Supporting Information

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SI Materials and Methods

Cell Culture and Treatments. Smooth-muscle cells (SMCs) from mesenteric arteries of WT mice and cystothionine γ-lyase (CSE) KO mice were cultured in DMEM (Sigma) containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. The experiments were performed when the cells reached 70–80% confluence between passages 6 and 16. In all studies, cells were incubated in the serum-free medium for 12 h to ensure synchronization at a quiescent state, and then 10% serum was added together with different agents, including A23187, LPS/H_2O_2, phenylephrine (Sigma), thapsigargin (Santa Cruz), and tunicamycin (VWR Scientific).

Separation of Mitochondria and Cytoplasm. Mitochondrial fractions were isolated by differential centrifugation (1). The cultured WT-SMCs were collected with PBS solution and resuspended in buffer A [250 mM sucrose, 10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT] supplemented with protease inhibitors (2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM tetrasodium pyrophosphate, 10 mg/mL aprotinin, 10 mg/mL leupeptin, and 250 mM phenylmethylsulfonyl fluoride) and disrupted by 15–20 strokes through a 26.5-gauge needle or homogenized with a glass-Teflon potter. The suspension was then centrifuged at 1,000 × *g* for 10 min at 4 °C to remove nuclei and unbroken cells. The supernatants were centrifuged again at 8,000 × *g* for 10 min at 4 °C to pellet the heavy membrane fractions containing mitochondria. The pellet fraction was washed with buffer B [250 mM sucrose, 1 mM EDTA, 50 mM Tris-HCl (pH 7.3), and 1 mM DTT] and saved at −80 °C for later analysis. The cytosolic fraction was obtained as supernatants by further centrifugation at 10,000 × *g* for 20 min at 4 °C and saved at −80 °C for later analysis.

Real-Time PCR. Total cellular RNA was isolated using TRIzol (Sigma), and treated with RNase-free DNase (New England BioLabs). Reverse transcription was performed using the SuperScript First Strand synthesis system (Invitrogen). The relative abundance of mRNA of CSE in each sample was measured by real-time PCR in a fluorescent temperature cycler (iQ5 Real-Time PCR Detection System, Bio-Rad, Mississauga, ON, Canada) with SYBR Green PCR Master Mix (Qiagen), as described previously (2). Controls containing no reverse transcriptase were used to safeguard for genomic DNA contamination in each sample. The primers for CSE were 5′-AGCGATCACACCACAGACCAAG-3′ (sense, position 432–453) and 5′-ATCAGCACCCAGAGCCAAAGG-3′ (antisense, position 589–609), which produced a product of 178 bp (2). The primers for *atf4* were designed as reported previously (3). The primers for β-actin (Ambion) produced a product of 295 bp. The specificity of PCR was determined by melt-curve analysis for each reaction. The relative difference in mRNA between samples was calculated using the arithmetic formula $2^{-\Delta\Delta CT}$ (2).

Measurement of Intracellular Ca^{2+} with Fluo-4 Pentaacetoxymethyl Ester (AM). Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in accordance with the device manufacturer's instructions (Molecular Probes). In brief, WT-SMCs (10^3 cellsper sample) cultured in a 96-well plate were initially washed with Hepes buffer containing 130 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM Hepes, and mM 11 glucose (pH 7.4). The cells were then stained with 5 μM fluo4-AM for $[Ca^{2+}]_i$ for 30 min at room temperature. A basal level of $[Ca^{2+}]_i$ was established during the first 10 s to measure the resting state of the cells. $[Ca^{2+}]_i$ was analyzed with an Olympus fluorescence microscope at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The fluorescence intensities emitted from the cells were recorded every 1 s for 5 min.

Measurement of Mitochondrial H_2S Production Rate and H_2S Concentrations. The H_2S production rate of the sonicated mitochondrial fractions was measured as described previously (4). Mitochondrial fractions were isolated and sonicated in 50 mM ice-cold potassium phosphate buffer (pH 6.8). The flasks containing the reaction mixture (100 mM potassium phosphate buffer, 10 mM L-cysteine, 2 mM pyridoxal 5-phosphate, and 10% wt/vol cell homogenates) and center wells containing trapping solution of 0.5 mL 1% zinc acetate and a piece of filter paper (2 × 2.5 cm) were flushed with N_2 and incubated at 37 °C for 90 min. The reaction was stopped by adding 0.5 mL of 50% trichloroacetic acid, and the flasks were incubated at 37 °C for another 60 min. The contents of the center wells were transferred to test tubes, each containing 3.5 mL of water, into which 0.5 mL of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 M HCl and 0.5 mL of 30 mM $FeCl_3$ in 1.2 M HCl were added. The absorbance of the resulting solution at 670 nm was measured 20 min later with a spectrophotometer (BMG Labtech). In this method, production of H_2S from the isolated and sonicated mitochondria was trapped and reacted with *N*,*N*-dimethyl-*p*-phenylenediamine sulfate and $FeCl_3$ to yield methylene blue.

H_2S concentrations of the intact mitochondria were measured as described previously (5). A total volume of 100 μL of the isolated mitochondria without sonication was transferred directly into a tube containing zinc acetate (1% wt/vol, 187.5 μL) and NaOH (12%, 12.5 μL) to trap the H_2S for 15 min at room temperature without addition of exogenous CSE substrates or effectors. The reaction was terminated by adding 1 mL of H_2O (pH 12.8), 200 μL of *N*,*N*-dimethyl-*p*-phenylenediamine sulfate (20 μM in 7.2 M HCl), and 200 μL of $FeCl_3$ (30 μM in 1.2 M HCl). After being kept in the dark for 15 min, 600 μL of the mixture was added to a tube with 150 μL of trichloroacetic acid (10% wt/vol) to precipitate protein. Then the mixture was centrifuged at 10,000 × *g* for 5 min, and absorbance at 670 nm of the resulting supernatant (200 μL) was determined using a 96-well microplate reader (BMG Labtech). The H_2S concentration of each sample was calculated against a calibration curve of NaHS.

Plasmid Construction and Transient Transfections. PCR was performed to amplify the ORF of CSE from the plasmid constructed in our laboratories as described previously (2, 6). The CSE fragment was cloned into the EcoRI-BamHI site of AcGFP1-N1 (Clontech) to obtain pCSE-GFP. The coding region of the construct was sequenced to ensure that no mutational error was introduced by PCR. WT-SMCs were transfected with pCSE-GFP or AcGFP1-N1 (as a control) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions and cultured on glass coverslips for 24 h. The cells were serum-starved overnight and then treated with 2 μM A23187 for another 24 h. The cells were then stained with Mitotracker Red (Invitrogen) at 150 nM for 45 min and fixed in paraformaldehyde for 15 min. Images were viewed and collected with an Olympus Fluoview300 confocal microscope equipped with BX-FLA and a narrow-band interference blue filter with excitation at 450–480 nm and emission at 510–530 nm. Colocalization of GFP-tagged CSE with mitochondria in living cells was identified by the overlap of fluorescent images.

The cDNA from mice liver was subjected to PCR amplification, using forward primers 5′-CGGGGATCCATGGTGGGCCGG-3′ and reverse primers 5′-CGGAATTCTCATTCCACATCAT-3′ to obtain the ORF of Tom20. The PCR product was then digested and cloned into the EcoRI-BamHI site of expression vector pcDNA3.1(+). The constructed recombinant plasmid (pTom20) was confirmed by DNA sequencing.

WT-SMCs were seeded at a density of 3.3×10^3 cells/cm^2 in 6-cm diameter plastic dishes in the presence of 2 mL of complete medium containing 10% FBS. The cells were transfected with pTom20 or control vector [pcDNA3.1(+)] using Lipofectamine 2000.

***Tom20* Gene Suppression by siRNA.** Predesigned *Tom20*-targeted siRNA (*Tom20*-siRNA), a pool of three target-specific 19- to 25-nt siRNAs designed to knock down *Tom20* gene expression, was purchased from Santa Cruz. The control siRNA was a nontargeting 20- to 25-nt siRNA designed as a RNA duplex with no known sequence homology with all genes (Santa Cruz). Transfection of WT-SMCs by siRNA was achieved using Lipofectamine 2000. In brief, *Tom20*-siRNA or control siRNA and the transfection reagent were incubated for 20 min to form complexes, which then were added to plates containing cells and medium. The cells were incubated at 37 °C in a CO_2 incubator for further analysis.

Coimmunoprecipitation and Western Blot Analysis. For coimmunoprecipitation experiments, the cell lysis containing equal amounts of protein from different groups were incubated with anti-Tom20 antibody (Santa Cruz; 1:100) overnight at 4 °C, after which protein A Sepharose CL4B beads (Sigma) were added and incubation was continued for another 1 h at 4 °C with gentle rocking. The beads were washed four times with lysis buffer and once with PBS, and the immunocomplexes were released by heating at 100 °C in loading buffer and analyzed by Western blot analysis for the indicated molecules using their specific antibodies. For Western blot analysis, whole cell, mitochondria, or cytoplasmic lysates (30–60 μg per lane) were separated by standard SDS/PAGE and then transferred onto PVDF membranes (Millipore) and probed with the following antibodies: CSE (PTG; 1:500); voltage-dependent anion channel (Cell Signaling; 1:1,000), lactate dehydrogenase (Cell Signaling; 1:1,000), β-actin (Sigma; 1:10,000), Tom20 (Santa Cruz; 1:1,000), and GFP (Clontech; 1:1,000). Isotype-matched, HRP-conjugated secondary antibodies (Sigma) were used, followed by detection with chemiluminescence (GE Healthcare).

Detection of Cysteine Levels. L-Cysteine was quantitated by the method of Gaitonde, with modifications (7–9). In brief, 200-μL samples were prepared from mitochondrial or cytosolic fractions and mixed with 60 μL of 6% perchloric acid, and the precipitated protein was removed by centrifugation. Then 200 μL of the supernatant was mixed with equal amounts of glacial acetic acid and ninhydrin reagent (i.e., 250 mg of ninhydrin dissolved in a mixture of 6 mL of glacial acetic acid and 4 mL of 12 N HCl) for 10 min at 100 °C. After heating, the sample mixture was cooled on ice for 3 min and then diluted to 1.0 mL with 95% ethanol. Sample absorbance was measured at 560 nm, and cysteine levels were quantified using a standard curve with known amounts of L-cysteine in reaction buffer, treated in the same way as the samples. References contained all buffer and reaction components with the exception of L-cysteine or L-cysteine–producing precursors. Under the study conditions, this method is specific for L-cysteine, generating no artifact reaction with L-homocysteine or L-methionine (7). L-Cysteine levels were expressed as micromolar concentration, as calculated according to the method of Wahlländer et al. (10).

Measurement of ATP Content and ADP/ATP Ratio. For the measurement in mitochondria, mitochondrial samples were allowed to react with 100 μL of ATP Assay Mix Reagent containing luciferin and luciferase. After a 10-min incubation at room temperature, luminescence was measured with a 0.5-s integration time using a FLUOstar OPTIMA microtiter plate reader (BMG Labtech). ATP content was quantified by measuring the amount of light produced by the samples and fitting the results to a standard intensity-vs.-ATP content calibration curve. All experiments were performed at room temperature in the dark. Mitochondrial ATP level was expressed as the percentage difference between treated and untreated WT-vascular SMC mitochondria. To measure ADP/ATP ratio, mitochondrial pellets were resolved in 100 μL of nuclear-releasing agent in accordance with the manufacturer's instructions for 5 min at room temperature with gentle shaking. Then 1 μL of ATP Detection Reagent was added to each sample. The plate was agitated and placed in the FLUOstar OPTIMA microtiter plate reader. The first luminescence readings were taken in 1 min (data A). The second reading was performed 10 min later (data B). Then, 1 μL of ADP-to-ATP Converting Reagent was then added to each sample, and the plate was agitated and placed into the plate reader. A third luminescence reading was obtained 1 min later (data C). ADP/ATP ratios were calculated according to the manufacturer's instructions and expressed as the percentage difference between hypoxic and normoxic samples.

For measurements in intact cells, a bioluminescence assay kit (Sigma) and a luminometric kit (Abcam) were used to measure ATP content and ADP/ATP ratio, respectively. In brief, cultured WT-SMCs were grown in 10-cm plates for 48 h. Freshly prepared mitochondria were resuspended with reaction buffer (10 mM Tris/HCl, 5 mM $MgCl_2$, 0.2 mM Pi, 0.02 mM EGTA/Tris, and 0.25 M sucrose; pH 7.4) (11) and incubated under hypoxic or normoxic conditions with or without treatment for 25 min. In other studies, SMCs were incubated under hypoxic conditions for 1 h, after which different concentrations of NaHS were added to the medium, which was kept under hypoxic conditions for another 1 h before measurement of intracellular ATP levels.

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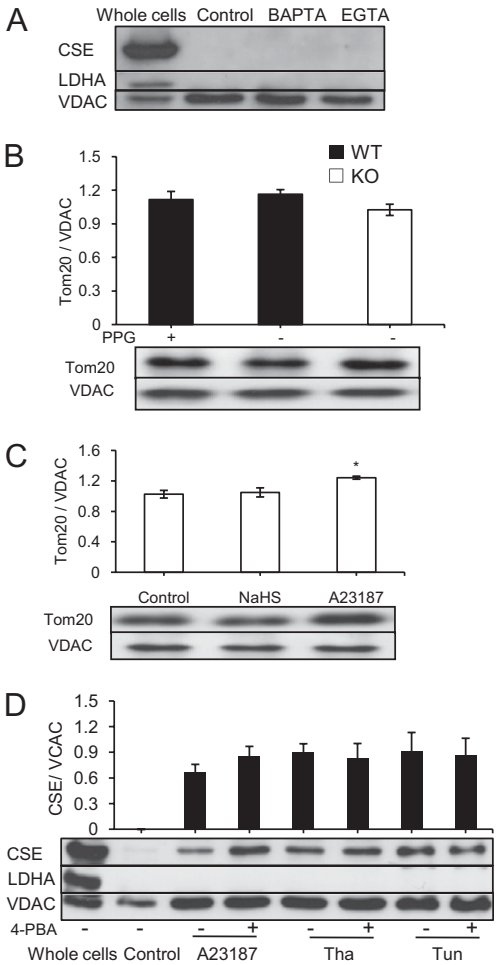

Fig. S1. (*A*) Effects of BAPTA (100 μM) and EGTA (1 mM) on CSE mitochondrial translocation. $n = 4$. (*B*) Correlation of CSE expression and activity with Tom20 expression level. (*C*) Effects of NaHS (100 μM) and A23187 (2 μM) on Tom20 expression in KO-SMCs ($n = 3$ for each group). *$P < 0.05$ vs. control. (*D*) Mitochondrial CSE translocation with different treatments. A23187 (2 μM), thapsigargin (Tha; 1 μM), and tunicamycin (Tun; 5 μg/mL) were applied in the presence or absence of 4-PBA (1 mM) ($n = 4$ for each group). The images in the panels are representative examples.

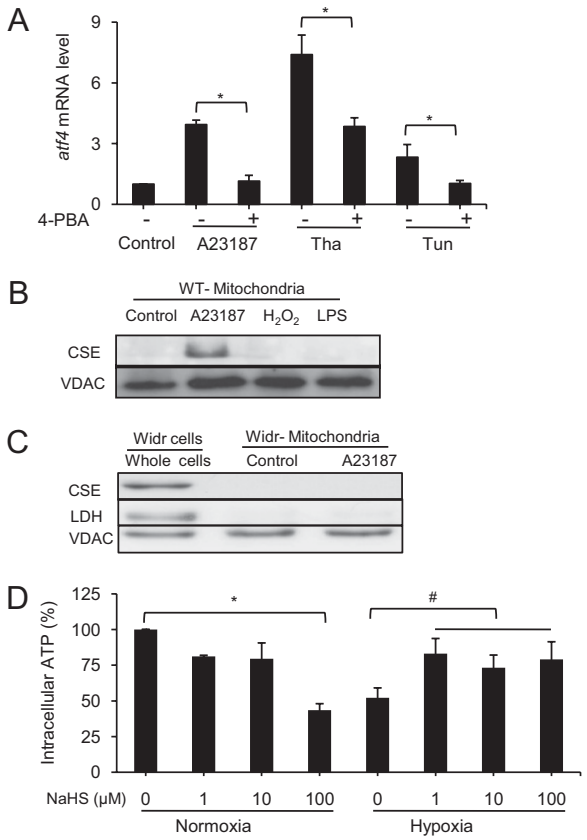

Fig. S2. (*A*) mRNA levels of ER stress marker gene *atf4* with different treatments ($n = 4$ for each group). (*B*) Effects of A23187 (2 μM), H_2O_2 (100 μM), and LPS (2 μg/mL) on mitochondrial CSE translocation in WT-SMCs ($n = 4$ for each group). (*C*) Effect of A23187 (2 μM) on mitochondrial CSE translocation in Widr cells ($n = 3$ for each group). (*D*) Effect of NaHS (1, 10, and 100 μM) on intracellular ATP in WT-SMCs under normoxic or hypoxic conditions ($n = 4$ for each group; $^{*}P < 0.05$, $^{\#}P < 0.05$ vs. the group without NaHS treatment).