull-down assay

HEK cells were co-transfected with 4 µg of pRSC-caspase-3 plasmid and with 8 µg of empty vector, pEBG-GST-Trx1 or pEBG-GST-Trx1(C35S). After 24 hours, cells were left untreated or treated with CysNO. Cell lysates were then prepared and incubated with GSH-agarose beads overnight at 4°C. Beads were washed 4 times in lysis buffer (with NaCl adjusted to 0.5M NaCl) and boiled in Laemmli sample buffer, and eluted proteins were analyzed by Western blotting.

Affinity labeling of active caspases

Untreated or Fas-stimulated 10C9 cells were lysed in 300 µl of lysis buffer that contained 10 μM biotin-Val-Ala-Asp(OMe) fluoromethyl ketone (bVAD-FMK). Lysates (1 mg protein) were incubated for 1 hour at room temperature to allow labeling of caspase active sites. Biotinylated proteins were captured by overnight incubation at 4°C with 30 μl streptavidin-agarose. Beads were washed 3 times in lysis buffer containing 0.5 M NaCl, and biotinylated proteins were eluted by incubation in 30 μl Laemmli sample buffer at 95°C for 10 min. Extracts and eluted, biotinylated proteins were analyzed by Western blotting with anti-caspase-3 antibodies.

Assessment of apoptosis by DNA fragmentation

Apoptotic DNA fragmentation was assessed 6 hours after cell treatment with anti-Fas CH11 antibody. The DNA fragmentation assay was performed as described (*9*) with minor modifications. In brief, 2-4 x 10^6 cells were lysed in 200 µl of lysis buffer (10 mM Tris, 10 mM EDTA, 0.5% Triton X-100, pH 8.0) and centrifuged at 15,000g for 15 min. To 180 µl of the supernatant was added 2 µg RNAse A for 2 hours at 37°C, followed by

2 μg proteinase K for 1 hour at 37°C. Isopropanol (180 μl) was added and DNA was precipitated for 12-18 hours at -20°C. Following centrifugation at 10,000g for 5 min, DNA pellets were washed with 70% ethanol and resuspended in 50 μl buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Aliquots of the re-solubilized DNA (30 μl) were analyzed by electrophoresis through a 1.2% agarose gel and visualized by ethidium bromide staining.

Statistical Analysis

Paired data were evaluated by Student's t-test. A one-way ANOVA with the Tukey post-hoc test was used for multiple comparisons. A value of $P < 0.05$ was considered statistically significant. Results are shown as mean ± SEM.

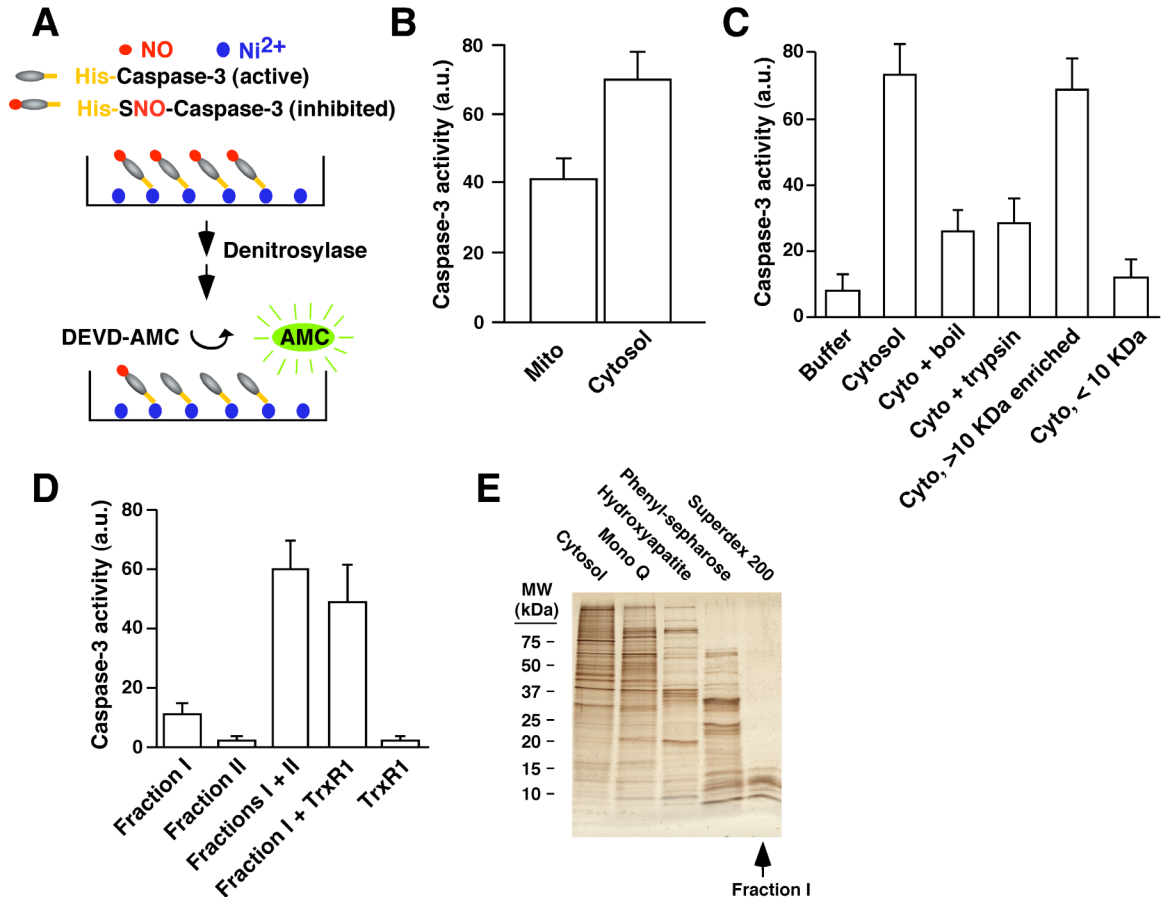


Fig. S1. Characterization of SNO-caspase-3 denitrosylating activities. (**A**) Strategy for biochemical identification of SNO-caspase-3 denitrosylase. Hexahistidine-tagged recombinant procaspase-3 was *S*-nitrosylated at the active site Cys and thereby inhibited before immobilization on nickel-coated microwell plates. Denitrosylation by cellular extracts was revealed by restoration of protease activity using N-acetyl-(Asp-Glu-Val-Asp)-7-amido-4-methylcoumarin (Z-DEVD-AMC) as substrate. (**B**) Caspase-3 activity after 30 min incubation of SNO-caspase-3 with mitochondria-enriched or cytosolic fractions (100 µg protein) prepared from Jurkat cells. (**C**) Caspase-3 activity after incubation of SNO-caspase-3 with cytosolic fraction prepared from Jurkat cells (Cyto) or with cytosolic extract boiled for 15 min, treated with trypsin (2 µg; 30 min), or enriched for high MW species by ultrafiltration (10 kDa cutoff) or for low MW species (the flow through after ultrafiltration). (**D**) Caspase-3 activity after incubation of SNO-caspase-3 with fractions obtained during the purification procedure (see Materials and Methods for details), and/or with recombinant rat TrxR1 (5 nM). (**E**) Representative SDS-PAGE gel corresponding to the chromatographic purification scheme of SNO-caspase-3 denitrosylating activity (see Table S1). Data (**B**, **C** and **D**) are presented as mean ± SEM; n = 3.

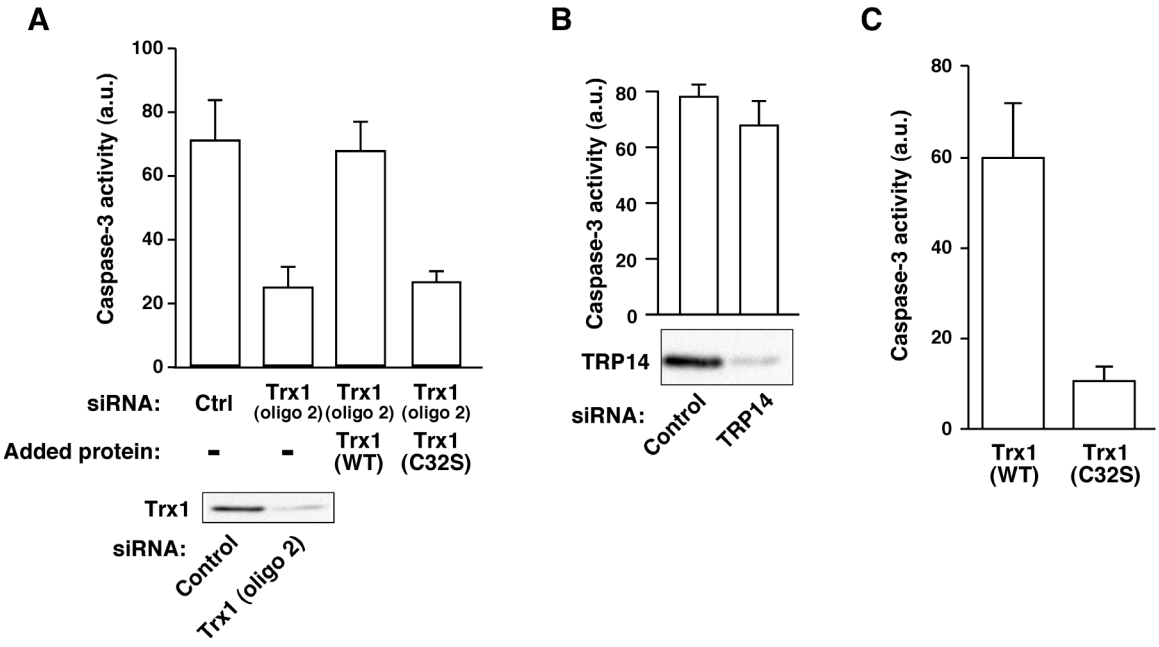


Fig. S2. Trx-mediated caspase-3 denitrosylation in vitro. (**A**) Caspase-3 activity was determined after incubation of SNO-caspase-3 with cytosolic fractions prepared from HeLa cells that were transfected with siRNA for Trx1 (oligo 2) or with control siRNA, or with Trx1-depleted cytosol supplemented with 100 nM recombinant wild-type Trx1 (Trx-WT) or with Trx1(C32S). (**B**) Caspase-3 activity after incubation of SNO-caspase-3 with a cytosolic fraction prepared from HeLa cells that were transfected with siRNA for TRP14 or with control siRNA. (**C**) Caspase-3 activity after incubation of SNO-caspase-3 with 100 nM recombinant Trx-WT or with Trx(C32S).

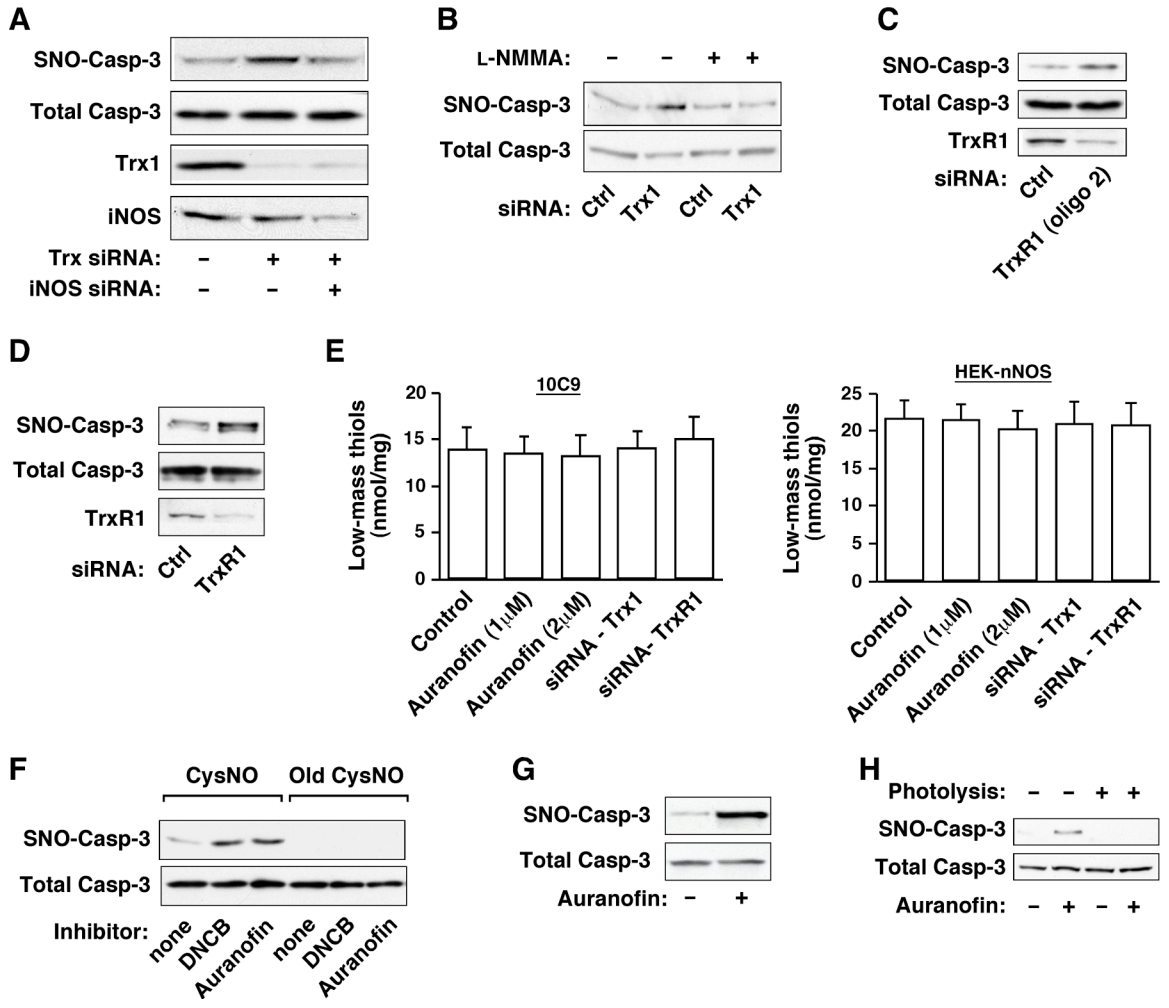


Fig. S3. Trx-TrxR inhibition increases the amount of SNO-caspase-3 in vivo. (**A**) 10C9 cells were transfected for 3 days with siRNA for Trx1 and siRNA for iNOS, as indicated. SNO-caspase-3 was assayed in whole cell extracts by biotin switch. (**B**) 10C9 cells were transfected with siRNA for Trx1 or with control siRNA, followed by treatment with L-NMMA (5 mM, 2 hours). SNO-caspase-3 was assayed in whole cell extracts by biotin switch. (**C**) 10C9 cells were transfected for 3 days with siRNA for TrxR1 (oligo 2) or with control siRNA, and the amount of SNO-caspase-3 was determined by biotin switch. (**D**) Neuronal NOS-expressing HEK cells (HEK-nNOS) were transfected for 3 days with siRNA for TrxR1 or with control siRNA, and for one day with caspase-3 cDNA. The amount of SNO-caspase-3 was determined by biotin switch. (**E**) 10C9 or HEK-nNOS cells were transfected with siRNA for Trx1 or TrxR1 or treated for 2 hours with auranofin. The amount of acid soluble thiols present in cell extracts was evaluated by DTNB colorimetric assay. (**F**) 10C9 cells were treated with DNCB (30 μM) or auranofin (2 μM) for 1 hour followed by exposure to CysNO (500 μM; 30 min). SNO-caspase-3 was assayed by biotin switch. Control samples were treated with decayed (old) CysNO. (**G**) HEK-nNOS cells were transfected for 1 day with caspase-3 cDNA, followed by 1 hour treatment with auranofin. The amount of SNO-caspase-3 was determined by biotin switch. (**H**) 10C9 cells were treated with auraonofin (2 μM; 2 hours). Lysates were then exposed or not to UV irradiation for 3 min (*4*) and SNO-caspase-3 was assayed by biotin switch.

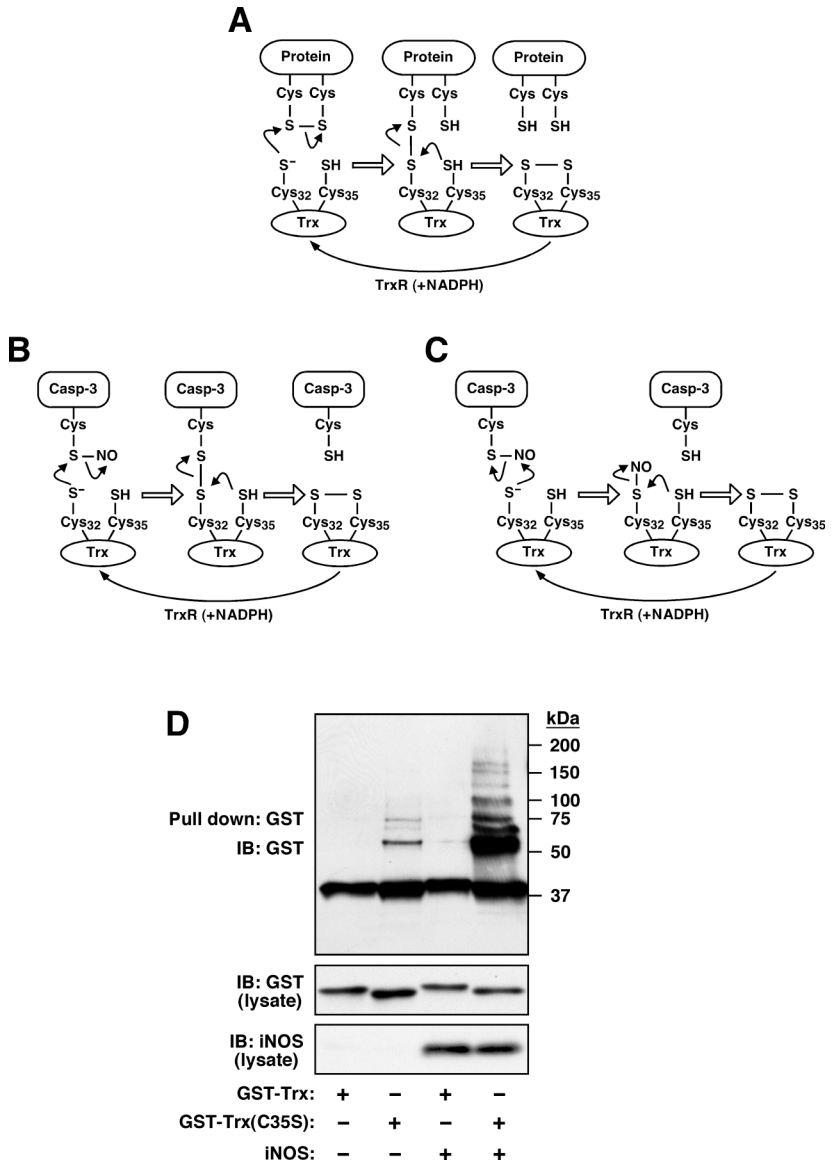


Fig. S4. Interaction of Trx with SNO-proteins. (**A**) Reaction mechanism of disulfide reduction by Trx. In the initial step, the N-terminal Cys thiolate (within the CXXC motif of Trx) attacks the target disulfide leading to formation of a mixed disulfide intermediate, which is followed by release of substrate as the disulfide is transferred to Trx via attack by the C-terminal Cys (*10*). (**B**) A possible reaction scheme for SNO-caspase denitrosylation by Trx-TrxR that is based on the results shown in figures 3E, 3F and S2C. (**C**) An alternative reaction scheme for SNO-caspase denitrosylation by Trx-TrxR that is based on transnitrosylation (*11*). (**D**) HEK cells were co-transfected (24 hours) with pIRES-iNOS and either GST-tagged-Trx1-WT or GST-tagged Trx1(C35S). Pull-down from lysates was with GSH-agarose. The complexes were resolved by electrophoresis under non-reducing conditions, and detected by western blotting with an anti-GST specific antibody.

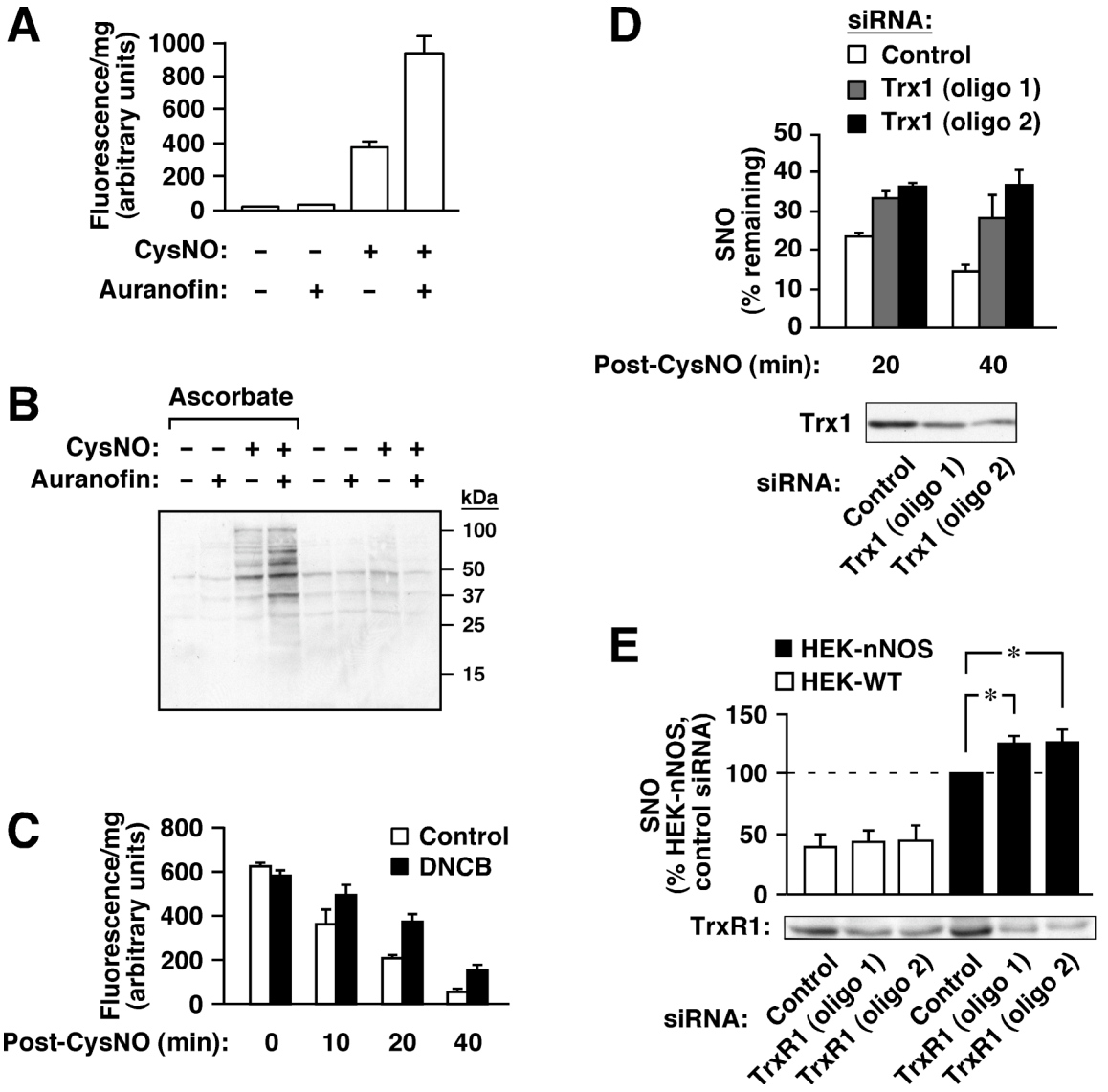


Fig. S5. Trx-TrxR inhibition increases the amount of SNO-proteins in vivo. (**A**) 10C9 cells were treated with auranofin (2 μM; 1 hour) followed by exposure to CysNO (500 μM; 30 min). Assessment of SNO in whole cell extracts was done by DAF-2 assay. CysNO rapidly decayed and thus resulted in insignificant SNO readings in this assay. (**B**) 10C9 cells were treated as in **A**. Protein *S*-nitrosylation in whole cell extracts was assessed by biotin-switch (*4*). (**C**) HEK cells were treated with DNCB (30 μM; 1 hour) then exposed to CysNO (500 μM). Levels of whole cell SNO-protein were determined using the DAF-2 assay at different intervals following CysNO exposure. Data are presented as mean ± SEM; n = 3. (**D**) HEK cells were transfected with siRNA for Trx1 or with control siRNA. After 3 days cells were treated with CysNO and analyzed for SNO content as in **C**. Data are presented as mean ± SEM; n = 3. (**E**) Wild-type HEK cells (HEK-WT) or HEK-nNOS cells were transfected for 3 days with siRNA for TrxR1 or with control siRNA. Assessment of SNO in whole cell extracts was done by DAF-2 assay. Data are presented as mean ± SEM; n=3. $^{*}P < 0.05$ by ANOVA.

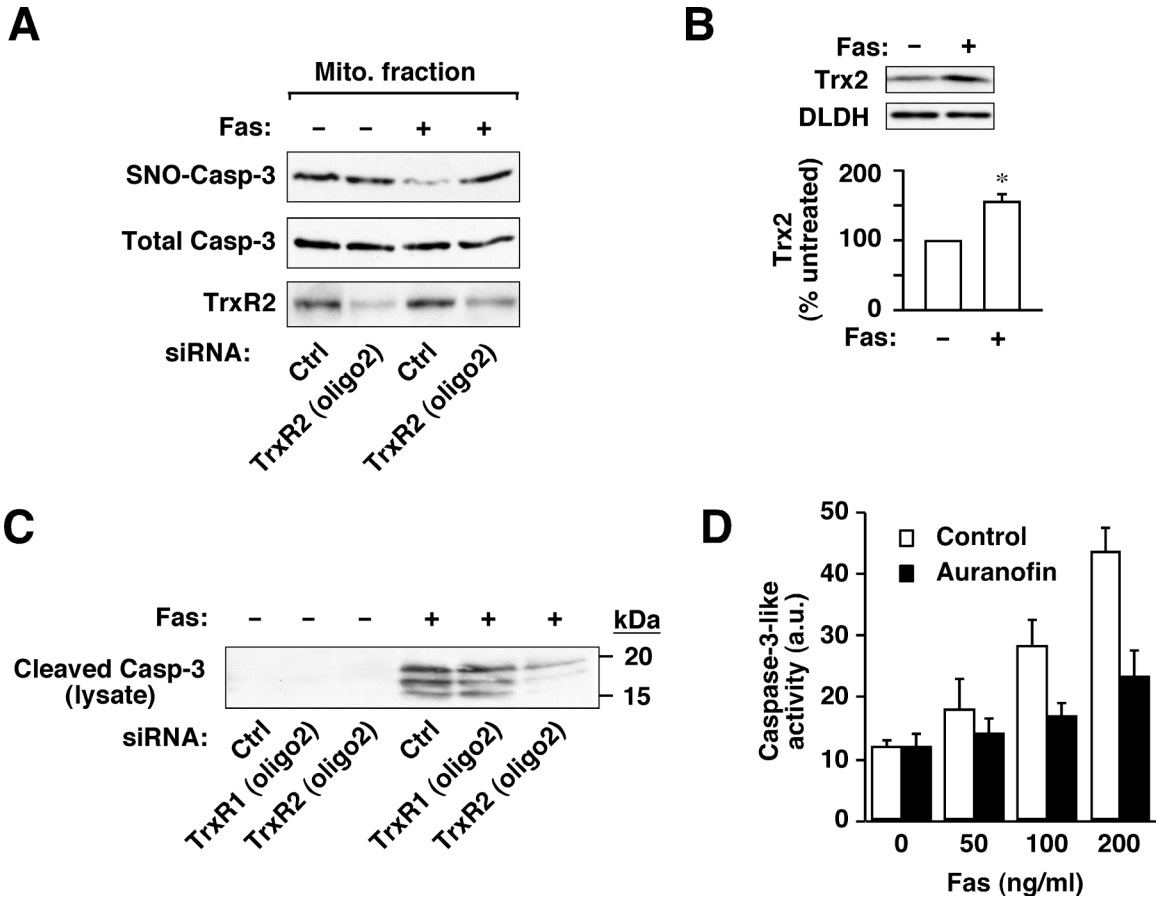
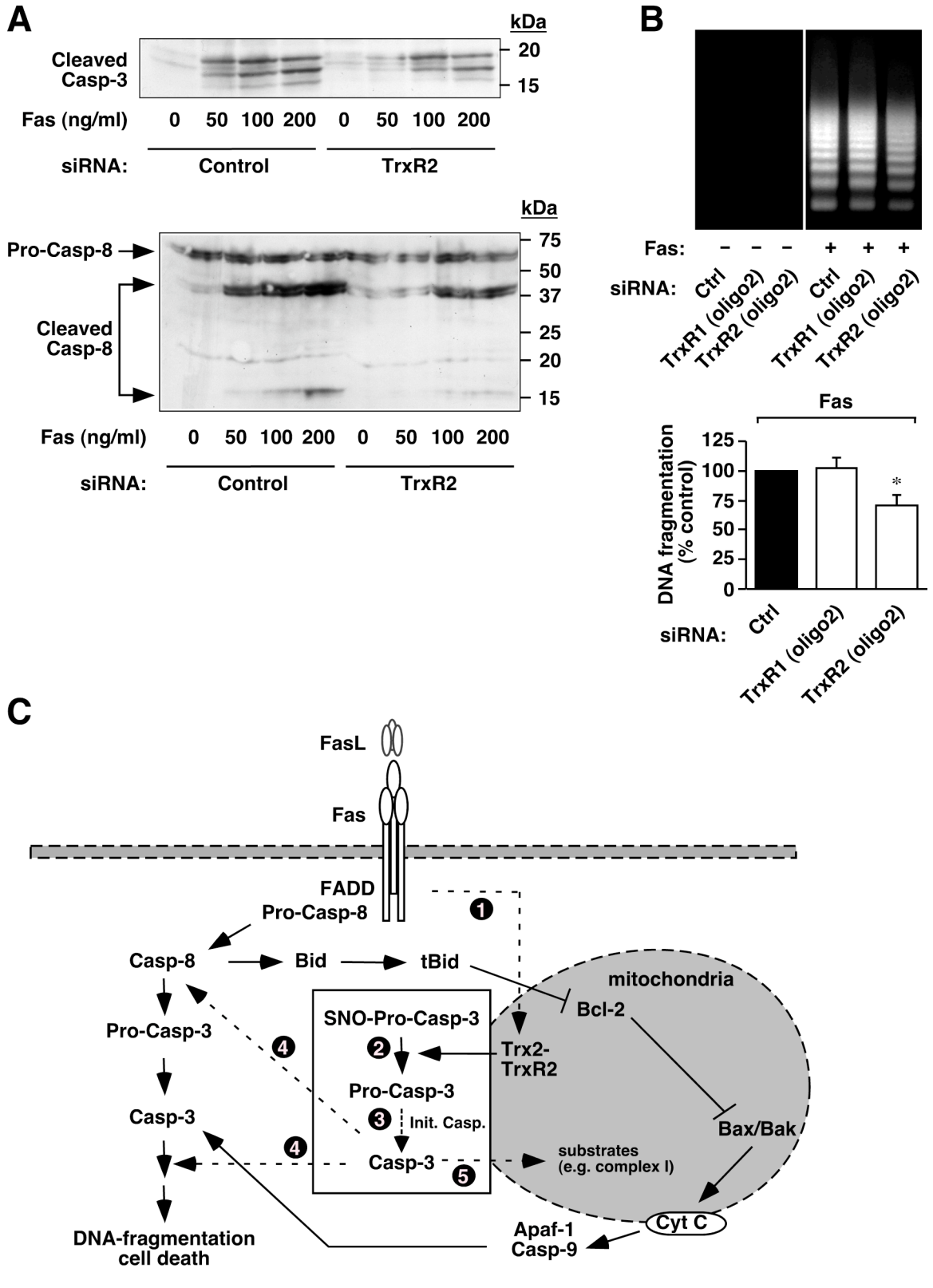


Fig. S6. Effects of TrxR2 inhibition on Fas-induced denitrosylation and activation of caspase-3. (**A**) 10C9 cells were transfected for 3 days with siRNA for TrxR2 (oligo 2) or with control siRNA before exposure to anti-Fas CH11 antibody (50 ng/ml) for 2 hours. The amount of SNO-caspase-3 in a subcellular fraction enriched for mitochondria was evaluated by biotin-switch. (**B**) 10C9 cells were treated with Fas ligand (50 ng/ml) for 2 hours, and the amounts of Trx2 and of the mitochondrial protein dihydrolipoamide dehydrogenase (DLDH) in a fraction enriched for mitochondria were determined by immunoblotting. The histogram summarizes results of 4 experiments (mean ± SEM); * $p<0.05$. (**C**) 10C9 cells were transfected with siRNA for TrxR1 (oligo 2), TrxR2 (oligo 2) or with control siRNA. Cells were treated with anti-Fas CH11 antibody (100 ng/ml; 2 hours) and caspase-3 cleavage was assessed by immunoblotting. (**D**) 10C9 cells were treated with auranofin (1 μM, 1 hour) followed by exposure to anti-Fas CH11 antibody for 2 hours. Caspase-3-like/DEVDase activity in whole cell lysates was measured by a fluorogenic assay using DEVD-AMC as a substrate. Data are presented as mean ± SEM; $n = 3$.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Casp-3
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4
Casp-3
5
substrates (e.g. complex I)
Bax/Bak
Cyt C
Apaf-1
Casp-9
DNA-fragmentation cell death

Fig. S7. Effects of TrxR2 inhibition on Fas-induced activation of caspase-3 and caspase-8 and on DNA fragmentation. (**A**) 10C9 cells were transfected for 3 days with siRNA for TrxR2 or with control siRNA before exposure to anti-Fas CH11 antibody for 2 hours. Cleavage of caspase-3 and of caspase-8 was assessed in whole cell lysates by immunoblotting. (**B**) 10C9 cells transfected with siRNA for TrxR1 (oligo 2) or TrxR2 (oligo 2) or with control siRNA. DNA fragmentation was assessed 6 hours after Fas treatment. Results (Fas-activated samples) are mean ± SEM of 3 experiments. $^{*}P < 0.05$ by ANOVA with Tukey post hoc test. Within-gel cropping of images is indicated by a white line. (**C**) A proposed model for involvement of caspase-3 denitrosylation in Fas-signaling. Engagement of Fas receptor triggers cytoplasmic and mitochondrial events that combine to induce apoptosis. Previous findings have demonstrated that activation of caspase-8 results in cleavage and activation of cytosolic caspase-3, inducing DNA fragmentation and cell death. In some cells, this cytosolic pathway only weakly activates caspase-3 and apoptosis is dependent upon a mitochondrial amplification loop (*12*), in which cleavage of proapoptotic Bid, followed by its translocation to the mitochondria, triggers the release of cytochrome c and activation of caspase-9. Our findings point to an additional mechanism whereby mitochondria may amplify the apoptotic signal, in which Fas up-regulates mitochondrial Trx2-TrxR2 (step 1) thereby inducing denitrosylation of mitochondria-associated SNO-procaspase-3 (step 2), required for activation. Initiator caspase-dependent cleavage of denitrosylated caspase-3 (in or at mitochondria or following translocation to cytosol (*13-16*)) completes the process of activation (step 3). The newly activated caspase-3 may then cleave cytosolic and/or mitochondrial substrates and may promote the activation of initiator caspases (steps 4 and 5) (*17-25*), amplifying the apoptotic cascade. *Dashed arrows* indicate indirect or incompletely elucidated interactions or sequences of events.

Table S1. Partial purification of a SNO-caspase-3 denitrosylating activity

Fraction	Total units (nmol/h)	Total protein (mg)	Specific activity (nmol/h/mg)	Yield (%)	Fold-purification
20000g supernatant	9412	150.0	63	100.0	1.0
Mono Q	1356	10.0	136	14.4	2.2
Hydroxyapatite	272	1.0	272	2.9	4.3
Phenyl-sepharose	182	0.1	1820	1.9	29.0
Superdex 200	146	0.005	29272	1.6	466.5

Table S2. Results of protein identification

Protein Name	Accession number (NCBI database)
1) PACAP protein	Q8WU39
2) Immunoglobulin lambda light chain variable region	AAM46219
3) Calgranulin B	EAW53334
4) Cytoskeleton associated protein, HUMCG22	BAA08572
5) Calgranulin A	EAW53330
6) Thioredoxin	NP_003320
7) Tetraubiquitin, chain B	1TBEB
8) Unnamed protein product, AX881592	CAE91323

Supplementary References

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