SUPPLEMENTAL MATERIAL

Detailed Materials and Methods

Materials and Reagents

DL-Homocysteine (Hcy), thapsigargin (Tg), serine protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), salubrinal, sodium bisulfite, and 14,15-EET were from Sigma-Aldrich (St. Louis, MO, USA). Primary antibody for GRP78, caspase-12, sEH, β-actin, phosphorylated eIF2α, GAPDH, ATF6, polyperoxidase-anti-mouse/rabbit IgG and protein A/G PLUS-agrous were from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescent detection (ECL) reagents (San Jose, CA). Cy3-labeled goat anti-rabbit IgG (H+L) was from Beyotime (Beijing, China) and DAPI was from Vector Labs (Burlingame, CA).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated and maintained in M199 (Gibco, USA) with 20% fetal bovine serum (FBS; Highclone, USA)[1]. Primary cultured human aortic endothelial cells (HAECs) were purchased from ScienCell (Carlsbad, CA) and maintained in Endothelial Cell Medium with 5% FBS. Experiments were performed with HUVECs or HAECs between 4 to 6 passages. Human endothelial cell lines EA.hy926 and HUV-EC-C (ATCC# CRL-1730) were cultured in DMEM supplemented with 10% FBS. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. For experiments, subconfluent ECs (80-90% density) were incubated with phosphate buffered saline (PBS; control), Tg, Hcy, AEBSF, 14,15-EET or a specific sEH inhibitor 1-(1-methanesulfonyl-piperidin-4-yl)-3-(4- trifluoro-methoxy-phenyl)-urea (TUPS) [2, 3].

Quantitative real-time RT-PCR (qRT-PCR)

We used qRT-PCR as in our previous study.[4] Total cellular or tissue RNA was extracted by the Trizol reagent method (Invitrogen, USA). The quantity and quality of RNA samples were determined by spectral absorption at 260 and 280 nm. The PCR primers used are in the Online Table I. mRNA levels were normalized to that of β-actin.

Western blot analysis

Treated HUVECs were washed with PBS and lysed in RIPA buffer supplemented with protease inhibitor cocktail. The protein concentration was determined by use of the BCA protein assay kit (NovasyGen, China). Equal amounts of protein (80 μg) underwent 10% SDS-PAGE and were transferred to PVDF membrane (Millipore, Germany). Immunoblotting involved primary antibodies against GRP78, JNK, phosphorylated eIF2α (p-eIF2α), caspase-12, VCAM-1, ICAM-1 sEH and GAPDH. Corresponding secondary antibodies were applied and blots were developed by use of Super ECL Plus Detection Reagent (NovasyGen, China).

Site-directed mutagenesis, transient transfection and luciferase activity assay

The plasmids of the human sEH promoter sEH-1000bp-Luc and deletion constructs[5] were used for transient transfection. The methyaltion site of "GCG" (-284 bp ~ -282bp, analyzed by

bioinformatic program) in ATF6 binding site on the human sEH-1000bp-Luc was mutated (primers contained the 3 bold mutations in Online Table I) with the TaKaRa MutanBEST Kit (TaKaRa Biotechnology, China) and were confirmed by sequencing. EA.hy926 cells grown to 80% confluence in a 24-well plastic dish were transfected with 1 μg pGL3-sEH deletion constructs by the Jet PEI method (Polyplus, San Marcos, CA). An amount of 0.5 μg CMV-β-galactosidase was co-transfected as a transfection control. At 24 hr after transfection, cells were treated with 200 μmol/L Hcy or 20 multiplicity of infection (MOI) adenovirus-coded N-terminal ATF6 [Ad-ATF6(N)] for 24 hr. Then cells were lysed and collected for luciferase activity assay (Luciferase Reporter Assay System, Promega).

Methylation-specific PCR (MSP)

Tissue or cellular genomic DNA was modified with sodium bisulfite and used as a template for PCR. The primers for MSP amplification were designed with use of a bioinformatic program (http://www.urogene.org/methprimer/index1.html). The MSP measurement involved use of primers specific for the methylated or unmethylated DNA sequence (Online Table I). PCR products were separated on agarose gels, and bands were visualized by staining with ethidium bromide. Methylation patterns were determined as we described previously[4]. All positive PCR products were ligated into the pGEM-T vector and confirmed by sequencing. The relative DNA abundance was quantified by densitometry with use of NIH Image J.

Transfection of siRNA

RNA interference assay was used to knock down the expression of SP1 or ATF6 in the HUV-EC-C. Small interfering RNA (siRNA) of SP1 or ATF6 and scramble siRNAs were obtained from GenePharma Co. (Shanghai). Then 100-nmol/L siRNA or vehicle was transfected into cultured HUV-EC-Cs (at 60% confluence) by the Jet PEI method (Polyplus, San Marcos, CA) for 48 hr. To confirm the effect of siRNA on the expression of SP1, transfected cells underwent real time RT-PCR analysis with an SP1 primer after transfection for 48 hr. siRNA primer sequences are in Online Table I.

Immunohistochemical (IHC) and immunofluorescence staining

Mice were killed and the left ventricle of thoracic aortas were perfused with PBS at 100 mmHg for 10 min and then perfusion-fixed with 4% paraformaldehyde for 30 min. Removed aortas were fixed overnight with 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm-thick cross sections. IHC staining of sections involved the VECTASTAIN ABC System (Vector Labs) with mouse primary antibodies for sEH, GRP78 and Caspase12 diluted in PBS (1:100) overnight at 4°C. After a rinsing, sections were incubated with polyperoxidase-anti-mouse/rabbit IgG by PV-9000 Polymer Detection System (GBI). Diaminobenzidine tetrahydrochloride was used for color development. Then sections were counterstained with hematoxylin and eosin and cover-slipped, and images were acquired by use of a digital camera. Negative controls were species-matched IgG. For immunofluorescence staining, tissue sections were incubated with rabbit anti-ICAM-1 and anti-VCAM-1 primary antibodies, then Cy3-labeled goat anti-rabbit IgG (H+L) and DAPI. Images were analyzed by confocal laser scanning microscopy (OLYMPUS, Japan).

Chromatin immunoprecipitation (ChIP) assay

HUVECs were cultured to 80% to 90% confluence on 100-mm plates. Cells were treated with 200 μmol/L Hcy, 20 MOI of Ad-ATF6(N) or 100 μmol/L AEBSF co-cultured with Hcy for 24 hr. ChIP assay was performed as described [6] with modification. In brief, HUVECs were treated with 1% formaldehyde to cross-link proteins to DNA, were sonicated, then underwent immunoprecipitation (IP) with polyclonal anti-ATF6 antibody. Normal IgG (Santa Cruz Biotechnology) was used as an IP control, and the supernatant was an input control. Immunoprecipitated complexes were collected by adding salmon sperm DNA/protein A/G–agarose for 2 hr at 4°C. The beads were then treated with RNase A (50 μg/mL) and proteinase K. DNA was extracted with phenol/chloroform and coprecipitated with glycogen, dissolved in 25 μL TE buffer, and underwent PCR amplification for ATF6 binding sites on the sEH promoter with specific primers (Online Table I). The resulting DNA was resolved on 1.5% agarose gel and stained with ethidium bromide.

Adenovirus infection

The protocol of HUVECs and EA.Hy926 cells infected with adenovirus sEH (Ad-sEH) or Ad-ATF6(N) was as described[3, 7, 8]. The adenovirus (MOI 20) was added to the cell culture and incubated for 24 hr. The infected cells underwent RNA and protein extraction to detect infection efficiency. Ad-GFP was an infection control.

Animal Model

The investigation conformed to the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (NIH Publication N0. 85–23, revised 1996). The animal experimental protocol was approved by the Shantou University Institutional Animal Care and Use Committee. Wild-type C57BL/6J and $sEH^{-/-}$ mice were fed standard laboratory chow and tap water *ad libitum* and bred under a 12-hr light/dark cycle. Male, 8-week-old C57BL/6J mice were divided into experimental groups (n=8 each): control, standard chow; HHcy: 2% (wt/wt) L-methionine (Sigma, USA) added to standard chow, HHcy+TUPS: 2% L-methionine diet plus TUPS (20 mg/L/day) in drinking water[3]. TUPS (0.65 mg/ml) was put in PEG 400 and vortexed until it completely dissolved, then added into deionized water. $sEH^{-/-}$ mice (n=8) were fed L-methionine for the hyperhomocystenemia model (HHcy+$sEH^{-/-}$). Four or 8 weeks later, all animals were anesthetized, blood was collected, and serum was harvested for measurement of total Hcy level in serum by the enzymatic recycling assay.

References

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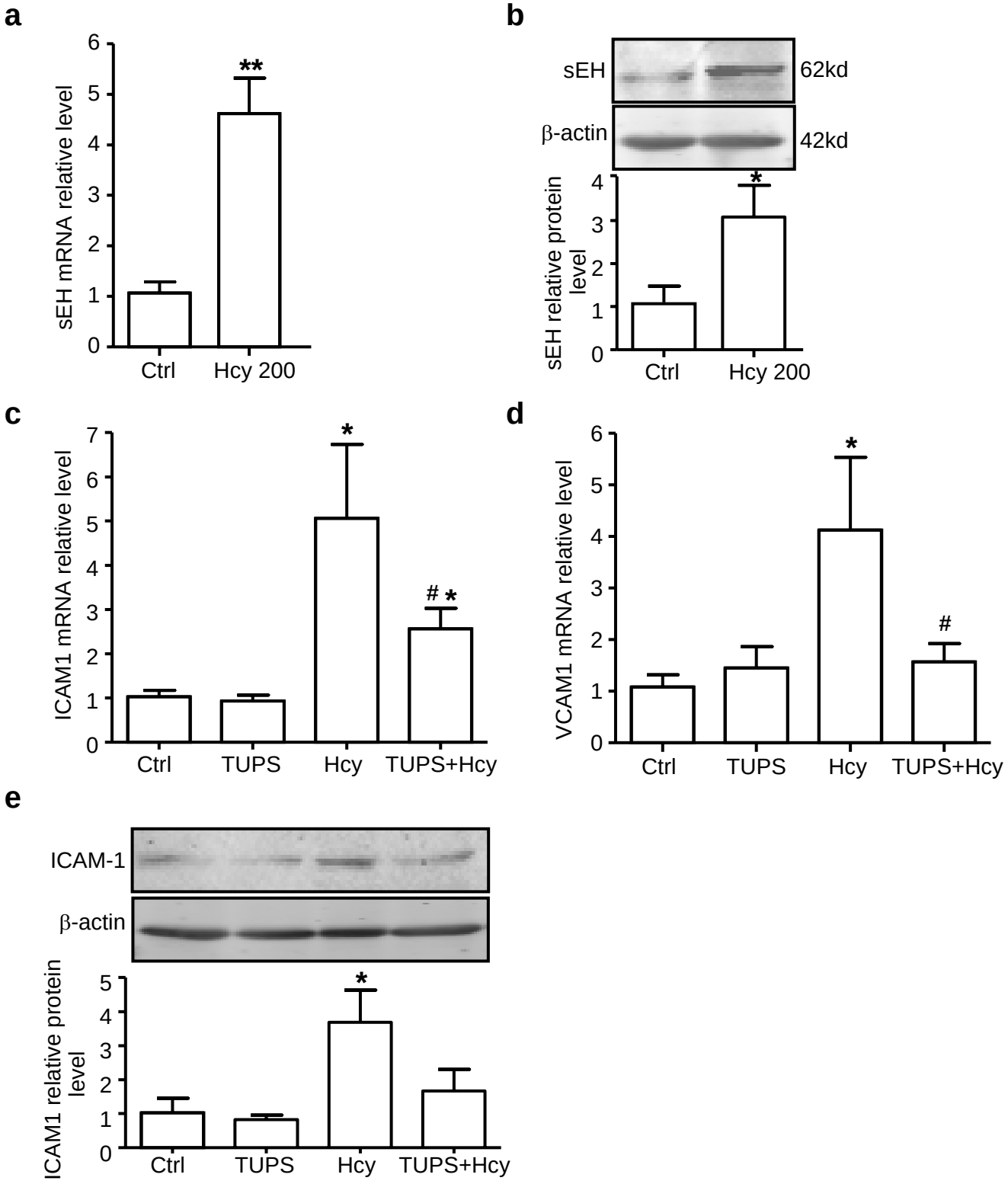
Online Tables

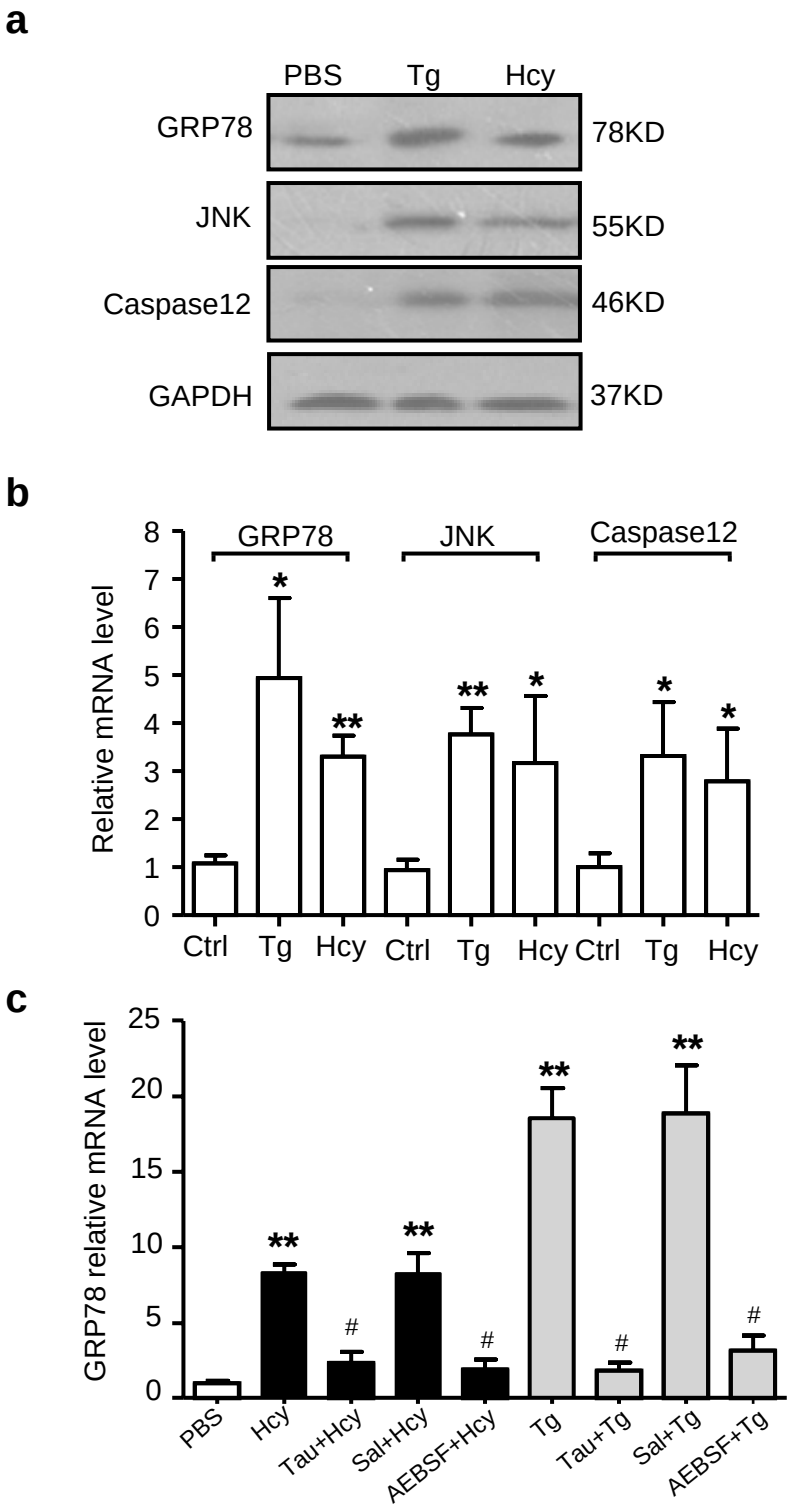
Online Table I. Primer sequences used in qRT-PCR, ChIP, siRNA, mutation and MSP assay.

Assay	Primer sequence (sense 5'-3')	Primer sequence (antisense 5'-3')
RT-PCR		
Human β-actin	AGCGAGCATCCCCCAAAGTT	GGGCACGAAGGCTCATCATT
Human GRP78	CTGGGTACATTTGATCTGACTGG	GCATCCTGGTGGCTTTCCA GCCATTC
Human ICAM-1	ATGTGCAAGAAGATAGCCACCCA	GTGCCAGTTCCACCCGTTC
Human VCAM-1	TCGTGATCCTTGGAGCCT	GCAAGTCAATGAGACGGAGT
Human SP1	GGTTCGCTTGCCTCGTCA	CCTGGGAGTTGTTGCTGTTCT
Human ATF6	TGATGAGCTGCAATTGGAAGCAGC	ACCACAGTAGGCTGAGACAGCAAA
Mouse β-actin	CTG TCC CTG TAT GCC TCT	ATG TCACGCACGATT TCC
Mouse sEH	GGACGACGGAGACAAGAGAG	CTGTGTTGTGGACCAGGATG
ChIP		
Human sEH	GCAAGTTCGCAGGGA	GGGCACAGGAAAGGGA
Mutation		
Human sEH	AGCTACTGCAGGG**AAA**TGGGGAG GGGGCATA	TATGCCCCCTCCCCA**TTT**CCCTGCAGT AGCT
siRNA		
Human SP1	GCCGUUGGCUAUAGCAAAUTT	AUUUGCUAUAGCCAACGGCTT
Human ATF6	GUGAGCUACAAGUGUAUUATT	UAAUACACUUGUAGCUCACTT
Negative control	UUCUCCGAACGUGUCACGUTT	AGGUGACACGUUCGGAGAATT
MSP		
Human sEH M	AAGTAGTTATTGCAGGGGCG	GTAGTTATTGTAGGGGTGGG
Human sEH U	ACACCCCCTTAACGAAACTC	CCACACCCCCTTAACAAAACTC
Mouse sEH M	TAGTGTTAGTATTGGGCGGGGCGG GGC	TTCGCTACGACGAAAAACCCCGACG
Mouse sEH U	GGGTAGTGTTAGTATTGGGTGGGG TGG	CCTCTTCACTACAACAAAAAACCCCA

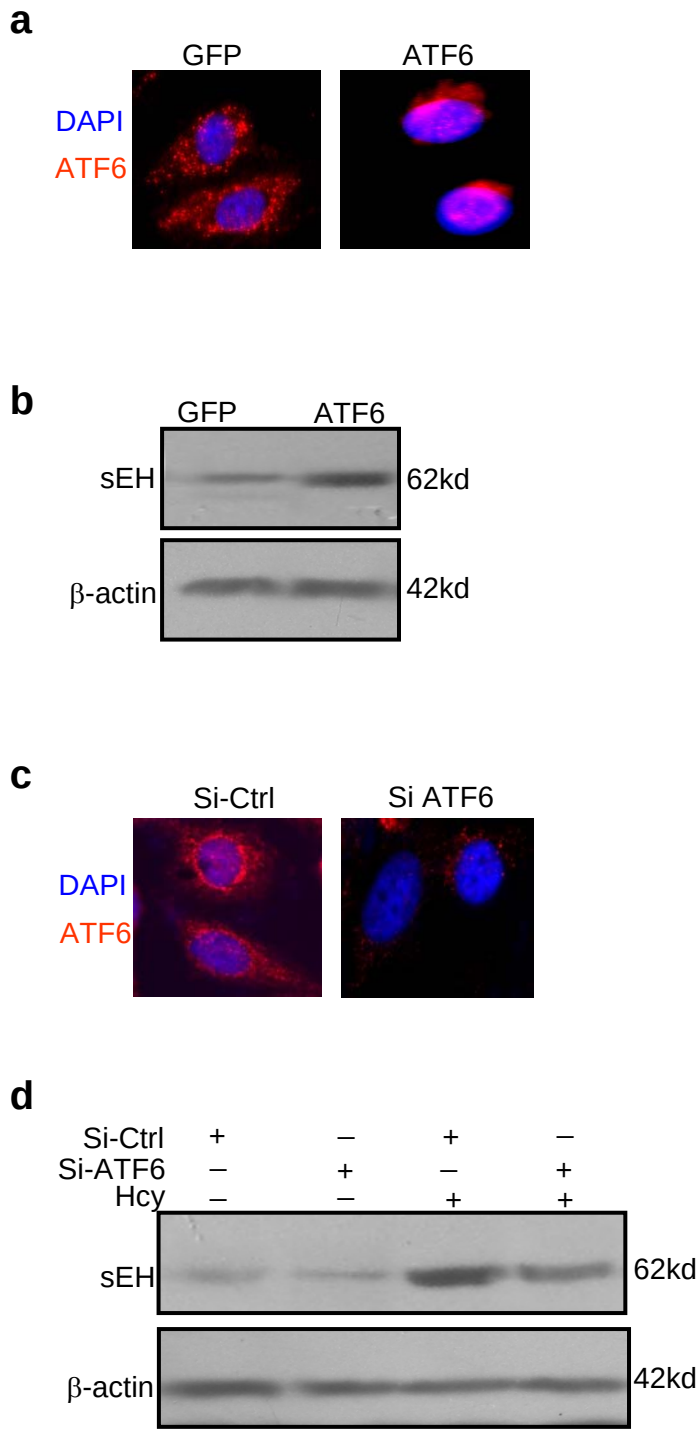
Online Figures and Figure Legends



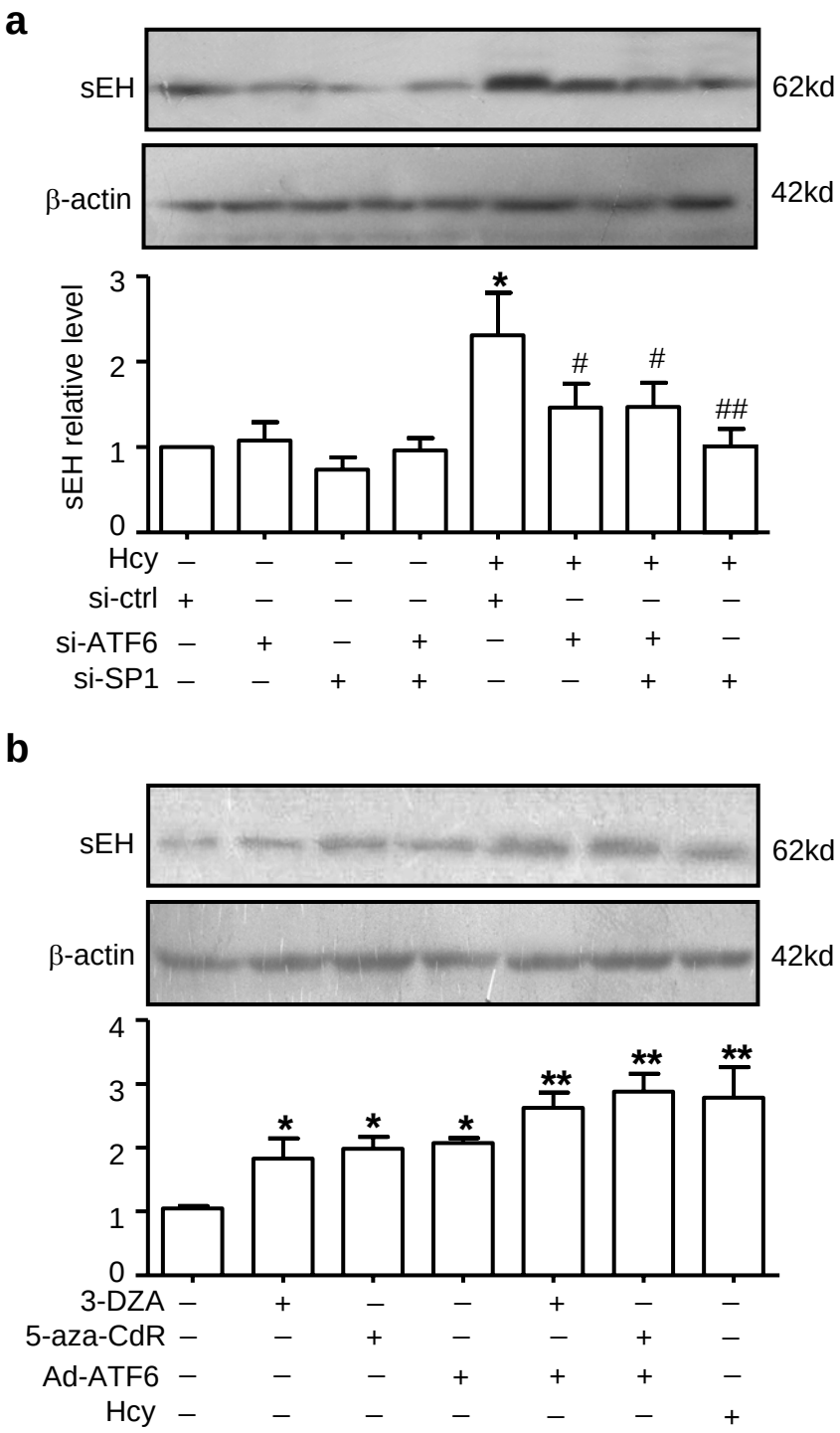
Online Figure I. Role of soluble epoxide hydrolase (sEH) in homocysteine (Hcy)-induced activation of human aortic endothelial cells (HAECs). (a,b) HAECs were treated with Hcy (200 μmol/L) for 24 hr, sEH mRNA (a) and protein (b) levels were detected by RT-PCR or western blot analysis, respectively. (c-e) HAECs were pretreated with TUPS (1 μmol/L) for 1 hr and then with Hcy (200 μmol/L) for 24 hr, VCAM-1 or ICAM-1 mRNA (c,d) and ICAM-1 protein (e) levels were detected. β-actin was an internal control. Data are means ± SD of the relative mRNA or protein normalized to that of β-actin from 3 independent experiments, respectively. *P<0.05, **P<0.01 vs. PBS controls, # P<0.05 vs. Hcy.



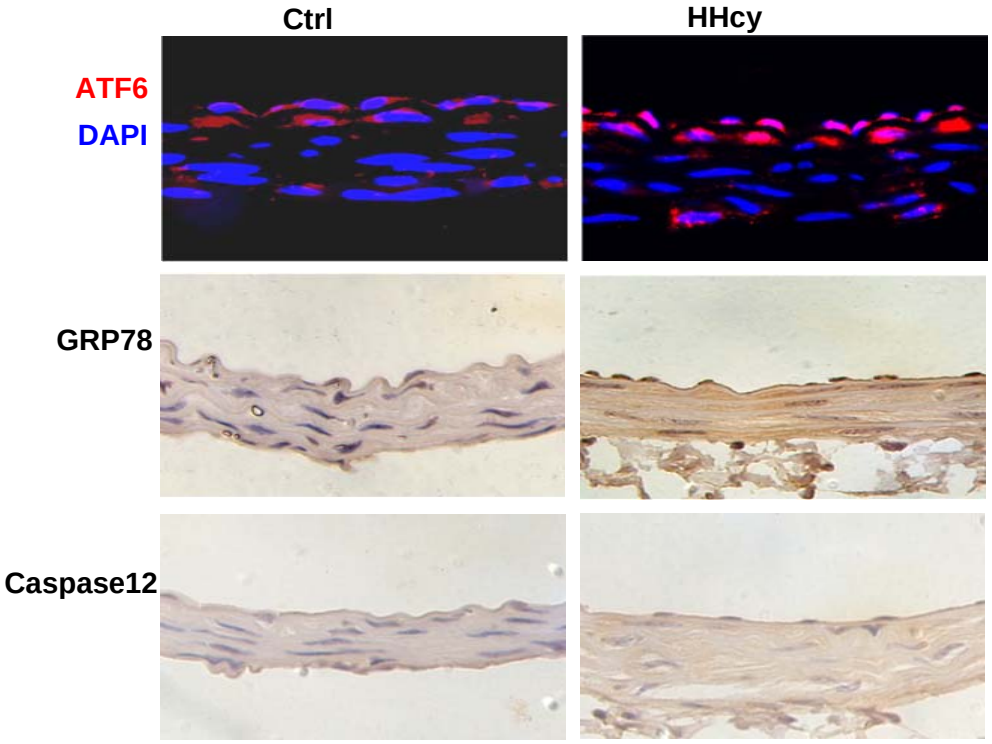
Online Figure II. Hcy-induced ER stress in ECs. (a,b) Human umbilical vein ECs (HUVECs) were treated with 200 μmol/L Hcy for 24 hr, or 4 μmol/L thapsigargin (Tg) for 8 hr. GRP78, JNK and caspase-12 protein (a) and mRNA (b) levels were detected by western blot analysis or RT-PCR, respectively. GAPDH was used as an internal control. Quantification of protein levels was by densitometry from 3 independent experiments. Data are means±SD. *P<0.05 vs. phosphate buffered saline (PBS) controls. (c) HUVECs were pre-treated with Tau (10 mmol/L), AEBSF (100 μmol/L) or Sal (50 μmol/L) for 1 hr before treatment with Hcy (200 μmol/L) and Tg (4 μmol/L). Quantitative RT-PCR analysis of mRNA expression of GRP78. β-actin was an internal control. *P<0.05, **P<0.01 vs. PBS control; #P<0.05 vs. Hcy.



Online Figure III. Activated transcription factor 6 (ATF6) is involved in Hcy-induced sEH expression in ECs. (a, b) Immunofluorescence analysis of ATF6 nuclear translocation and western blot analysis of sEH protein expression in HUVECs infected with adenovirus ATF6(N) [Ad-ATF6(N)] or Ad-GFP for 24 hr. Nuclei were visualized by DAPI staining (blue). (c, d) HUV-EC-C cells were transfected with siRNA of ATF6 (si-ATF6) or scramble siRNA control (si-Ctrl) for 48 hr. ATF6 and sEH protein expression was measured by immunofluorescence staining (c) and western blot analysis (d). β-actin was an internal control. Data are representative of at least 3 independent experiments.



Online Figure IV. ATF6 and SP1 co-contributed to Hcy-induced sEH expression. (a) si-ATF6 and/or si-SP1 and their corresponding si-Ctrl were transfected into cultured HUV-EC-Ccells for 48 hr and then treated with Hcy (200 μmol/L) for 24 hr. (b) HUVECs were exposed to Hcy (200 μmol/L), 5-aza-CdR (8 μmol/L), 3-DZA (100 μmol/L), or Ad-ATF6(N) for 24 hr, then underwent western blot analysis of sEH protein level. Quantification of protein levels was by densitometry. Data are mean±SD from 3 repeated experiments. *P<0.05, **P<0.01 vs. PBS controls, # P<0.05 vs. Hcy.



Online Figure V. ER stress was induced in the aortic intima of HHcy mice. Male C57BL/6J mice (8 weeks old) were fed standard chow diet with 2% (wt/wt) L-methionine for 4 weeks (n=8). Representative confocal images of immunofluorescence staining of ATF6 (red) and nuclei (blue), and immunohistochemical staining of GRP78 and caspase-12 expression in cross sections of pectoral aortas from HHcy and Ctrl mice.