



## Supporting Online Material for

### **H<sub>2</sub>S as a Physiologic Vasorelaxant: Hypertension in Mice with Deletion of Cystathionine $\gamma$ -Lyase**

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Figs. S1 to S6  
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## Supplementary Methods

### Targeted disruption of the CSE gene and generation of CSE knockout mice

A conventional mouse CSE gene knockout procedure was performed with assistance from inGenious Targeting Laboratory, Inc. (Stonybrook, NY). A Bacterial Artificial Chromosome (BAC) Library containing a 129SvEv mouse strain genomic DNA was screened with a CSE gene probe (Genbank accession number NT\_078406). A ~14.6 kb region containing CSE 5' untranslated region to exon 4 was obtained from positively identified BAC clone and subcloned into backbone of a homemade gene targeting vector. To introduce a neomycin resistance gene ( $neo^f$ ) cassette that flanks CSE exon 1 (including the ATG start codon), exon 2, and exon 3, a selection cassette flanked by *BsiWI* endonuclease sites was firstly inserted by homologous recombination (1). The selection cassette was then removed by cutting with *BsiWI* and the  $neo^f$  cassette digested with *KpnI* was ligated in antisense orientation to the CSE targeting vector. The CSE 5' untranslated region served as the long homology arm which fused with 3' end of the  $neo^f$  cassette. The CSE gene sequence between exon 3 and exon 4 served as the short homology arm, which fused its 5' end with 5' end of the antisensely oriented  $neo^f$  cassette. The vector construct was confirmed with both restriction analyses (*BamHI* and/or *HindIII*) and bi-directional DNA sequencing using T7 (5'-ATTATGCTGAGTGATATCCCTCT-3'), N7 (5'-ATGTGTCAGTTTCATAGCCTGAAG-3') and N1 (5'-TGC GAGGCCAGAGGCCAGTTGTGTAGC-3'), P6 (5'-ATTTAGGTGACACTATAGAAC TC-3') sequencing primers. CSE gene targeting vector was linearized with an appropriate endonuclease and transfected into the 129SvEv-derived embryonic stem (ES) cells with electroporation. ES cells were then selected with G418 (300  $\mu\text{g ml}^{-1}$ ) for two

weeks. About 300 G418 resistant cell clones were subjected to a PCR base screening using N1/A1 primers (A1: 5'-TCAGTCCAAATTCAGATGCCACCC-3'). Positive cell clones were expanded to microinject into C57BL/6J mice blastocysts, and then planted back to a surrogate mother. Two litters of chimeras carrying disrupted CSE were estimated by coat colors (i.e. C57BL/6J is black and 129SvEv is agouti in color). The chimeras were chosen for mating to generate F1. Two males and 8 females were identified as heterozygous CSE knockout mice ( $CSE^{-/+}$ ) and they were set up to generate F2 mice. Unless otherwise stated, the second generation of male homozygous and heterozygous CSE knockout ( $CSE^{-/-}$  and  $CSE^{-/+}$ ) offspring and age-matched male wild-type littermates ( $CSE^{+/+}$ ) on C57BL/6J  $\times$  129SvEv background were used. All animal experiments were conducted in accordance with approved protocols by the Animal Health Care Committee of the University of Saskatchewan, Canada. All animals were maintained on standard rodent chow, and had free access to food and water.

### **Determination of mouse genotypes**

PCR-genotyping of CSE knockout mice was performed using a three-primer assay in two reactions (2). N1 primer is specific for the targeted allele and F1 primer (5'-TGTTTCATGGTAGGTTTGGCC-3') is for wild-type allele. The reverse primer, R1 (5'-TCAGAACTCGCAGGGTAGAA-3'), lies downstream of the  $neo^r$  insertion of targeted allele or exon 3 of wild-type allele, anneals to both wild-type and targeted alleles. Southern blotting analysis was also used to confirm PCR-genotyping results. A set of primers, 5'-TCTACACCACCCGTTCTCCATTGA-3' and 5'-AGTCCACCTCCAGCCC CATCTAT-3' were first used to clone a 0.74-kb probe, which was sequenced to prove

correct sequence. Mouse genomic DNA was digested with *Bam*HI and hybridized with probe.

### **Quantitative analysis of gene expression**

The mRNA expression levels of CSE were quantitatively analyzed by real-time PCR with an iCycler IQ apparatus (Bio-Rad) as described before (3). The primers for CSE are: 5'-GGGCCAGTCCTCGGGTTTTGAATA-3' and 5'-TAATCGTAATGGTGGCAG CAAGAC-3'.

### **Western immunoblotting**

Mouse tissues were lysed in a lysis buffer (0.5 mol/L EDTA; 1 mol L<sup>-1</sup> Tris-Cl, pH 7.4; 0.3 mol L<sup>-1</sup> sucrose; 1 µg ml<sup>-1</sup> antipain hydrochloride; 1 mmol L<sup>-1</sup> benzamidine hydrochloride hydrate; 1 µg ml<sup>-1</sup> leupeptin hemisulfate; 1 mmol L<sup>-1</sup> 1,10-phenanthroline monohydrate; 1 µmol L<sup>-1</sup> pepstatin A; 0.1 mmol L<sup>-1</sup> plenylmethylsulfonyl fluoride, and 1 mmol L<sup>-1</sup> iodoacetamide). The extracts were separated by centrifugation at 14,000 ×g for 15 min at 4°C. SDS-PAGE and Western blot analysis were performed as described previously (4). Briefly, equal amounts of proteins were boiled in 1× SDS sample buffer (62.5 mmol L<sup>-1</sup> Tris-Cl, pH6.8; 2% SDS; 10% glycerol; 50 mmol L<sup>-1</sup> DTT; and 0.01% bromophenol blue) and resolved on a 10% SDS-PAGE gel, and transferred onto the PVDC nitrocellulose membrane. The primary antibodies were diluted at 1:1000 for CSE (5) and 1:5000 for β-actin. HRP-conjugated secondary antibody was used at 1:5000. Immunoreactions were visualized by ECL and exposed to X-ray film (Kodak Scientific Imaging film). Membranes were stripped by incubating in a buffer containing 100 mmol L<sup>-1</sup> β-mercaptoethanol, 2% SDS, and 62.5 mmol L<sup>-1</sup> Tris-HCl (pH 6.8).

## **Immunohistochemistry**

Polyclonal antibody against bacterially expressed full-length mouse CSE was raised in rabbits. Specificity was shown by Western blot analysis in many tissues from CSE<sup>+/+</sup> and CSE<sup>-/-</sup> mice including the heart, aorta, mesenteric artery, liver and kidneys. 8-10 week CSE<sup>+/+</sup> and CSE<sup>-/-</sup> mice were used to obtain cross sections of the aorta and mesenteric arteries using a cryostat. Sections were fixed by immersion in 4% paraformaldehyde and incubated with antisera to CSE for 12 h at 4°C. Staining was developed with an immunoperoxidase ABC Elite kit (Vector Laboratories) by using 3,3'-diaminobenzidine as substrate.

## **Chemicals**

H<sub>2</sub>S stock solution or the stock solution of NaHS as precursor of H<sub>2</sub>S were used (6). Calcium ionophore A23187, calmodulin antagonist N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) and calmidazolium chloride (CC), NaSH, calmodulin and all other chemicals were purchased from Sigma (Oakville, Ontario). Unless otherwise specified, calcium concentration of the culture medium was 2 mM.

## **Protein Purification and co-immunoprecipitation**

Mouse full-length CSE DNA was subcloned into pET-28c+ His vectors (Novagen, San Diego, CA), which encodes a 6x-histidine tag and introduced into BL21 codon plus bacteria (Stratagene). Expression of CSE was induced by 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (Sigma) for 12 h at 30°C. Cells were collected by centrifugation and disrupted by sonication in medium containing 20 mM Tris-HCl (pH 7.4), 15 μM PLP, 10 mM imidazole and 400 mM NaCl. After addition of 1% Triton X-100, the suspension was cleared by centrifugation (40,000 x g for 15 min), and CSE was purified from the

supernatant by binding to Talon resin (Clontech) according to the manufacturer's instructions.

For immunoprecipitation experiments, purified recombinant CSE was incubated with purified calmodulin from Sigma (St. Louis, MO) with and without calcium, EGTA and W7 overnight at 4°C. Following which, TALON resin was added for 1 h and the beads then washed three times with buffer containing 20 mM Tris pH 7.4, 150 mM NaCl and 0.1% Triton-X 100. LDS loading buffer was then added and a western blot run for CSE and calmodulin using antibody to calmodulin purchased from Upstate (Lake Placid, NY).

### **Cell culture**

Bovine aortic endothelial cells (BAECs) and human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. For the determination of H<sub>2</sub>S production, cells were seeded in 48-well plates with equal amount of cells in each well and cultured until confluence.

### **Measurement of blood pressure, H<sub>2</sub>S, L-cysteine, homocysteine and oxobutanoate**

Systolic blood pressure of mice was measured using a standard tail-cuff noninvasive measurement system or intra-arterial catheterization method as described previously (6, 7). Standard protocols were employed to measure H<sub>2</sub>S, plasma L-cysteine (6), homocysteine (8) and oxobutanoate (9). In particular, H<sub>2</sub>S production was measured using an ion-selective electrode (Lazar Research Laboratories, Los Angeles, CA) on a Fisher Accumet Model 10 pH meter (Fisher Scientific, Pittsburgh, PA) following the modified manufacturer's directions. Standards were prepared from NaHS solution.

### **Myograph measurement of vascular tension development**

The third order division segments (0.5-2.0 mm in length) of mesenteric arteries from 10-13 week CSE knockout mice and their control wild-type littermates were used for endothelial function study as described before (10). Endothelium removal was performed as described before (11).

### **Superoxide anion levels in samples**

Superoxide anion levels were measured as described before (12).

### **Glutathione (GSH) levels in samples**

GSH levels were measured as described before (13).

### **Statistical Analysis**

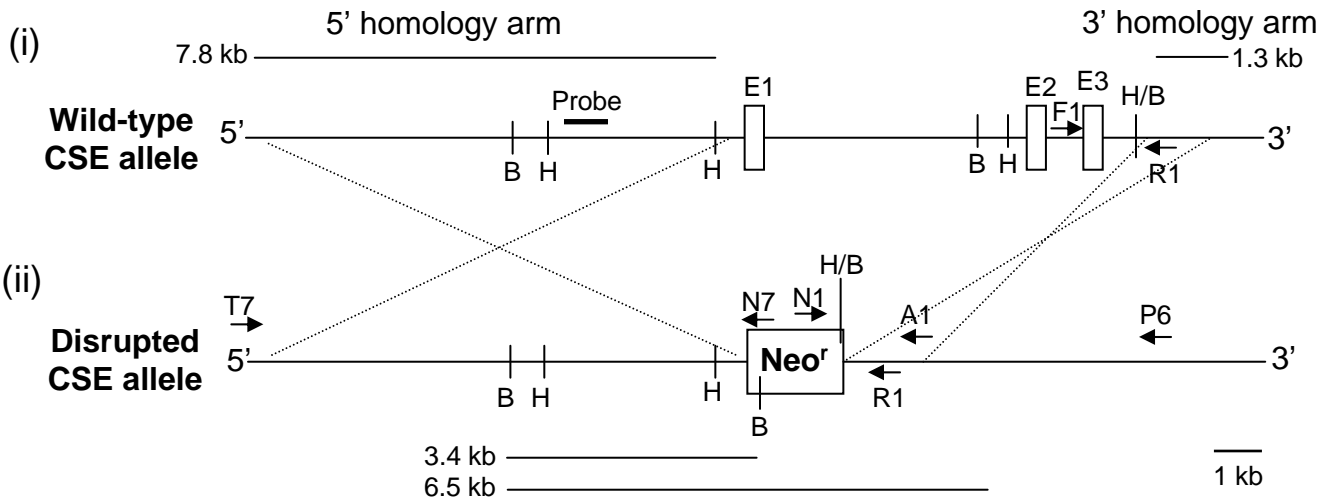
All data are expressed as means  $\pm$  SEM from at least three independent experiments performed in duplicate except as otherwise stated. Where appropriate, statistical analyses employed an unpaired Student's *t*-test or analysis of variance in conjunction with the Newman-Keuls test.

### **References**

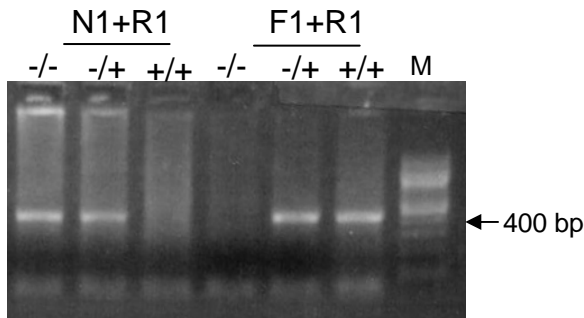
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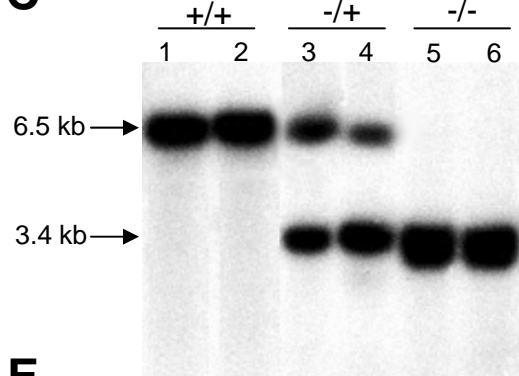
## A



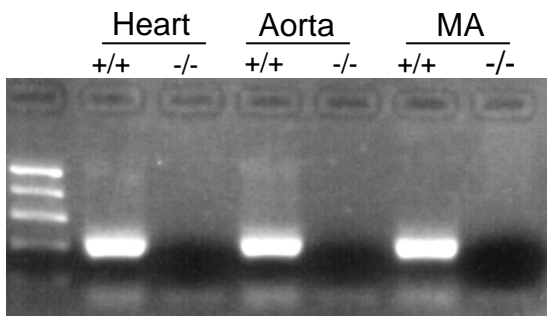
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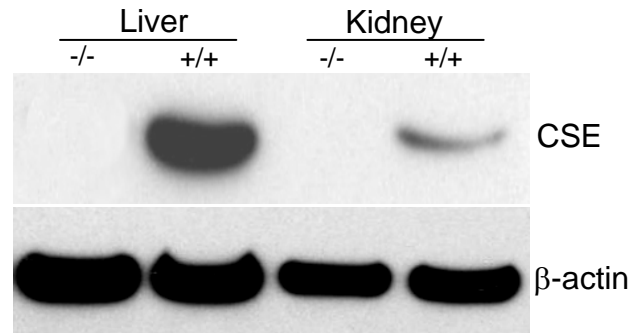
## C



## D

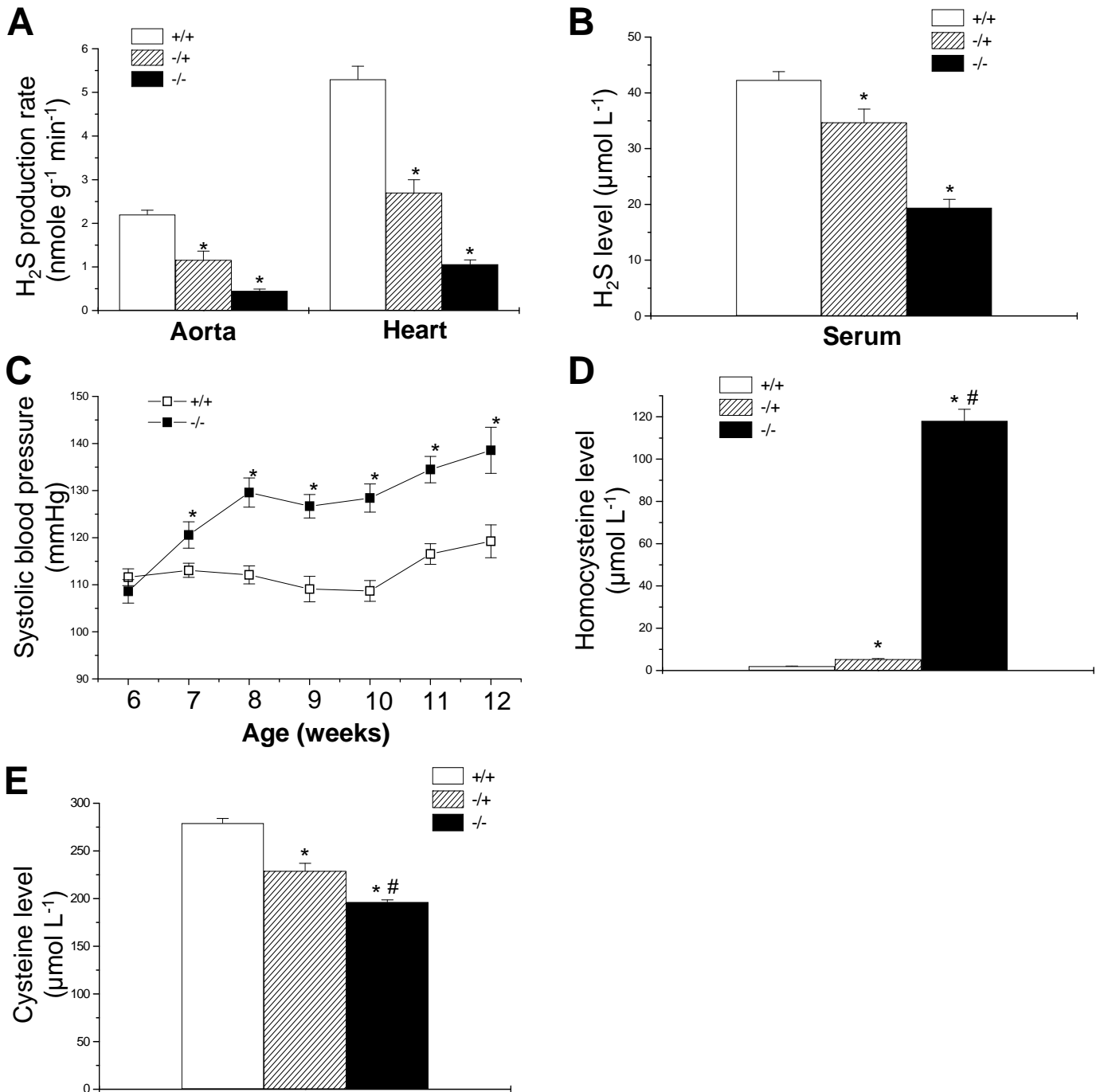


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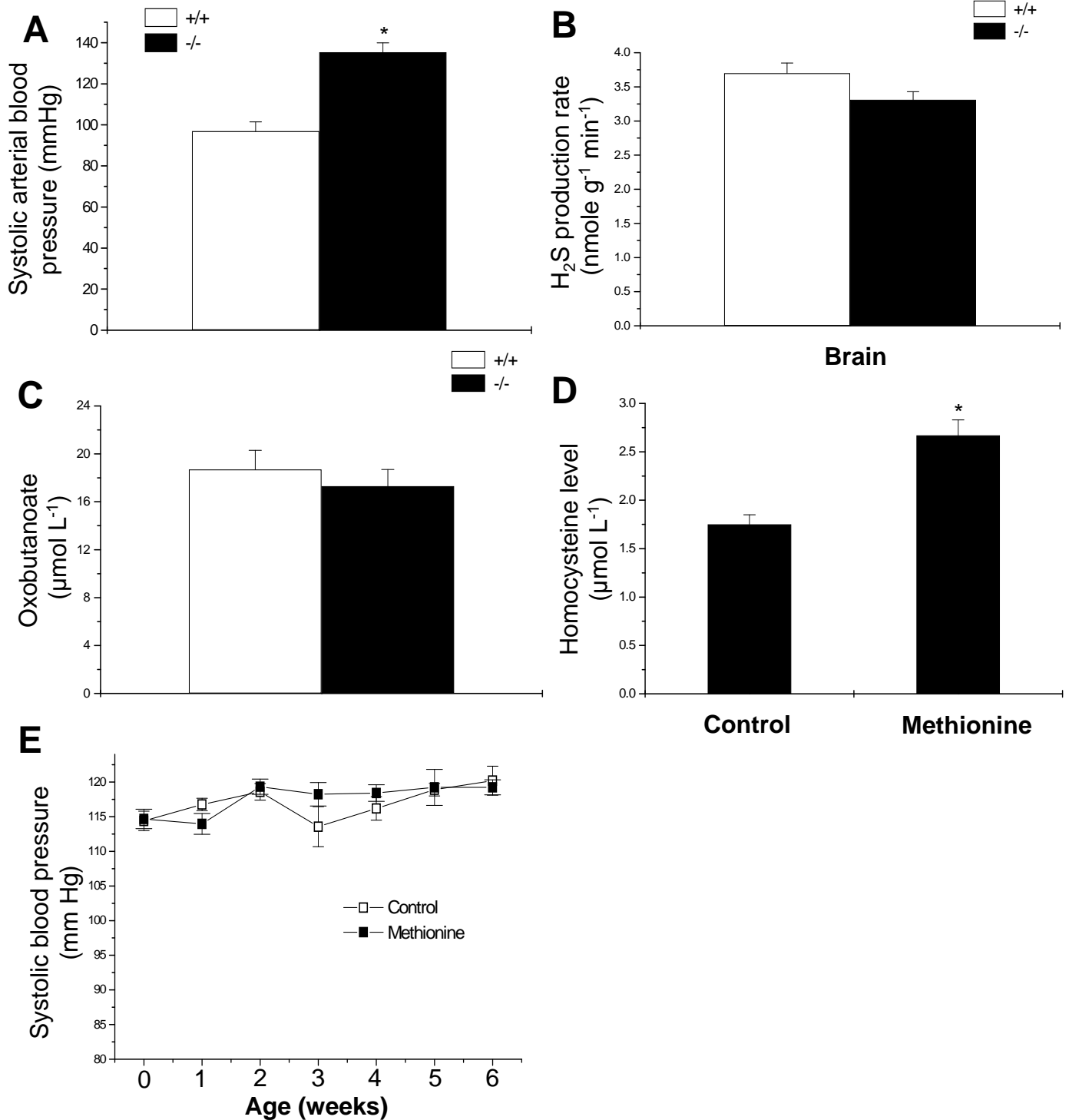
**Targeted disruption of the CSE gene and analysis of expression. (A)** Gene targeting strategy. (i) Wild-type genomic structure of CSE gene with exons 1-3; (ii) Expected structure of the mutant allele made by replacing 5.5-kb of exons 1-3 with a 1.8-kb a neomycin resistance gene (*neor*) cassette by homologous recombination. E1, E2 and E3 are exon 1, exon 2 and exon 3. T7, N7, P6, F1, R1, and N1 indicate the position of primers used in sequencing and genotyping. B, *Bam*HI; H, *Hind*III. The horizontal bars show *Bam*HI restriction fragments of wild type and mutant alleles detected by probe. **(B)** Genotyping of CSE<sup>-/-</sup> mice by PCR using three primers in two reactions. The expected sizes of wild-type and mutant alleles are shown. +/+, wild-type; +/-, heterozygote; -/-, homozygote. M, DNA marker. **(C)** Southern blotting analysis of genotyping. Mouse genomic DNA was digested with *Bam*HI and hybridized to a 0.74-kb probe. The positions of hybridizing fragment for the wild-type and the disrupted CSE gene are shown. Lack of CSE mRNA **(D)** and proteins **(E)** expression in CSE<sup>-/-</sup> mice. MA, mesenteric artery.

# Supplementary Figure 2

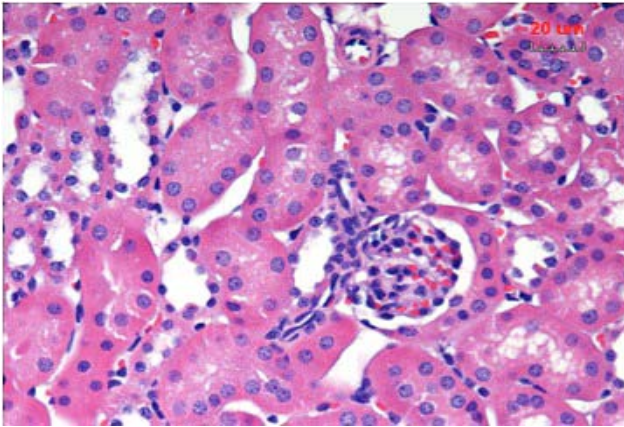
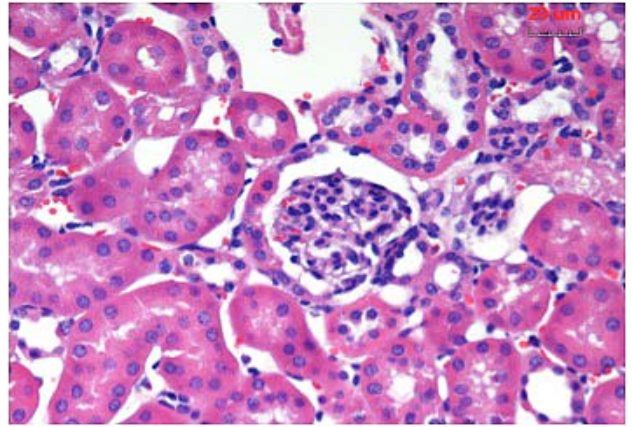


**Phenotype of CSE female knockout mice.** (A) Reduced H<sub>2</sub>S production from aorta and heart tissues in female CSE<sup>-/-</sup> mice and CSE<sup>+/-</sup> mice. Sixteen mice for each group. (B) Reduced serum H<sub>2</sub>S level in female CSE<sup>-/-</sup> mice and CSE<sup>+/-</sup> mice. Eight to ten mice for each group. (C) Age-dependent increase in blood pressure of female CSE<sup>-/-</sup> mice. Fifteen mice for each group. (D) Increased plasma homocysteine level in female CSE<sup>-/-</sup> mice and CSE<sup>+/-</sup> mice. Twelve mice for each group. (E) Decreased plasma L-cysteine level in female CSE<sup>-/-</sup> mice and CSE<sup>+/-</sup> mice. Ten mice for each group. +/+, wild-type; +/-, heterozygote; -/-, homozygote. All results are mean ± s.e.m. \*, p<0.05 versus wild-type; #, p<0.05 versus heterozygote.

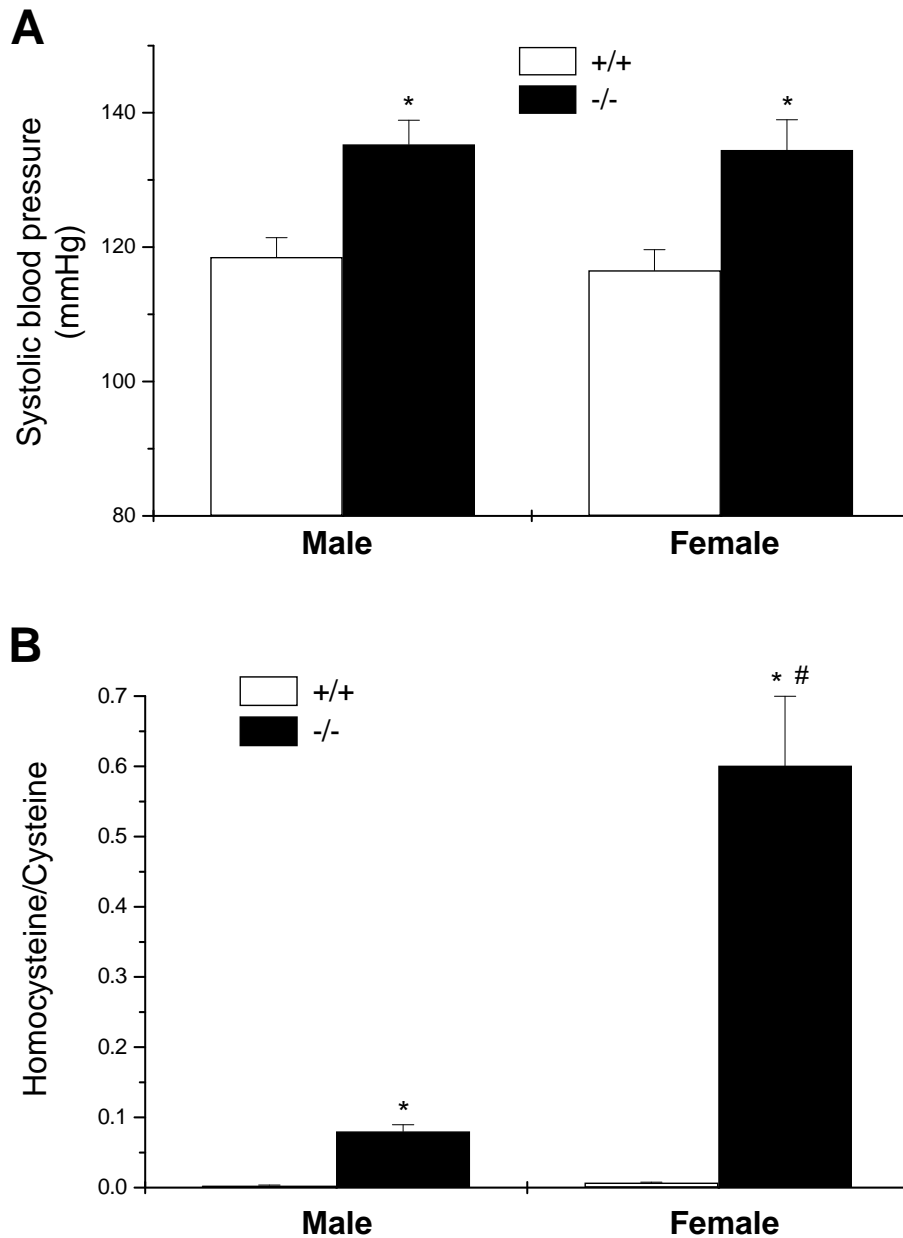
# Supplementary Figure 3



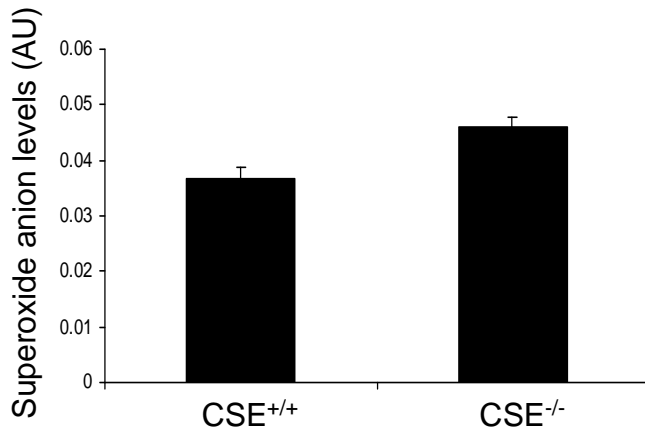
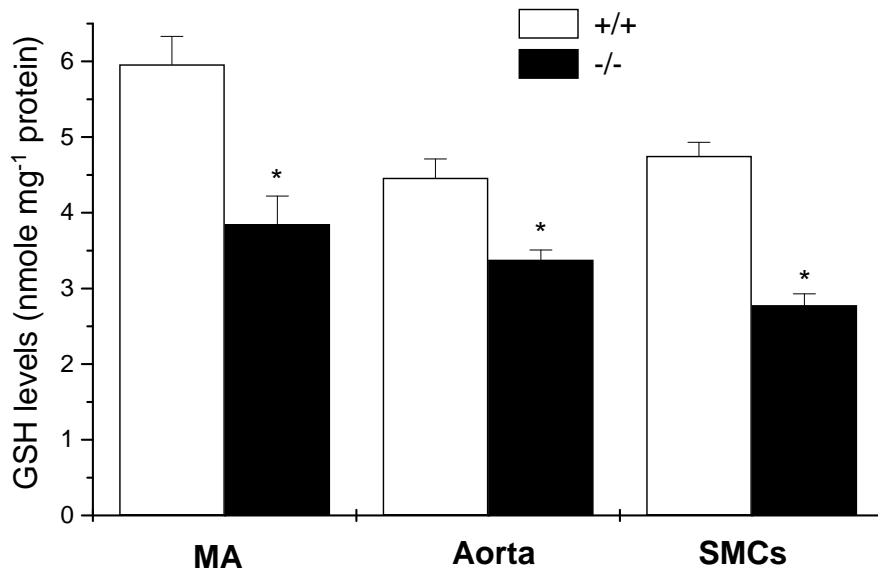
**Direct increases in CSE<sup>-/-</sup> blood pressure is not a result of brain H<sub>2</sub>S or two important members of the CSE pathway, oxobutanoate and methionine.** (A) The increased arterial blood pressure in 12-week CSE<sup>-/-</sup> mice was directly monitored through intra-carotid artery catheterization. Eight mice for each group. (B) No change of endogenous H<sub>2</sub>S levels from brains of CSE<sup>-/-</sup> mice and CSE<sup>+/+</sup> mice. Six mice for each group. (C) Plasma oxobutanoate levels are the same in CSE<sup>-/-</sup> and CSE<sup>+/+</sup> mice. Six mice for each group. (D) L-methionine (0.5% in drinking water for 6 weeks) increases plasma homocysteine levels in CSE<sup>+/+</sup> mice.. Five mice for each group. (E) Methionine feeding fails to alter blood pressure of CSE<sup>+/+</sup> mice. Five mice for each group. All results are mean  $\pm$  SEM \*,  $p < 0.05$ .

**A****B**

**Examination of kidney tissue sections.** Kidneys were isolated from male CSE+/+ (**A**) and CSE-/- (**B**) mice. Histology tissue sections (5  $\mu\text{m}$ ) from kidney cortex did not reveal significant structural differences between the samples (H&E staining, original magnifications, x 200).



**Change of blood pressure and homocysteine/cysteine ratio in CSE male and female deficient mice. (A)** No difference between male and female CSE<sup>-/-</sup> mice blood pressure at 12 weeks. Fifteen mice for each group. **(B)** Higher homocysteine/cysteine ratio in female CSE<sup>-/-</sup> mice at 10-12 weeks. Ten to twelve mice for each group. +/+, wild-type; -/-, homozygote. All results are mean  $\pm$  s.e.m. \*,  $p < 0.05$  versus wild-type; #,  $p < 0.05$  versus male homozygote.

**A****B**

**Changes in vascular redox state in CSE deficient mice. (A)** Superoxide anion levels detected by fluorescence probe dihydroethidium (DHE) did not change significantly between CSE<sup>+/+</sup> and CSE<sup>-/-</sup> SMCs. n=3. **(B)** Decreased glutathione (GSH) levels in the vasculature of CSE<sup>-/-</sup> mice. GSH levels in mesenteric artery (MA), aorta, and SMCs of mesenteric arteries isolated from CSE<sup>+/+</sup> as well as CSE<sup>-/-</sup> mice were determined using HPLC. n=5 for each group. \* p<0.05.