SUPPLEMENTAL MATERIAL

Cystathionine γ lyase sulfhydrates the RNA binding protein HuR to preserve endothelial cell function and delay atherosclerosis development

Materials and Methods

Materials

Cell culture media were from Gibco (Invitrogen; Darmstadt, Germany). OCT Tissue Tek was from Sakura (Staufen, Germany) and the protease inhibitor cocktail was from Roche (Mannheim, Germany). Sulfane Sulfur Probe 4 (SSP4) was from Dojindo (GERBU Biotechnik GmbH, Heidelberg, Germany) and both interleukin (IL)-1β and the rabbit anti-CSE antibody were from Proteintech (Manchester, UK). Actinomycin D, the β-actin and the Cy3-conjugated α smooth muscle actin antibodies and all other chemicals (unless otherwise specified), were from Sigma-Aldrich (Darmstadt, Germany).

The antibodies against CD31 (rat), CD62E (rabbit) and eNOS (mouse) were from BD Transduction (Heidelberg, Germany), the CD144 (goat) and HuR (mouse) antibodies were from Santa Cruz (Heidelberg, Germany), the Von Willebrand factor (sheep) and the V5 (rabbit) antibodies were from Abcam (Cambridge, UK), anti-CBS (rabbit) was from Abnova (Biozol, Echnig, Germany), and anti-3MST (rabbit) was from Atlas (Bromma, Sweden). The phospho-serine (mouse) antibody was from Biomol GmbH (Hamburg, Germany), the phospho-tyrosine (mouse) antibody was from Millipore (Merck, Darmstadt, Germany) and the phospho-threonine (rabbit) antibody was from Cell Signaling Technologies (Europe B.V., Frankfurt, Germany). The phospho-specific S377 CSE (rabbit) antibody was raised by Pacific Immunology Corp (Ramona, CA). Secondary antibodies were from Calbiochem (Darmstadt, Germany). The PE-CD62E and APC-CD144 antibodies used for FACS analysis were from Biolegend (Koblenz, Germany). Alexa fluor secondary antibodies and Phalloidin were from Thermo Scientific (Dreieich, Germany).

Animals

Floxed CSE ($CSE^{fl/fl}$) mice were generated as described[1], and crossed with tamoxifen-inducible Cdh5-CreERT2 mice[2] in the C57/BL6J background or with Cdh5-Cre mice in the apolipoprotein E-deficient ($ApoE^{-/-}$) background; $ApoE^{-/-}$ mice were originally purchased from Charles River Laboratories Sulzfeld, Germany. Mice were housed in conditions that conform to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH publication no. 85-23). Animals received the usual laboratory diet and all studies were approved by the animal research ethics committees in Athens (790/13-02-2014) and Darmstadt (FU1177 and FU1189). Littermates of both genders were used. To induce robust Cre activity, animals were treated with tamoxifen (75 mg/kg i.p., Sigma-Aldrich) for 5 days. Sufficient knock down of CSE was observed 7 days post-injection.

Partial carotid artery ligation

Animals were randomized following the block randomization method to ensure similar sample sizes per group. Groups were age and sex matched. Experiments were performed in a double blinded manner. Partial ligation of the left carotid artery was performed as described[3]. Briefly, anesthesia was induced by intraperitoneal injection of a mixture of xylazine (5 mg/kg) and ketamine (100 mg/kg). The left carotid artery was exposed by blunt dissection and three of the four caudal branches (left external carotid, internal carotid and occipital artery) were ligated with a 6.0 silk suture while the superior thyroid artery was left intact. A single subcutaneous injection of buprenorphine (0.05 mg/kg) was given 30 minutes prior to and 4-6 hours after partial ligation (plus twice a day for consecutive two days) for pain relief. Post-operative animals were monitored for wealthness every day and their body weight was monitored weekly. For atherosclerosis studies, mice were fed a Paigen diet (energy 35 kJ% fat, containing 12.5 mg/kg cholesterol) immediately following partial ligation until the end of the experiments i.e. 2 days, one or three weeks after ligation. Ligated left carotid arteries and non-ligated right carotid arteries were used for immunostaining studies. Rescue studies

were perform with the addition of sodium polysulthionate (SG1002, Sulfagenix Inc, USA) 400-600 ng/day in the drinking water starting one day prior to partial carotid ligation. Exclusion criteria included post-operative death (0 animal excluded in the end of the study) and negative flox or cre genotype (1 animal excluded as was found CSE flox negative after re-genotyping).

Ultrasound imaging of the carotid arteries

Anesthesia was induced and maintained using isoflurane (induction 3%, maintenance 1.0–1.5% in room air supplemented with 100% O_2) delivered using a vaporizer (Visualsonics, Toronto, Canada). Mice were placed on a heating platform and limbs were taped to electrocardiogram electrodes to monitor heart rate. Body temperature was monitored using a rectal thermometer (Indus Instruments, Houston, TX). The neck area of each mouse was shaved and treated with a chemical hair remover to reduce ultrasound attenuation. To provide a coupling medium for the transducer, a pre-warmed ultrasound gel was spread over the neck area. Blood flow velocity of the left and right carotid arteries was measured using the Vevo3100 imaging system equipped with a 32-55 MHz transducer (MX550D, Visualsonics, Canada) in pulse wave Doppler mode 2, 7 and 21 days after surgery. The peak systolic velocity was calculated offline by an experienced sonographer using the VisualSonics Vevo3100 system.

***In vivo* microCT**

Experiments were performed as described previously[4]. In brief, animals were anesthetized with isoflurance (1%) and the neck was fixed by polystyrene foam. AuroVist (100 µl/28g bodyweight - nanoparticles 15 nm; Nanoprobes, NY, USA) was injected into the tail vein and a 13 minute scan was performed with the following settings: 50 kV, aluminium 1 mm filter, 250 µA source current, exposure time 750 ms, 18 µm isotopic resolution, 1 projection image per 0.5° gentry rotation step, rotation range 360° and a field of view covering the neck region (microCT, Skyscan. Kontich, Germany). Data were reconstructed with the NRecon/ InstaRecon CBR Server – Premium software (Skyscan, Kontich, Belgium/ InstaRecon, Champaign, Illinois, USA). Image analysis, segmentation and quantification of carotid artery lumen area were performed with the Imalytics Preclinical Software (Gremse-IT, Aachen, Germany). Discrimination of contrast agent and soft tissue was achieved by applying a fixed threshold. The lumen areas along the carotid arteries were calculated with the virtual elastic sphere tool and averaged over nine equidistant parts. The start point was set shortly behind the aorta in the carotid artery and the end point shortly before the bifurcation. For demonstration purpose, 3D-Models were fit to scale and freed of artefacts with Imalytics Preclinical.

Vascular reactivity studies

Vascular function was assessed as previously described[5]. Aorta from ApoE and ApoExCSE$^{\Delta EC}$ mice were cleaned of fat and connective tissue, and cut into 2 mm long segments. The presence of a functional endothelium was assessed in all preparations by the ability of acetylcholine (1 µmol/L) to induce more than 60% relaxation of vessels pre-contracted with phenylephrine (1 µmol/L) and only arteries with an intact endothelium were used for further studies. A concentration-relaxation curve to acetylcholine was generated using arteries pre-contracted to 80% of their maximal response to phenylephrine in the presence of the cyclooxygenase inhibitor diclofenac (10 µmol/L).

Cell isolation and culture

Human umbilical vein endothelial cells were isolated and cultured as described[6], and confluent cells up to passage 2 were used for the different experiments. The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki and the isolation of endothelial cells was approved in written form by the ethics committee of the Goethe University Hospital.

Murine lung endothelial cells were isolated from wild-type or $CSE^{i\Delta EC}$ mice, cultured as described[7] and used up to passage 8. To induce CSE deletion *in vitro,* cells (passage 5-6) were treated with 4-OH-tamoxifen (10 µmol/L) for 7 days. Tamoxifen was removed and the cells passaged a further 2-3 times before experiments were performed. Cells isolated from wild-type mice were treated identically.

HEK-293 were cultured in MEM containing 8% heat inactivated FCS, gentamycin (25 µg/mL), non-essential amino acids (MEM NEA, Thermo Fisher Scientific, Schwerte, Germany) and Na pyruvate (1 mmol/L). Cultures were kept in a humidified incubator at 37°C containing 5% CO2. THP-1 monocytic cells were purchased from ATCC (VA, USA) and cultured in RPM1 1640 containing 2 mmol/L glutamine; 10 mmol/L HEPES; 1mmol/L sodium pyruvate; 4.5g/L glucose; 1.5g/L sodium bicarbonate and 10% FCS.

Shear stress

Human endothelial cells (1st passage) or murine lung endothelial cells (5th-7th passage) were transferred to culture medium containing 2% FCS and either maintained under static conditions or exposed to shear stress (12 dyne cm^{-2}) in a cone-plate viscosimeter, as described[8].

Cell transfection

Endothelial cells were transfected with small interfering RNAs (siRNA) directed against CSE, HuR, or a scrambled negative control (Eurogentec), using Lipofectamine RNAiMAX (Invitrogen, Karlsruhe, Germany). Cells were then kept in culture for a further 48 hours.

Adenoviral transduction

GFP and CSE adenoviruses were generated as described [9,10]. Adenoviruses (10 MOI) were incubated with AdenoBoost (Sirion Biotech, Martinsried, Germany) for 30 minutes before being added to endothelial cells (80% confluent) in EGM containing 0.1% BSA for 4 hours at 37°C. Cells were then washed, and cultured for an additional 36 hours in the presence of 20% FCS.

Cell adhesion

Endothelial cells (cultured in 96 well plates) were stimulated with IL-1β (30 ng/ml, 3 hours). Thereafter, THP-1 cells (100.000) were added and left undisturbed for 30 minutes. Thereafter, non-adherent cells were removed by gentle washing and adherent cells were imaged. In some studies a control purified mouse IgG1 and a purified anti-mouse CD62E neutralizing antibody (1:200; Biolegend, Koblenz, Germany) were included.

Generation of point mutants

Mutations of CSE and HuR were generated using a QuickChange kit (Stratagene, Waldbronn, Germany) with point mutation primers for Ser377 and S282 to alanine or aspartic acid, Y60 and Y114 to phenylalanine or aspartic acid for CSE, or cysteine to alanine at positions 13, 245 and 284 for HuR.

Cell transfection

HEK-293 cells were transfected with pcDNA 3.1, CSE, myc-WT CSE, myc-S377A CSE, myc-S377D CSE, WT HuR, C13A HuR, C245A HuR and C284A HuR plasmids using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Cells were then maintained in culture and studied 24 hours after transfection. In the case of HuR transfection in HEK-293 cells, endogenous HuR levels were silenced by adding a small interfering RNA directed against HuR (in the presence of Lipofectamine RNAimax) 72 hours prior to transfection. Cells transfected with a control oligonucleotide were used as a positive control.

Immunohistochemistry

Wild-type or $CSE^{i\Delta EC}$ aortae were embedded in OCT Tissue TeK (Sakura, Staufen, Germany) and frozen on dry ice. Subsequently, sections (6 µm) were prepared on a microtome (Microm HM 650, Thermo Scientific, Darmstadt, Germany) and placed on positively charged glass slides. The samples were re-hydrated with phosphate–buffered saline (PBS) for 5 minutes and incubated for 2 hours at room temperature (RT) in a blocking buffer consisting of Triton X-100 (0.3%), horse serum (5%) and BSA (0.5%) in PBS. Samples were washed with PBS and primary antibodies against CSE and CD31 were added in final dilution of 1:500 in Triton X-100 (0.2% in PBS) overnight at 4°C. Thereafter, secondary antibodies against rabbit and rat were diluted 1:200 in PBS supplemented with DAPI (10 ng/ml) and a Cy3-congugated anti α-smooth muscle actin antibody (1:500). After washing, sections were mounted with Dako fluorescent mounting medium (Dako, Glostrup, Denmark).

Ligated and non-ligated carotid were dissected, perfused with PBS and fixed in 4% paraformaldehyde overnight. Samples were stained with oil O red for evaluation of intima-media ratio.

For *en face* immunostaining, whole aortae from wild-type or $CSE^{i\Delta EC}$ were cleaned of perivascular fat and fixed for 30 minutes at room temperature with 4% paraformaldehyde (PFA) before blocking and staining with antibodies against CSE and CD144 as described above. In some experiments, the surface expression of CD144 and CD62E was determined in non-permeabilized samples i.e. Triton X-100 was omitted from the buffers. Human samples were fixed in 4% paraformaldehyde and sectioned in paraffin. After demasking with sodium citrate buffer (10 mmol/L, pH 6.0), staining was performed as for the murine samples.

Proximity ligation was performed using the Duolink assay according to manufacturer's instructions (Sigma), usign antibodies against phosphoserine (mouse) and CSE (rabbit). Images were taken using a confocal microscope (LSM-780; Zeiss, Jena, Germany) and ZEN software (Zeiss).

Modified *in situ* biotin switch assay-proximity ligation assay

For the modified *in situ* biotin switch assay-proximity ligation assay, samples of carotid artery were permeabilized with Triton X-100 (1%) followed by blocking of free thiols with methane thiosulfonate (MMTS, 20 mmol/L). Thereafter, sulfhydrated proteins were labeled with the iodoacetyl-PEG2-biotin diluted in phosphate buffer saline solution at a final concentration of 10 mg/ml. After 5 washing steps of 30 minutes with phosphate buffer saline (pH=7.4), samples were blocked with the Duolink buffer (Sigma) and incubated with antibodies against biotin (rabbit), HuR (mouse), CD31 (rat) and Cy-α-smooth muscle actin. Proximity ligation was performed according to manufacturer's instructions.

Immunoblotting

Samples (cells or tissues) were lysed in ice-cold RIPA buffer (50 mmol/L Tris HCl-pH 7.5, 150 mmol/L NaCl, 25 mmol/L NaF, 10 mmol/L $Na_4P_2O_7$, 1% Triton X-100 and 0.5% sodium deoxycholate) supplemented with 0.1% SDS and protease and phosphatase inhibitors. Protein concentrations were determined using the Bradford assay, and detergent-soluble proteins were solubilized in SDS-PAGE sample buffer, separated by SDS-PAGE and subjected to Western blotting as described[7]. Proteins were visualized by enhanced chemiluminescence using a commercially available kit (Amersham, Freiburg, Germany).

Human samples were lysed in ice-cold RIPA buffer supplemented with 0.1% SDS as well as protease and phosphatase inhibitors. Proteins were then precipitated in acetone (4/1 v/v) overnight at -80°C. Following centrifugation (16000 g, 15 minutes, 4°C) the pellets were re-suspended in ice cold RIPA buffer containing 1% SDS and protease and phosphatase inhibitors. Samples (1 mg/ml) were passed through desalting columns (Thermo Scientific) and the recovered proteins were used for subsequent evaluation. Proteins were separated by SDS-PAGE and subjected to Western blotting.

To evaluate the dimeric form of HuR or the tetrameric form of CSE, samples were lysed in non-reducing non-denaturating conditions using RIPA buffer without the addition of SDS and

boiled in a DTT free Laemli lysis buffer. Western blotting was performed in the absence of SDS at 4°C.

Immunoprecipitation of CSE

Samples were lysed in a HEPES lysis buffer (20 mmol/L HEPES pH 7.5, 1.5 mmol/L $MgCl_2$, 5 mmol/L EGTA, 150 mmol/L NaCl, 1% Triton X-100, 0.5% glycerol buffer containing a protease inhibitor cocktail). After centrifugation at 16,000xg for 15 minutes supernatants were pre-incubated with protein G agarose beads (Pierce, IL, USA) for 2 hours. Supernatants were incubated with protein G agarose beads coated with antibody against V5 or CSE overnight at 4°C. Samples were washed with lysis buffer and analyzed by SDS–PAGE or washed 3 times with HEPES lysis buffer without Triton X-100 and glycerol and evaluated in mass spectrometry for interactions.

IL-1β ELISA

Circulating levels of IL-1β were evaluated with a commercially available kit according to manufactures instructions (R&D, Invitrogen).

Sample preparation for the identification of CSE-interacting proteins

Beads were resuspended in 50 µl 6M guanidine hydrochloride (GdmCl), 50 mmol/L Tris/HCl, pH 8.5 and incubated at 95°C for 5 minutes. Samples were diluted with 25 mmol/L Tris/HCl, pH 8.5, 10% acetonitrile to obtain a final GdmCl concentration of 0.6 mol/L. Proteins were digested with 1 µg Trypsin (sequencing grade, Promega) overnight at 37°C under gentle agitation. Digestion was stopped by adding trifluoroacetic acid to a final concentration of 0.5%. Peptides were loaded on multi-stop-and-go tip (StageTip) containing three strong cation exchange (SCX) disks and a stack of three C18-disks on top. SCX fractionation by StageTips was performed as described[11]**.** Three fractions of each sample were eluted in wells of microtiter plates and peptides were dried and resolved in 1% acetonitrile, 0.1 % formic acid.

Mass spectrometry of CSE interacting proteins

Liquid chromatography/mass spectrometry (LC/MS) was performed using a Thermo Scientific™ Q Exactive Plus equipped with an ultra-high performance liquid chromatography unit (Thermo Scientific Dionex Ultimate 3000) and a Nanospray Flex Ion-Source (Thermo Scientific). Peptides were loaded on a C18 reversed-phase pre-column (Thermo Scientific) followed by separation on a 2.4 µm Reprosil C18 resin (Dr. Maisch GmbH) in-house packed picotip emitter tip (diameter 100 µm, 15 cm long from New Objectives) using a gradient from mobile phase A (4% acetonitrile, 0.1% formic acid) to a 30 % mobile phase B (99% acetonitrile, 0.1% formic acid) for 25 minutes followed by a second step to 60% B for 5 minutes (IP). MS data were recorded by data dependent acquisition Top10 method for HCD fragmentation in positive mode. Lock mass option was enabled to ensure high mass accuracy between multiple runs. The full MS scan range was 300 to 2000 m/z with resolution of 70000, and an automatic gain control (AGC) value of $3x10^6$ total ion counts with a maximal ion injection time of 160 ms. Only higher charged ions (2+) were selected for MS/MS scans with a resolution of 17500, an isolation window of 2 m/z and an automatic gain control value set to 10^5 ions with a maximal ion injection time of 150 ms. Selected ions were excluded in a time frame of 30 s. Fullscan data were acquired in profile and fragments in centroid mode.

Data analysis for CSE interacting proteins

For data analysis MaxQuant 1.5.3.30 (Cox and Mann, 2008, Nat. Biotechnology), Perseus 1.5.6.0[12] and Excel (Microsoft Office 2013) were used. For protein immunoprecipitation experiments the following settings were used: N-terminal acetylation (+42.01), oxidation of methionine (+15.99) and carbamidomethylation (+57.02) on cysteines were selected as modifications. The mouse reference proteome set (Uniprot, February 2016, 79950 entries supplemented with the human CTH, P32929) was used to identify peptides and proteins with a false discovery rate (FDR) less than 1%. Minimal ratio count for label-free quantification was 2. Reverse identifications and common contaminants were removed and the data-set

was reduced to proteins that were identified in all 5 samples in one experimental group. Missing values were replaced by a background value which reflects the smallest value of data set. Significant interacting proteins were determined by permutation-based FDR calculation.

RNA Immunoprecipitation

Endogenous RIP studies were performed using the MagnaRIP Kit according to the manufacturer's protocol (Millipore). Briefly, to cross-link RNA and proteins, cells or tissues were treated with formaldehyde (0.1% in PBS) at 4 °C for 30 minutes and subsequently lysed with RIP lysis buffer for 40 minutes on ice. The whole-cell lysates were incubated at 4 °C overnight with magnetic protein A–protein G beads coupled with 5 µg of either normal mouse IgG (Millipore) or HuR monoclonal antibody (mouse monoclonal antibody; clone: 3A2, sc-5261, Santa Cruz Biotechnology, Inc.). Beads were then washed three times followed by RNA isolation from the immunoprecipitates. cDNA was prepared in each case as described above. RT–qPCR was performed by amplifying a 300bp region in the 3′ UTR of both CTSS transcript variants and CD62E (primers presented on the qPCR section). To ensure efficient binding of the antibody to its antigen, the presence of the HuR protein was monitored in the post-immunoprecipitation supernatant as well as in the immunoprecipitate.

Sulfhydration

Sulfhydration was detected using a modified biotin switch assay. In brief, samples were precipitated with 20% trichloroacetic acid (TCA) and stored at -80°C. Precipitates were washed with 10% and then 5% TCA and then centrifuged (16000g, 30 minutes, 4°C) before being suspended in HENs buffer (250 mmol/L HEPES-NaOH, 1 mmol/L EDTA, 0.1 mmol/L neocuproine, 100 µmol/L deferoxamine, 2,5% SDS) containing 20 mmol/L methanethiosulfonate (MMTS) to block free thiols and protease and phosphatase inhibitors. Acetone precipitation was performed and pellets were re-suspended in 300 µL qPerS-SID lysis buffer (6 mol/L urea, 100 mmol/L NaCl, 2 % SDS, 5 mmol/L EDTA, 200 mmol/L Tris pH 8.2; 50 mmol/L iodoacetyl-PEG2-biotin, 2.5 mmol/L dimedone), sonicated and incubated for 2 hours at room temperature in the dark. Lysates (500 µg) were precipitated with acetone and protein pellets were re-suspended in 50 µl Tris/HCl (50 mmol/L, pH 8.5) containing guanidinium chloride (GdmCl 6 mmol/L), and incubated at 95°C for 5 minutes. A negative control was generated for each sample by adding DTT (1 mmol/L) during biotin cross-linking. Biotin was then immunoprecipitated overnight (4°C) using a high capacity streptavidin resin (Thermo Scientific, Heidelberg, Germany). Elution was performed by addition of 3% SDS, 1% β-mercaptoethanol, 8 mol/L Urea and 0.005% bromophenol blue in PBS for 15 minutes at room temperature followed by 15 minutes at 95°C. Sulfhydrated proteins were then detected by SDS PAGE and Western blotting.

Enzymatic purification and activity of CSE mutants

Wild-type or mutated GST-CSE were purified and the activity evaluated using the methylene blue assay, as described[13].

H_2S measurements.

Intracellular levels of H_2S were measured by monitoring the selective reaction of SSP4 with H_2S. In brief, cells were seeded in 12 or 48 well plates and cultured to confluency. The culture medium was replaced with phenol red-free Endothelial Growth Medium (EGM, PeloBiotech, Martinsried, Germany) supplemented with 0.1 % BSA. After 2 hours, SSP4 (10 µmol/L), L-cysteine (100 µmol/L) and pyridoxal phosphate (10 µmol/L) were added for 60 minutes. In case of tissues substrates and co-factors were added for 60 minutes in homogenates of 1 mg/ml protein in 1% Triton X-100 lysis buffer supplemented with protease and phosphatase inhibitors. Samples were incubated for 60 minutes at 37°C. Thereafter, the tissue supernatant or cell supernatant was collected and floating cells were removed by centrifugation (16000 g, 10 minutes, 4°C). The specific products of the reaction of H_2S with SPP4 were quantified by LC-MS/MS.

Nitrite measurements

Plasma samples from ApoExCSE$^{\Delta EC}$ mice 21 days after carotid artery ligation were prepared as described[14]. Circulating nitrites were measured using a Nitric Oxide Analyzer (Sievers 280_max 1150W, GE Analytical Instruments, Colorado, USA) after reaction with iodide and acetic acid under nitrogen at room temperature.

FACS analyses

Murine endothelial cells were treated with solvent or IL-1β (30 ng/ml, 4 hours). After washing, cells were re-suspended in PBS and labeled with a PE-conjugated anti-CD62E antibody and an APC-conjugated anti-CD144 Biolegend (Koblenz, Germany). The cell suspension was washed, re-suspended in PBS and analyzed in a FACscan flow cytometer using the CellQuest software (Becton Dickinson, CA, USA).

RT-qPCR

Total RNA was extracted using an RNeasy kit (QIAGEN, Hilden, Germany) and equal amounts (1 µg) of total RNA were reverse transcribed (Superscript III; Invitrogen). Gene expression levels were detected using SYBR Green (Absolute QPCR SYBR Green Mix; Thermo Fisher Scientifc). The relative expression levels of the different genes studied was calculated using the formula $2^{-\Delta Ct}$ ($\Delta C_t = C_{t\ (gene)} - C_{t\ (housekeeping\ gene)}$) with the 18S RNA as a reference. The primer sequences used were as follows:

18s	forward 5´-CTTTGGTCGCTCGCTCCTC-3´
	reverse 5´-CTGACCGGGTTGGTTTTGAT-3´
hCD62E	forward 5´- CCGAGCGAGGCTACATG AAT-3´
	reverse 5´- GCCAGAGGAGAAATGGTGCT-3´
mCD62E	forward 5´- ATGGAAGCCTGAACTGCTCC-3´
	reverse 5´- CCATTCCCCTCTTGGACCAC-3´
hCSE	forward 5´- ACTTCAGGCAAGTGGCATCTG-3´
	reverse 5´- GCCAAAGGGCGCTTGGTTT-3´
mCSE	forward 5´- AGGGTGGCATCTGAATTTGG-3´
	reverse 5´- GTTGGGTTTGTGGGTGTTTC-3´
ICAM-1	forward 5´- CTTCCAGCTACCATCCCAAA-3´
	reverse 5´- CTTCAGAGGCAGGAAACAGG -3´
VCAM-1	forward 5´- CAATGGGGTGGTAAGGAATG-3´
	reverse 5´- ACCTCCACCTGGGTTCTCTT-3´
eNOS	forward 5´- GCTGTTCCAGATTCG-3´
	reverse 5´- GCTGCAGGTGTTCGATG-3´
3'UTR CTSS	forward 5´- GGCTCCTTCTCCATAAAGCA-3´
	reverse 5´- AAAGTAGGCTGGGCTCAGTG -3´

mRNA stability assay

The decay rate of CD62E mRNA was assessed at regular intervals following transcriptional inhibition using actinomycin D. Briefly, endothelial cells from wild-type or CSE$^{i\Delta EC}$ mice were treated with actinomycin D (1 µg/mL) for up to 24 hours. RNA was then extracted and levels of *CD62E* mRNA were determined by RT–qPCR. Residual mRNA levels were calculated based on the mathematical formula $2^{(-Ct(gene,\ \text{each time point}) + Ct(gene,\text{time point } 0))}$.

Human Samples

Carotid plaques were prospectively collected from 24 random patients, who had internal carotid artery (ICA) stenosis of 75-90% and underwent carotid endarterectomy (see **Table I** for details of the study cohort). Arteriographical evaluation of the carotid bifurcation stenosis was performed and the degree of luminal stenosis was determined according to North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria. Peal systolic velocity was monitored by using a Philips HD11 ultrasound platform (Philips, Netherlands). Eight additional samples of healthy thyroid arteries were used as the control group. Thyroid arteries were chosen from aged matched subjects without additional comorbidities as described in **Table I**. Samples were collected post-mortem and were evaluated by a pathologist for the possibility of atherosclerotic lesions. Arteries that showed no pathological characteristics were snap frozen for additional analysis. Tissue samples were either frozen and used for biochemical analyses or embedded in paraffin for immunostaining purposes.

Plasma from a further 70 patients characterized with internal carotid artery stenosis of 75-90% before carotid endarterectomy and 32 age matched healthy donors was used for the amino acid profiling, H_2S measurements and assay of IL-1β levels (see **Supplemental Table 2** for details of the study cohort).

All studies followed the Code of Ethics of the World Medical Association (Declaration of Helsinki). The study protocols were approved by the Institutional Ethics Committee (Scientific and Ethic Committee of Hipokrateion University Hospital, PN1539) and all patients enrolled gave their informed consent.

Assessment of endothelial function in humans

Flow-mediated dilation (FMD) and low-flow-mediated constriction (L-FMC) were measured in 12 subjects; 6 with atherosclerosis and 6 without (**Supplemental Table 6**), as described[15]. L-FMC corresponds to the constriction observed during a 4.5 minute occlusion of a pneumatic cuff placed distal to the site of arterial diameter measurement. FMD corresponds to the maximal dilation observed in the 5 minutes following deflation of the cuff, i.e. during reactive hyperaemia. All data were acquired digitally and analyzed in a randomized, blinded fashion. All participants provided informed consent for their participation in the study, which was approved by the Scientific and Ethic Committee of Hipokrateion University Hospital (extension to PN1539).

Amino acid profiling in plasma and arterial samples

Blood samples from the subjects participating in endothelial function studies were used for amino acid profiling. Sample preparation was performed using the EZ:faast LC-MS free amino acid analysis kit (Phenomenex, Aschaffenburg, Germany) according to the manufacturer's instructions, with minor modifications. Internal standards (10 µl) were applied to all samples and to the standard curve. Sample pH was adjusted to be between pH 1.5-6.0 with hydrochloric acid. Analysis of metabolites was performed by LC-MS/MS using the EZ:faast AAA-MS HPLC column (inner diameter 2 mm) on an Agilent 1290 Infinity LC system (Agilent, Waldbronn, Germany) coupled to a QTrap 5500 mass spectrometer (Sciex, Darmstadt, Germany). Electro spray ionization in positive mode was employed. The intensity of the measured metabolite was normalized to internal standards. Analyst 1.6.2 and MultiQuant 3.0 (Sciex, Darmstadt, Germany), were used for data acquisition and analysis, respectively.

Statistics

Data are expressed as mean ± SEM. Statistical evaluation was performed using Student's t-test for unpaired data. The Mann-Whitney test was used if the sample size was lower than 8 or populations followed non-Gaussian distribution. One-way ANOVA followed by Newman-Keuls test and two-way ANOVA with a Bonferroni post-test were used where appropriate. A linear Pearson model was used for correlation statistics. ANOVA repeated measures with a Bonferroni post test was used where appropriate. Statistical tests are described in the figure

legend for each experiment. Central tendency and dispersion of the data were examined for replicates below 6. Values of $P<0.05$ were considered statistically significant. MetaboAnalyst[16] was used to construct the heat map and perform hierarchical clustering based on amino acid profile.

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Supplemental Table 1. Clinical and demographic data from the studied tissues.

Characteristics	*Plaque subjects*	*NP subjects*
Demographic data		
No.	24	8
Mean age (range)	73.6 (55–86)	65.6 (53–82)
Male /female	14M/10F	6M/2F
Smokers	4	0
Clinical data		
Hypertension	20	0
Diabetes	4	0
Hyperlipidemia	24	0
Coronary disease	0	0
Myocardial Infarction	0	0
Valve insufficiency	0	0
Renal disease	0	0
Heart failure	0	0
Angiographic carotic stenosis		
<90%	24	N/A
Plaque histopathology		
Unstable	12	N/A
Stable	12	N/A
Medication		
Statins	0	0
ACE inhibitors	0	0
β-blockers	0	0

Supplemental Table 2. Clinical and demographic data from the studied plasma.

Characteristics	***Plaque subjects***	***NP subjects***
Demographic data		
No	90	32
Mean age (range)	70.3 (51–87)	64.6 (50–85)
Male /female	60M/30F	20M/12F
Smokers	18	0
Clinical data		
Hypertension	30	0
Diabetes	9	0
Hyperlipidemia	75	0
Coronary disease	34	0
Myocardial Infarction	20	0
Valve insufficiency	0	0
Renal disease	0	0
Heart failure	0	0
Angiographic carotic stenosis		
<90%	90	N/A
Plaque histopathology		
Unstable	45	N/A
Stable	45	N/A
Medication		
Statins	0	0
ACE inhibitors	0	0
β-blockers	0	0

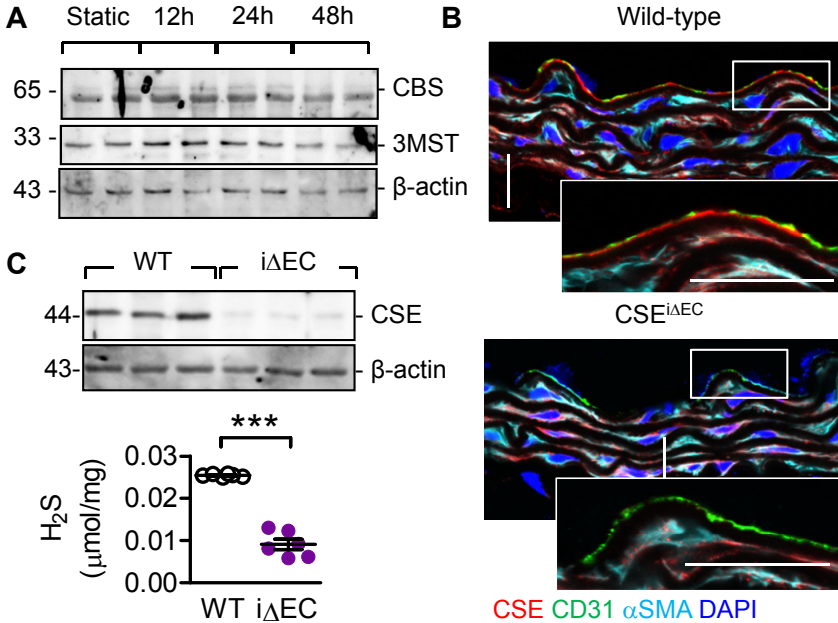
Supplemental Table 3. CSE interacting proteins identified by MS.

Supplemental Table 4. Amino acid profiling of human plasma

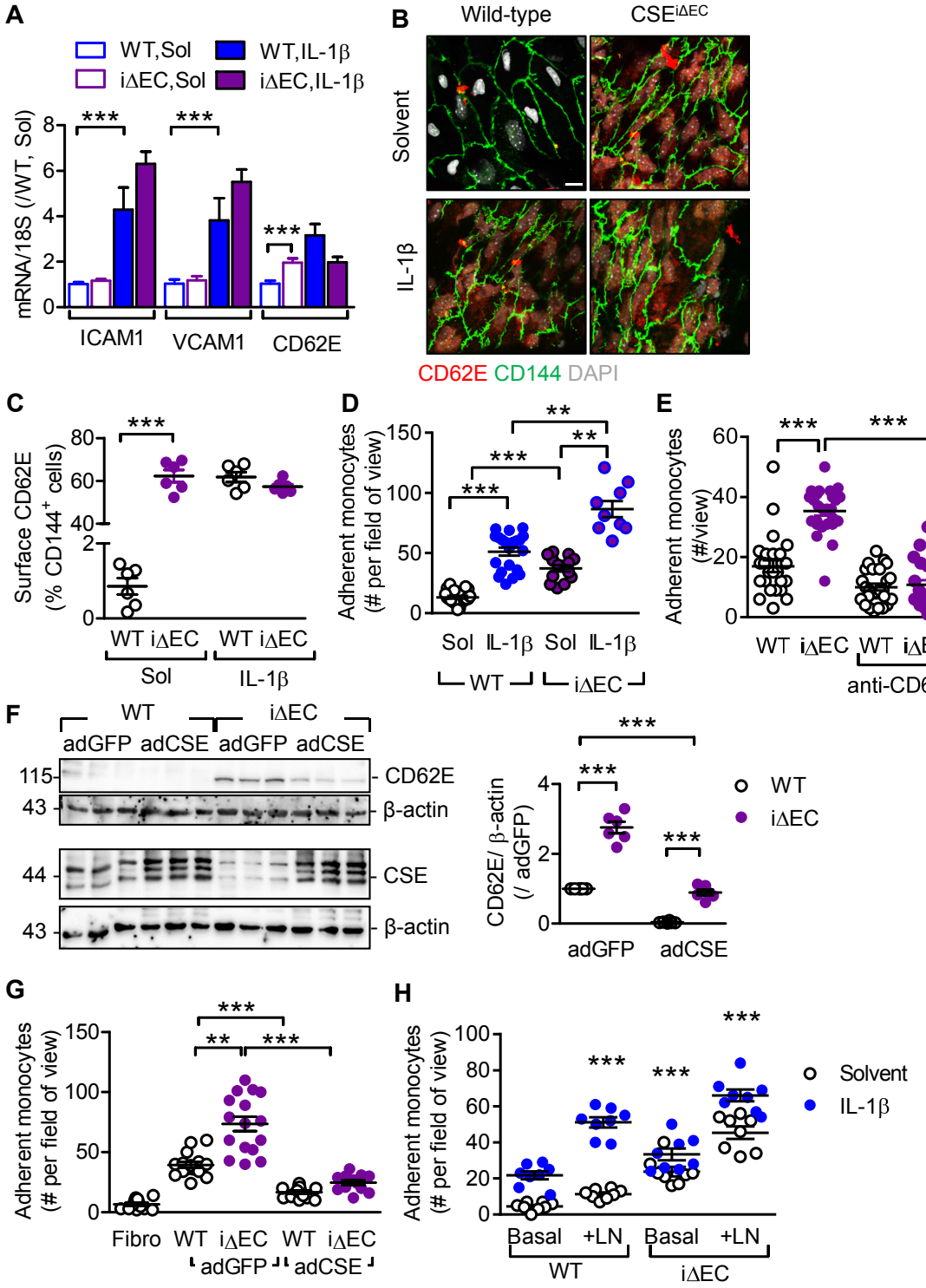
Supplemental Table 5. Amino acid profiling from ApoExCSE$^{\Delta EC}$ mice treated with vehicle or SG2001

Supplemental Table 6. Clinical and demographic data from the vascular function studies.

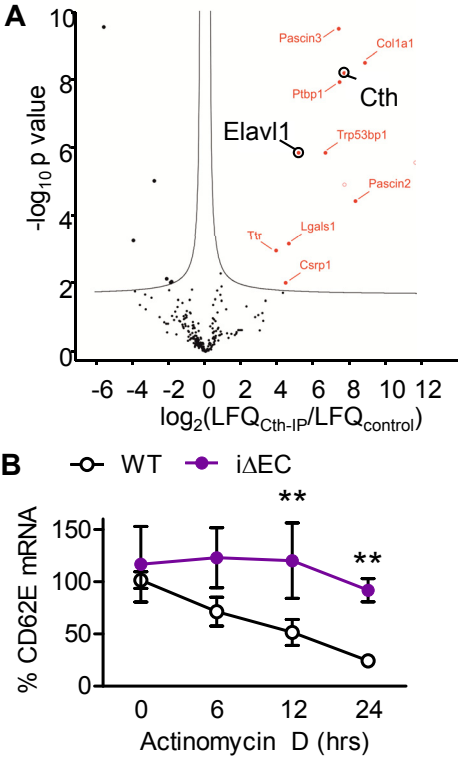
Characteristics	***Plaque subjects***	***NP subjects***
Demographic data		
No	6	6
Mean age (range)	75.8 (57-88)	58.8 (28-76)
Male /female	5M/1F	4M/2F
Smokers	0 (6 ex smokers)	0
Clinical data		
Hypertension	5	0
Diabetes	3	0
Hyperlipidemia	4	0
Coronary disease	2	0
Myocardial Infarction	2	0
Valve insufficiency	0	0
Renal disease	2	0
Heart failure	1	0
Angiographic carotic stenosis		
<90%		N/A
Plaque histopathology		
Unstable	5	N/A
Stable	1	N/A
Medication		
Statins	4	0
ACE inhibitors	1	0
b-blockers	2	0
Blood flow at rest, mL/min	56 ± 4	56 ± 7
During cuff inflation	12 ± 1	12 ± 1
After cuff deflation	131 ± 9	120 ± 7



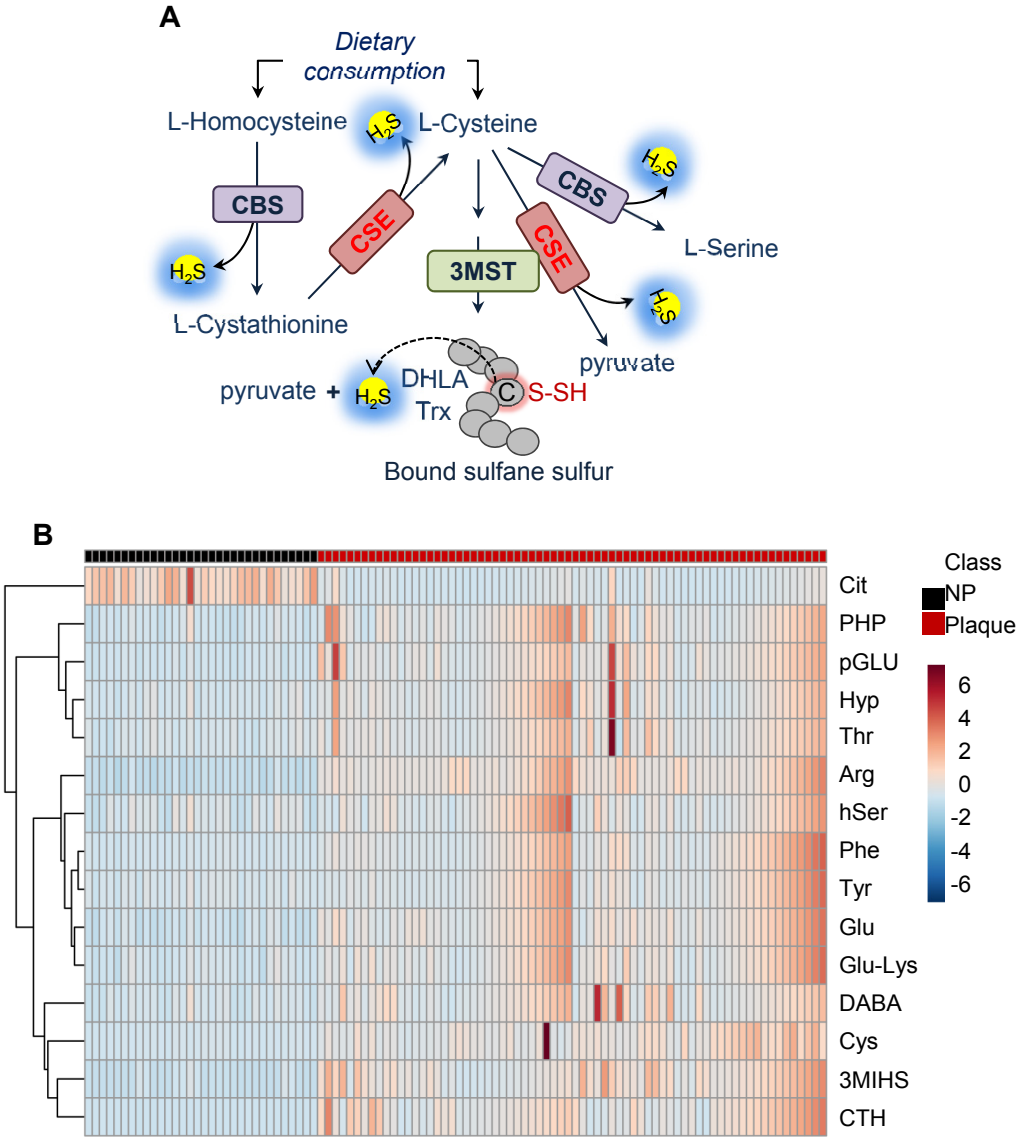
Supplemental Figure 1. **Effect of shear stress on CBS and 3MST levels and effect of endothelial CSE deletion in H_2S production. A**| CBS and 3MST levels in cultured endothelial cells exposed to fluid shear stress (12 dynes/cm^2) for up to 48 hours. Representative Western blot of additional 5 independent experiments of human endothelial cells (ANOVA, Newman-Keuls). **B**| CSE (red), CD31 (green) and α-smooth muscle actin (SMA; cyan) in cross sections of aortae from wild-type and CSE$^{i\Delta EC}$ mice; bar = 20 μm. Comparable images were obtained in samples from 5 additional animals per genotype. **C**| Expression of CSE in (upper panel), and generation of H_2S by (lower panel) endothelial cells from wild-type (WT) and CSE$^{i\Delta EC}$ (iΔEC) mice, n=6 animals per group (Student's t test). ***P<0.001.



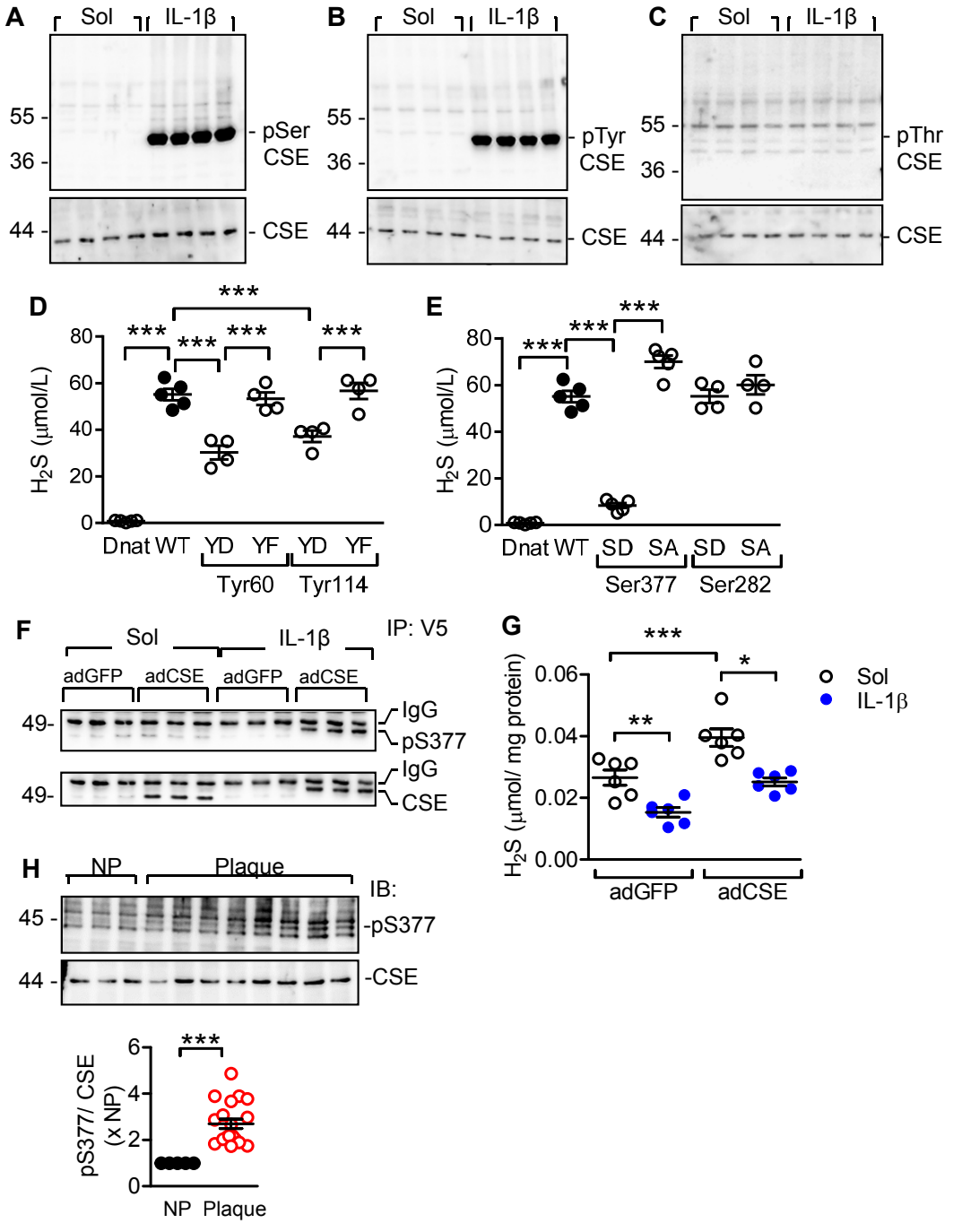
Supplemental Figure 2. **Consequences of CSE deletion on monocyte adhesion. A**| Pulmonary endothelial cells were isolated from wild-type (WT) and CSE$^{i\Delta EC}$ (iΔEC) mice and stimulated with solvent or IL-1β (30 ng/ml) for 3 hours. mRNA levels of ICAM1, VCAM1 and CD62E in cells treated with Sol or IL-1β (30 ng/ml, 3 hours). The graphs summarize n=6-9 experiments from 6 different batches of endothelial cells (ANOVA, Newman-Keuls). **B**| *en face* staining of CD62E (red) and CD144 (green) in descending aortae from wild-type and CSE$^{i\Delta EC}$ mice. Similar results were obtained using 5 additional animals per group. **C**| Surface expression of CD62E in cultured endothelial cells from wild-type and CSE$^{i\Delta EC}$ mice, n=6 different cell batches (2 way ANOVA , Bonferonni). **D**| Adherence of THP-1 monocytes to endothelial cells from wild-type (WT) and CSE$^{i\Delta EC}$ (iΔEC) mice treated with solvent (Sol) or or IL-1β (30 ng/ml) for 3 hours. n=6-9 experiments from 6 different batches of endothelial cells (ANOVA, Newman-Keuls). **E**| Adherence of monocytes to endothelial cells from wild-type (WT) and CSE$^{i\Delta EC}$ (iΔEC) mice in the absence and presence of a CD62E neutralizing antibody, n=6 experiments each performed in triplicate or quadruplicate using 6 different cell batches (2 way ANOVA , Bonferonni). **F**| CD62E expression in endothelial cells from wild-type (WT) and CSE$^{i\Delta EC}$ (iΔEC) mice after transduction with adenoviruses encoding GFP (adGFP) or CSE (adCSE), n=6 experiments using 6 different endothelial cell batches (ANOVA, Newman-Keuls). **G**| Monocyte adherence to endothelial cells from wild-type (WT) and CSE$^{i\Delta EC}$ (iΔEC) mice following transduction with adGFP or adCSE. Adherence on fibronectin (Fibro) was included as a negative control, n=6 experiments (each in duplicate or triplicate) using 6 different endothelial cell batches (ANOVA, Newman-Keuls). **H**| Adherence of monocytes to endothelial cells from wild-type (WT) and CSE$^{i\Delta EC}$ (iΔEC) mice and treated with solvent or IL-1β (30 ng/ml, 3 hours). Experiments were performed in the presence of L-sepiapterin (10 μmol/L) and in the absence (Basal) and presence of L-NAME (+LN; 300 μmol/L); n=6 experiments using 3 different endothelial cell batches (2 way ANOVA, Bonferonni). $^{**}P<0.01$, $^{***}P<0.001$.



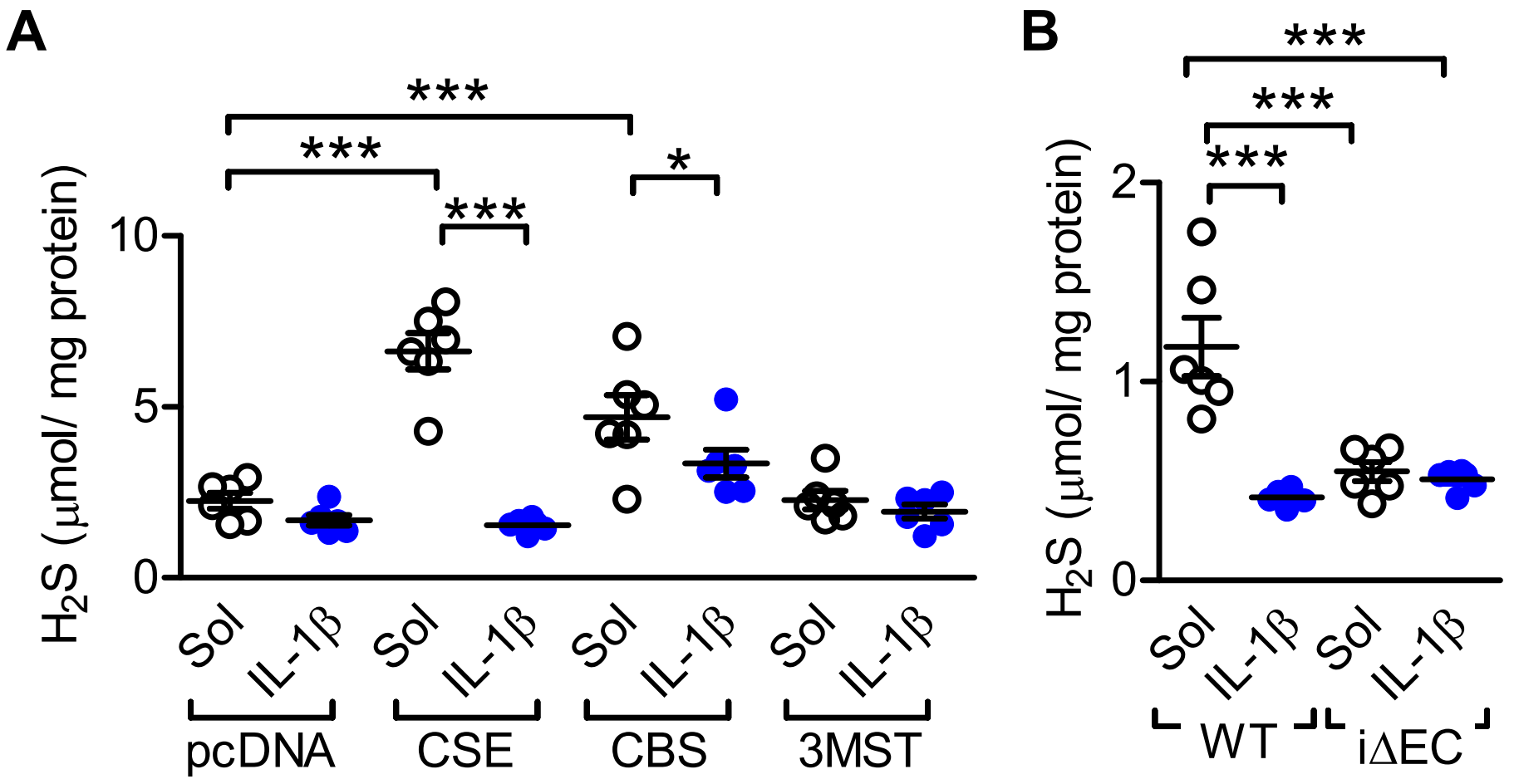
Supplemental Figure 3. Identification of the CSE interactome and link to CD62E mRNA stability. **A**| Volcano plot showing proteins co-precipitated with CSE (Cth); Elavl1= HuR. **B**| CD62E mRNA levels in wild-type (WT) and CSE$^{i\Delta EC}$ (iΔEC) endothelial cells following incubation with actinomycin D (1µg/ml), n=6 experiments using 4-6 different cell batches (2 way ANOVA, Bonferonni). ***P*<0.01.



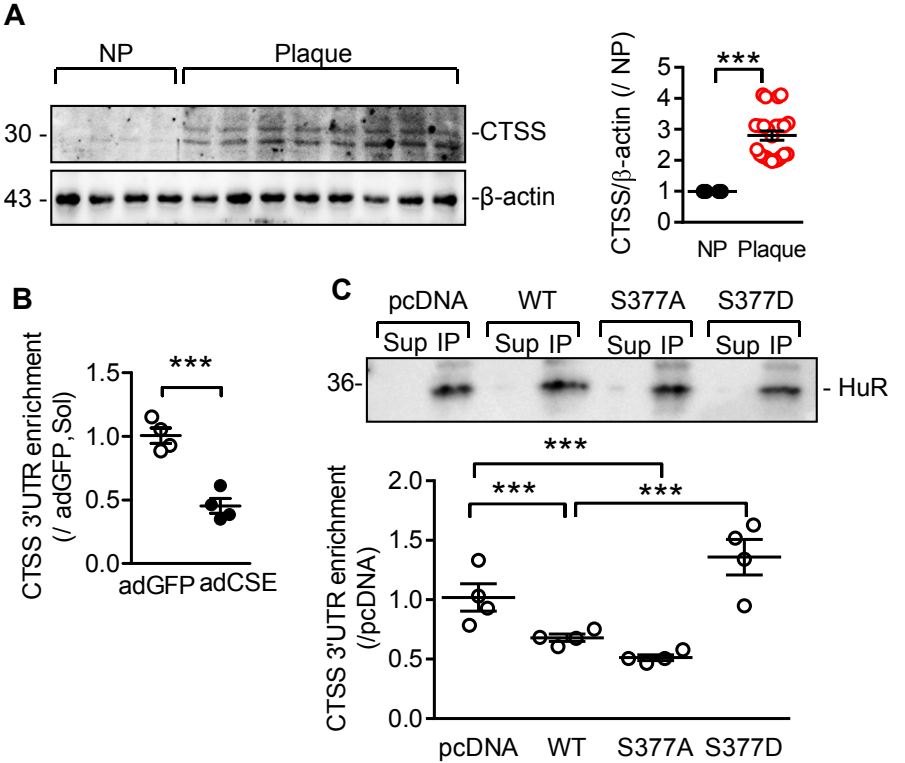
Supplemental Figure 4. **CSE substrate bioavailability in human samples. A**| L-Cysteine metabolism in mammalian cells. CBS catalyzes β-replacement of homocysteine and L-cysteine to produce L-cystathionine, H_2S and L-serine. CSE catalyzes the hydrolysis of L-cystathionine and L-cysteine. 3-MST produces bound sulfane sulfur from 3-mercaptopyruvate, which is generated from L-cysteine by aminotransferase (CAT). Thioredoxin (Trx) and dihydrolipoic acid (DHLA) are endogenous reducing cofactors that facilitate the release of H_2S from 3MST. **B**| Hierarchical clustering analyses of the top 15 plasma amino acids from the human cohort studied. The color key indicates the average fold change, blue: lowest; red: highest, n=102 plasma samples.



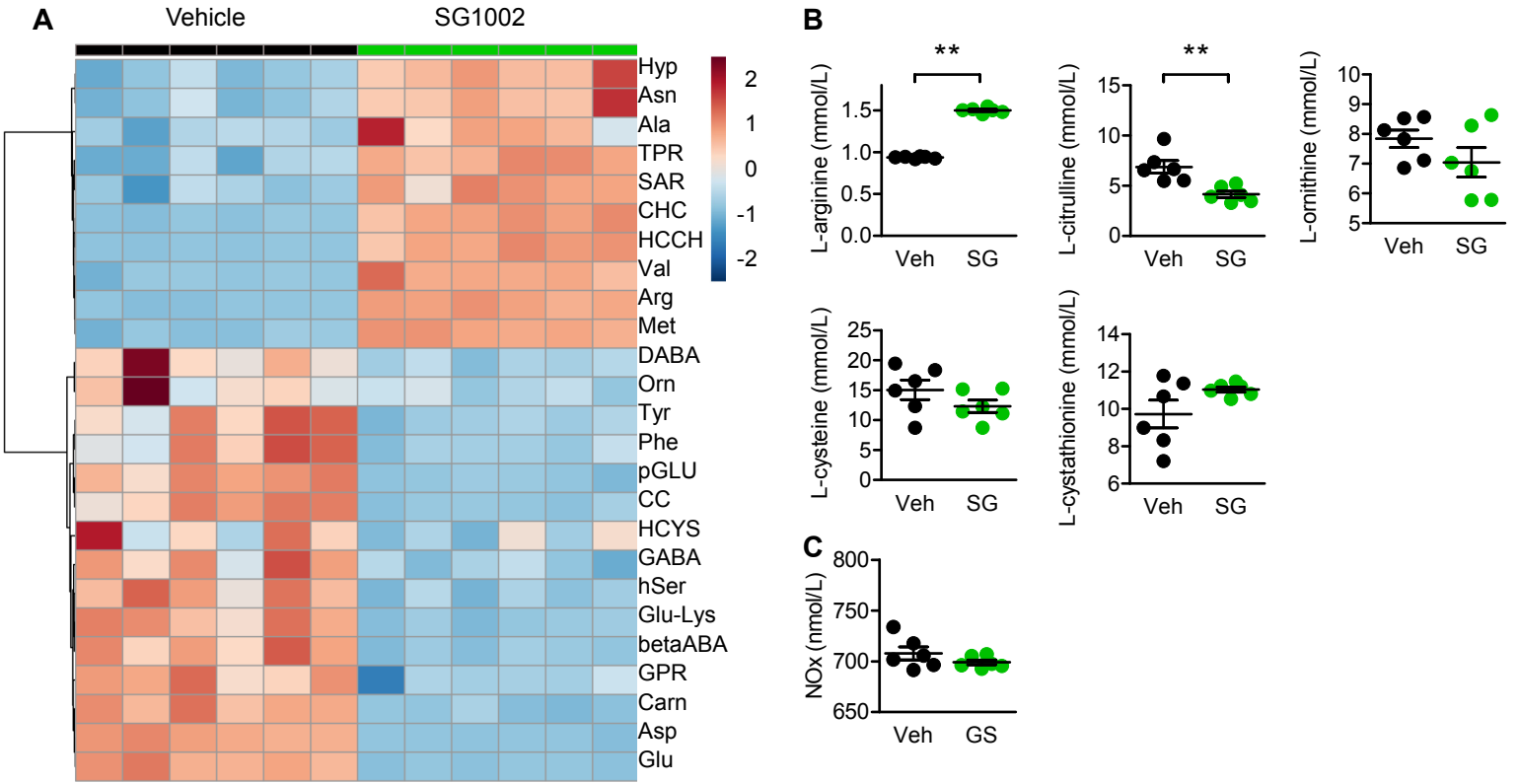
Supplemental Figure 5. **CSE phosphorylation on Ser377 and its consequences on CSE activity during inflammation. A-C**| Human endothelial cells were treated with solvent or IL-1β (30 ng/ml) for 18 hours. CSE was immunoprecipiated and immunobloting was performed using antibodies directed against phospho-serine (A), phospho-tyrosine (B) and phospho-threonine (C). The results are representative of experiments performed using 4 different cell batches. **D-E**| H_2S production *in vitro* by the wild-type CSE as well as the Tyr60 and Tyr144 mutants (D) and Ser377 and Ser282 mutants (E) assessed using the methylene blue assay. A denaturated wild-type CSE (Dnat) was used as negative control, n=4-5 independent experiments (ANOVA, Newman-Keuls). **F**| CSE phosphorylation on Ser377 in human endothelial cells transduced with adGFP or adCSE and treated with solvent (Sol) or IL-1β, n=6 different cell batches. **G**| H_2S production by the cells shown in panel h , n=6 independent cell batches (ANOVA, Neuman-Keuls). **H**| CSE phosphorylation on Ser377 in samples from non-plaque ateries (NP; n=5) versus atherosclerotic plaques (n=20; Mann Whitney). *P<0.05, **P<0.01, ***P<0.001.



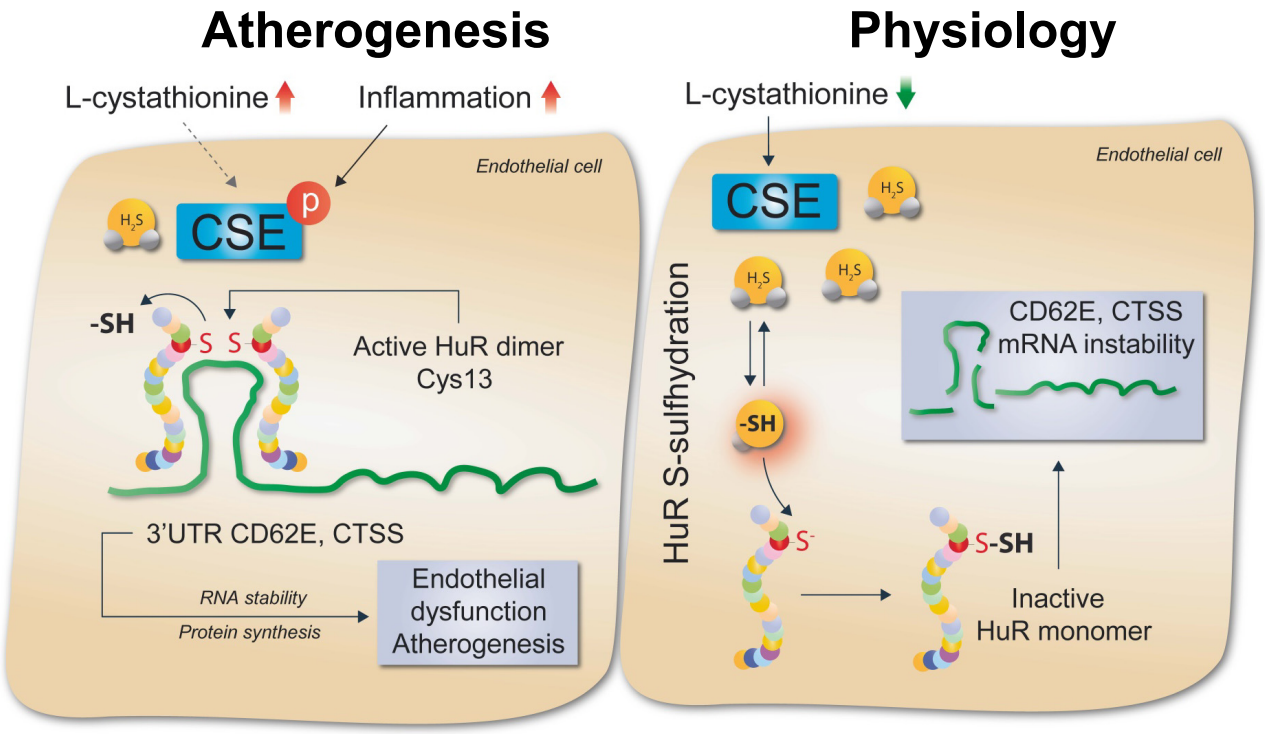
Supplemental Figure 6. **Effects of inflammation on CBS and 3MST. A|** Effect of IL-1β (30 ng/ml, 18 hours) on H_2S production by HEK-293 cells transfected with an empty vector (pcDNA), CSE, CBS or 3MST, n=5 independent experiments, (ANOVA; Newman-Keuls). **B|** Effect of IL-1β (IL, 30 ng/ml, 18 hours) on H_2S production by endothelial cells from wild-type (WT) and $CSE^{i\Delta EC}$ mice; n=6 independent experiments each using a different cell batch (ANOVA; Newman-Keuls). *P<0.05, ***P<0.001.



Supplemental Figure 7. Link between HuR sulfhydration in human plaques and CTSS levels and link between CSE activity and HuR RNA binding capacity. A| CTSS expression in non-plaque (NP) material as well as in atherosclerotic plaques from human subjects. **B|** Levels of *CTSS* 3'UTR RNA co-precipitated with HuR from human endothelial cells, n=4 different cell batches (Students's t test). **C|** CTSS RNA immunoprecipitated from HEK cells transfected with an empty vector or CSE wild type and mutant plasmids, n=4 experiments using 4 different cell batches (ANOVA, Newman-Keuls). The blots demonstrate the equivalent immunoprecipitation (IP) of HuR and are representative of 3 additional experiments. Sup = supernatant after IP. ***P<0.001.



Supplemental Figure 8. Effect of SG1002 on circulating amino acid and nitrite levels. A| Hierarchical clustering analyses of (the top 25) amino acids in plasma from ApoExCSE$^{\Delta EC}$ mice after carotid ligation and treated with vehicle (Veh) or SG1002 (SG) for 21 days. The colour key indicates metabolite average fold change, blue: lowest; red: highest. **B|** Circulating levels of L-arginine, L-citrulline, L-ornithine, L-cystathionine and L-cysteine, n=6 animals per group (Mann Whitney). **C|** Nitrite levels (NOx, as a readout of NO availability) in plasma from the same mice studied in panel B. **P<0.01



Supplemental Figure 9. Graphical abstract. In physiological conditions circulating L-cystathionine is metabolized by cystathionine gamma lyase (CSE) to generate intracellular H_2S. At physiological pH more than 80% of H_2S is in its ionic form (HS^-) which attacks the highly nucleophilic Cys13 of the RNA binding protein HuR to preserve its monomeric form and maintain its activity low, leading to increased instability of the target mRNAs i.e. *CD62E* and *CTSS*. This results in the preservation of endothelial function in atheroprone regions. However, in the presence of excessive circulating pro-inflammatory cytokines, CSE is phosphorylated on S377 and inhibited. L-cystathionine is elevated in the circulation, whilst intracellular and circulating H_2S levels are rapidly reduced. The absence of H_2S and subsequently HS^- allows the formation of the disulfide bond in Cys13 and homodimeritation of HuR. The HuR dimer exerts increase activity and elevated capacity to bind to the target 3´UTRs leading to increased stability of the *CD62E* and *CTSS* mRNAs, elevated protein synthesis and further development of endothelial dysfunction coupled with an enhanced and accelerated pro-atherogenic phenotype.