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ORIGINAL RESEARCH

Endothelial Cell Cystathionine γ-Lyase Expression Level Modulates Exercise Capacity, Vascular Function, and Myocardial Ischemia Reperfusion Injury

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BACKGROUND: Hydrogen sulfide (H_2S) is an important endogenous physiological signaling molecule and exerts protective properties in the cardiovascular system. Cystathionine γ-lyase (CSE), 1 of 3 H_2S producing enzyme, is predominantly localized in the vascular endothelium. However, the regulation of CSE in vascular endothelium remains incompletely understood.

METHODS AND RESULTS: We generated inducible endothelial cell-specific CSE overexpressed transgenic mice (EC-CSE Tg) and endothelial cell-specific CSE knockout mice (EC-CSE KO), and investigated vascular function in isolated thoracic aorta, treadmill exercise capacity, and myocardial injury following ischemia-reperfusion in these mice. Overexpression of CSE in endothelial cells resulted in increased circulating and myocardial H_2S and NO, augmented endothelial-dependent vasorelaxation response in thoracic aorta, improved exercise capacity, and reduced myocardial-reperfusion injury. In contrast, genetic deletion of CSE in endothelial cells led to decreased circulating H_2S and cardiac NO production, impaired endothelial dependent vasorelaxation response and reduced exercise capacity. However, myocardial-reperfusion injury was not affected by genetic deletion of endothelial cell CSE.

CONCLUSIONS: CSE-derived H_2S production in endothelial cells is critical in maintaining endothelial function, exercise capacity, and protecting against myocardial ischemia/reperfusion injury. Our data suggest that the endothelial NO synthase—NO pathway is likely involved in the beneficial effects of overexpression of CSE in the endothelium.

Key Words: cardioprotection ■ cystathionine γ-lyase ■ endothelial function ■ hydrogen sulfide ■ nitric oxide

Hydrogen sulfide (H_2S), a highly toxic gas, is now recognized as an important endogenous physiological signaling molecule. At physiological levels, H_2S exerts many protective properties in the cardiovascular system, including vasodilation, antioxidant, and anti-inflammation.[1–4]

The endogenous production of H_2S is catalyzed by 3 enzymes: cystathionine β-synthase, cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase.[5] While, all 3 enzymes have been found in the cardiovascular system, CSE is believed to be expressed primarily in the heart and vasculature and responsible for the majority endogenous H_2S production in the cardiovascular system.[6] Therefore, it appeared that CSE is a critical enzyme that regulates H_2S generation, and ultimately modulates cardiovascular function and health. Indeed, decreased CSE expression results in a global decrease

CLINICAL PERSPECTIVE

What Is New?
- Cystathionine γ-lyase is a critical enzyme responsible for generating hydrogen sulfide in endothelial cells.
- Hydrogen sulfide production in endothelial cells plays important role in maintaining cardiovascular function and protecting against myocardial ischemia/reperfusion injury.

What Are the Clinical Implications?
- Modulation of endogenous endothelial hydrogen sulfide production could be a potential therapeutic strategy in the treatment of cardiovascular disease.

Nonstandard Abbreviations and Acronyms

CSE	cystathionine γ-lyase
EC-CSE KO	endothelial cell-specific CSE knockout
EC-CSE Tg	endothelial cell-specific CSE overexpressed transgenic
eNOS	endothelial NO synthase
H_2S	hydrogen sulfide
INF	infarct size
tTA	tetracycline-controlled trans-activator

in H_2S bioavailability and is associated with multiple cardiovascular diseases.[7,8] Genetic deletion of CSE globally leads to age-dependent hypertension, impaired vascular relaxation, early development of atherosclerosis, and exacerbated myocardial and renal ischemia/reperfusion injury.[6,9–11] Conversely, genetic overexpression of CSE in cardiomyocytes improves cardiac function and attenuates myocardial infarction following myocardial ischemia reperfusion injury,[12,13] suggesting the important role of CSE-derived H_2S in cardioprotection and cardiovascular homeostasis.

Endothelial cells are a critical component of blood vessels that are responsible for regulating vascular tone and downregulating oxidative stress, inflammation, and smooth muscle cell proliferation.[14] Endothelial dysfunction is strongly associated with cardiovascular diseases including hypertension, peripheral vascular disease, coronary artery disease, and heart failure.[2,15,16] Previous studies have shown that H_2S protects endothelial function through various mechanisms, and augmentation of NO signaling is 1 of the major mechanisms.[2,16] CSE is predominantly localized to the endothelial layer of blood vessels where it has been shown to produce H_2S and mediate smooth muscle relaxation and subsequent vasodilation.[6,17] However, the regulation of CSE in vascular endothelium remains incompletely understood.

The purpose of this study was to investigate the impact of endothelial CSE-derived H_2S on cardiovascular function. We first generated inducible endothelial cell-specific CSE overexpressed transgenic (EC-CSE Tg) mice and investigated the effect of endothelial CSE overexpression on vascular function, exercise and myocardial protection following myocardial-reperfusion injury. We then similarly investigated the effect of endothelial CSE deficiency on vascular function, exercise, and myocardial injury following ischemia-reperfusion using endothelial cell-specific CSE knockout (EC-CSE KO) mice.

METHODS

An expanded methods section is available in Data S1.

Data Sharing
The authors declare that all supporting data are available within the article and its online supplementary files.

Experimental Animals
EC-CSE Tg mice were generated by breeding tetracycline response element (TRE)-CSE mice as shown in Figure 1A with Cdh5-tTA (tetracycline-controlled trans-activator) mice. Tre-CSE was developed using human CSE transgene under the control of a tetracycline operator sequence, which contains a minimal promoter, TRE. Cdh5-tTA mice, which express tTA under the control of Cdh5 promoter, were purchased from Jackson Laboratory. As a result, EC-CSE Tg mice overexpress CSE selectively in endothelial cells.

EC-CSE KO mice were generated by breeding CSE^flox+/fox+ mice[18] with Cdh5-Cre mice. CSE flox mice were developed by GenOway (Lyon, France), where CSE gene was loxp-flanked. The Cdh5-Cre mice (Jackson Laboratory) express Cre recombinase under the control of Cdh5 promoter. By breeding with Cdh5-Cre, the loxp-flanked CSE gene in CSE flox mice was deleted in endothelial cells specifically.

EC-CSE Tg and EC-CSE KO mice and age-matched control littermates (C57Bl/6J, male, 10–12 weeks old) were used in the following studies. Cdh5-tTA mice were used as controls for EC-CSE Tg, and CSE^flox+/flox+ mice were used as controls for EC-CSE KO mice. All experimental protocols were approved by the Institute for Animal Care and Use Committee at Louisiana State University Health Sciences Center-New Orleans
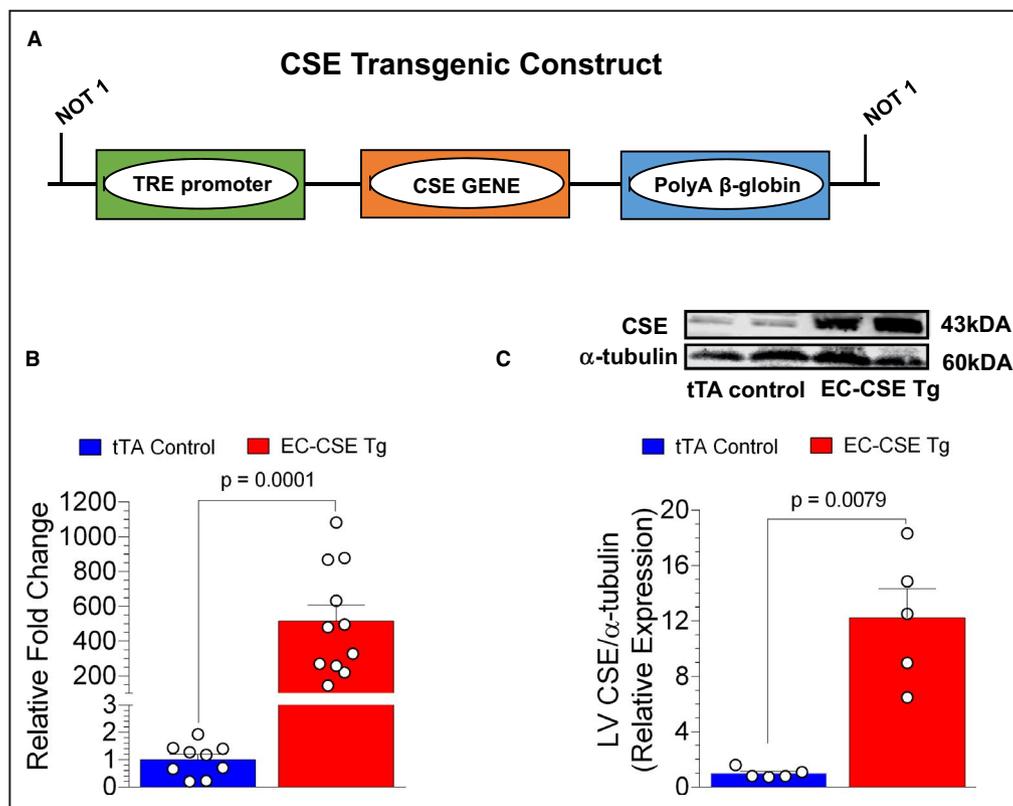
Figure 1. Increased cardiac cystathionine γ-lyase mRNA and protein expression in endothelial cell-specific cystathionine γ-lyase overexpressed transgenic mice.

A, Cystathionine γ-lyase (CSE) transgenic construct. CSE gene is under the control of the tetracycline response element (TRE). In the presence of tTA (tetracycline trans-activator), CSE transgene expression is induced. **B**, Endothelial cell-specific CSE transgenic mice (n=11) had a 500-fold increase in cardiac CSE mRNA expression compared with tTA controls (n=9). **C**, CSE protein expression in the left ventricle was increased by 12-fold in endothelial cell-specific CSE transgenic mice (n=5) compared with tTA controls (n=5), as shown in the immunoblot and calculated optical density. Data are presented as mean±SEM, Student *t*-test (**B**), Mann–Whitney *U* test (**C**). CSE indicates cystathionine γ-lyase; EC-CSE Tg, endothelial cell-specific CSE transgenic mice; LV, left ventricle; TRE, tetracycline response element; and tTA, tetracycline trans-activator.

and conformed to the *Guide for the Care and Use of Laboratory Animals*. All experimental animals received humane care in accordance with National Society of Medical Research and National Institutes of Health tenets.

H₂S, Nitrite, and Cyclic Guanosine Monophosphate Measurements

Plasma and myocardial free H_2S and sulfane sulfur were measured using gas chromatography-chemiluminescence (7890 Series GC, G660XA Series Chemiluminescence Detector; Agilent, Santa Clara, CA, USA), as previously described.[19,20]

Nitrite content of plasma and myocardial specimens were measured by an automated ion chromatography system (ENO30 Analyzer; Eicom San Diego, CA, USA), as previously described.[20,21] Tissue levels of H_2S and nitrite were adjusted based on protein concentration.

Cardiac cyclic guanosine monophosphate (cGMP) levels were measured by radio-immunoassay, as previously described.[9]

Real Time-Polymerase Chain Reaction for mRNA Expression

RNA isolation was performed by using TRIzol reagent (Thermo Fisher 15596018). RNA was transcribed using I-script cDNA synthesis kit (Bio-Rad 1708891). Real-time quantitative polymerase chain reaction were performed on a Thermocycler (StepOnePlus, Applied Biosystem) by using TaqMan Gene Expression assay generated for 18S or CSE (Thermo Fisher 4331182).

Western Blot Analysis

Flash-frozen tissue was homogenized in radio-immunoprecipitation assay buffer supplemented with Halt

protease and phosphatase inhibitor cocktail (Thermo Fisher). Protein concentration was determined using a bicinchoninic acid protein assay (Thermo Fisher). Immunoblotting used specific antibodies for the N terminus of CSE (Proteintech 60231-4-lg) and α-tubulin (Abcam ab7291).

Vascular Reactivity Studies

Vascular function was measured in isolated segments (3 mm in length) of thoracic aorta as previously described.[16,21] Briefly, vascular rings were equilibrated in oxygenated Krebs-Henseleit solution (118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.25 mmol/L $CaCl_2$, 1.2 mmol/L KH_2PO_4, 25 mmol/L $NaHCO_3$, and 11 mmol/L Dextrose) at 0.5 g tension for 60 minutes. And then the rings were pre-contracted with phenylephrine (1 μmol/L) then treated with increasing concentrations of acetylcholine (10^{-9} to 10^{-5} mol/L), calcium ionophore A23187 (10^{-10} to 10^{-7} mol/L), and sodium nitroprusside (SNP, 10^{-11} to 10^{-7} mol/L). Data are reported as percent of relaxation from maximum contraction to phenylephrine.

Exercise Capacity Assessment

Treadmill exercise capacity was measured using a rodent treadmill (IITC, Woodland Hills, California) as previously described.[16] Briefly, immediately before the test, mice were placed on the treadmill to acclimate for 15 minutes. Then, the mice were subjected to a slow walk/run period to be familiarized with treadmill running. Finally, in the test phase, the treadmill was set to a 30°-incline. The starting speed was set to 10 m/min and programed to increase to 18 m/min in 3 minutes, then treadmill maintained at its speed of 18 m/min. All mice were run at this setting until they reached a state of exhaustion. Distance and duration of the running and shocks per minute were recorded. Shocks per minute is a measurement of endurance. As mice fell behind the speed of the treadmill, they received an electrical shock by the shocking grid. Higher shocks per minute indicated lower endurance. In a blinded fashion, a mouse was deemed to be exhausted when it spent >5 consecutive seconds at the back of the treadmill despite receiving electrical shocks (1.5 mA) repeatedly.

Myocardial Ischemia/Reperfusion Protocol and Myocardial INF Determination

Surgical ligation of the left coronary artery and myocardial INF determination were performed as previously described.[13,22] Briefly, mice were fully anesthetized with ketamine (60 mg/kg) and pentobarbital sodium (50 mg/kg), intubated, and connected to rodent ventilator (model 845, MiniVent, Hugo Sachs).

A median sternotomy was performed, and the left coronary artery was ligated with 7-0 silk suture and a small piece of polyethylene-10 tubing. Mice were subjected to 45 minutes of left coronary artery ischemia. After 24 hours of reperfusion, the left ventricle area-at-risk and INF were determined by Evan's blue and 2,3,5-triphenyltetrazolium chloride staining at 37°C.

Troponin I Measurement

Plasma was collected at 4 hours following reperfusion from the tail vein. Plasma levels of the cardiac-specific isoform of troponin-I were assessed using an ELISA kit from Life Diagnostics (West Chester, PA), as previously described.[13]

Statistical Analysis

Data were analyzed in a blinded manner to the genetic background of the animals in each study until all measurements were complete. All data in this study are graphically represented as mean±SEM. Prism 6 (GraphPad Software Inc) was used for data analysis. Normal distribution was tested with the D'Agostino-Pearson test. For 2-group comparisons, we used a Student unpaired 2-tailed t-test, or a Mann–Whitney test when the data did not follow a normal distribution. For multiple group comparison, data were analyzed using ordinary 1-way ANOVA analysis, or Kruskal–Wallis test when the data did not follow a normal distribution. Two-way ANOVA analysis followed by a Bonferroni multiple comparison test was used when appropriate. A P value of <0.05 was considered statistically significant. Presented data may have different numbers of animals per end point because of procedural complications, limited sample collection (ie, plasma volume), or lack of participation in involuntary treadmill running. Before conducting statistical analysis, an outlier test was performed using "robust regression and outlier removal" method developed by Prism 6 with 1% aggressiveness to identify and remove any outlier in the data set.

RESULTS

Cardiac CSE mRNA and Protein Expression in EC-CSE Tg

Real time-quantitative polymerase chain reaction analysis revealed a 500-fold increase in CSE mRNA expression in the heart of EC-CSE Tg mice compared with tTA control mice (Figure 1B). This increase in CSE RNA translated to a 12-fold increase in protein expression as quantified by western-blot (Figure 1C).

Increased Circulating and Myocardial H$_2$S Production in EC-CSE Tg Mice

Figure 2 depicts plasma and myocardial levels of free H$_2$S and sulfane sulfur. Free H$_2$S levels were (P=0.01) increased in the plasma as shown in Figure 2A but not significantly changed in the heart as shown in Figure 2B when comparing EC-CSE Tg mice with tTA controls. There was no statistically significant difference in free myocardial H$_2$S levels between the 2 groups (P=NS). However, sulfane sulfur, the storage form of H$_2$S, was increased in both plasma ($P<0.0001$) as shown in Figure 2C and heart tissue ($P=0.0028$, Figure 2D) of the EC-CESE Tg mice compared with control. These data indicate that overexpression of CSE specifically in

endothelial cells increases H$_2$S levels and storage both locally and systemically.

Increased Circulating and Myocardial NO Production and cGMP Levels in EC-CSE Tg Mice

Several previous studies have shown that H$_2$S promotes endothelial NO synthase (eNOS) activity and increases NO bioavailability.[9,23] We investigated whether NO levels were affected by overexpression of CSE in endothelial cells. Nitrite, a stable breakdown metabolite of NO, was measured in plasma and heart tissues. EC-CSE Tg mice displayed increased nitrite levels in
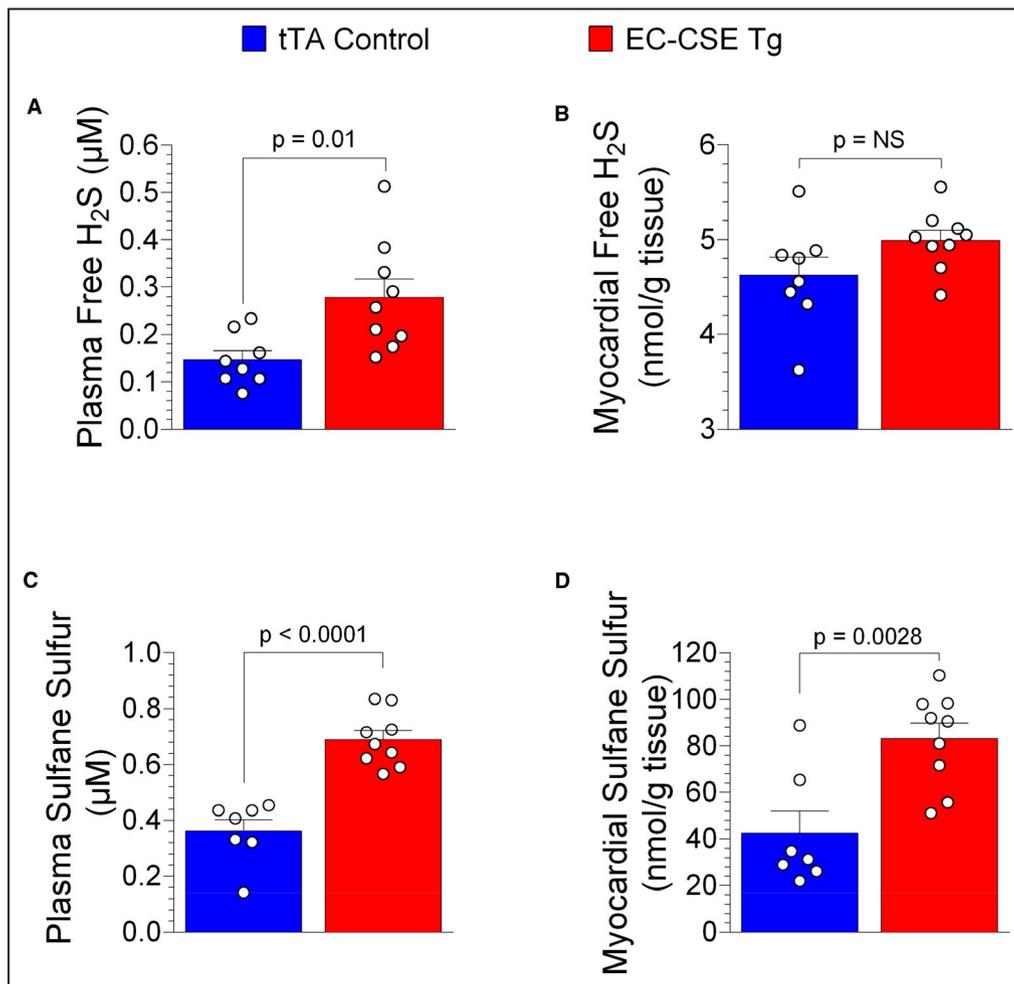

Figure 2.   Overexpression of cystathionine γ-lyase in endothelial cells increased circulating and myocardial hydrogen sulfide production.
Free hydrogen sulfide levels (μmol/L) were higher in plasma (**A**) but not significantly changed in cardiac tissue (nmol/g) (**B**) of endothelial cell-specific cystathionine γ-lyase transgenic mice (n=9) compared with tTA (tetracycline trans-activator) controls (n=8). Sulfane sulfur levels (μmol/L) were elevated in both circulating (**C**) and cardiac tissue (nmol/mg) (**D**) of endothelial cell-specific cystathionine γ-lyase transgenic mice (n=9) compared with tTA controls (n=7). Data are presented as mean±SEM, Student *t*-test (**A** and **B**), Mann–Whitney *U* test (**C** and **D**). EC-CSE Tg indicates endothelial cell-specific cystathionine γ-lyase transgenic mice; H$_2$S, hydrogen sulfide; and tTA, tetracycline trans-activator.

both plasma (P=0.013, Figure 3A) and heart tissue (P=0.026, Figure 3B), suggesting increased NO production in mice with endothelial cell overexpression of CSE.

NO activates soluble guanylyl cyclase to form cGMP. Myocardial cGMP levels were also increased in EC-CSE Tg mice compared with control (P=0.012, Figure 3C), confirming increased myocardial NO signaling in these mice.

Improved Endothelium-Dependent Vasorelaxation Responses in EC-CSE Tg Mice

We evaluated vascular reactivity of isolated thoracic aorta vascular rings to acetylcholine, A23187, and SNP and these data are presented in Figure 4. Aortic rings from EC-CSE Tg mice displayed augmented vasorelaxation responses to acetylcholine as shown

in concentration-response curve and half maximal effective concentration values (Figure 4A). Aortic vascular rings from EC-CSE Tg mice also displayed enhanced responses to the eNOS-activating calcium ionophore, A23187 as shown in Figure 4B. In contrast, the endothelium-independent vasorelaxation responses to SNP in thoracic aortas were not significantly different in EC-CSE Tg mice and tTA controls (Figure 4C). These data suggest that overexpression of CSE in endothelial cells increases endothelium-dependent vasodilation responses, possibly through activation of eNOS.

Increased Exercise Capacity in EC-CSE Tg Mice

Exercise capacity reveals important information regarding the integration of cardiac and vascular function and is important to consider as an index of overall
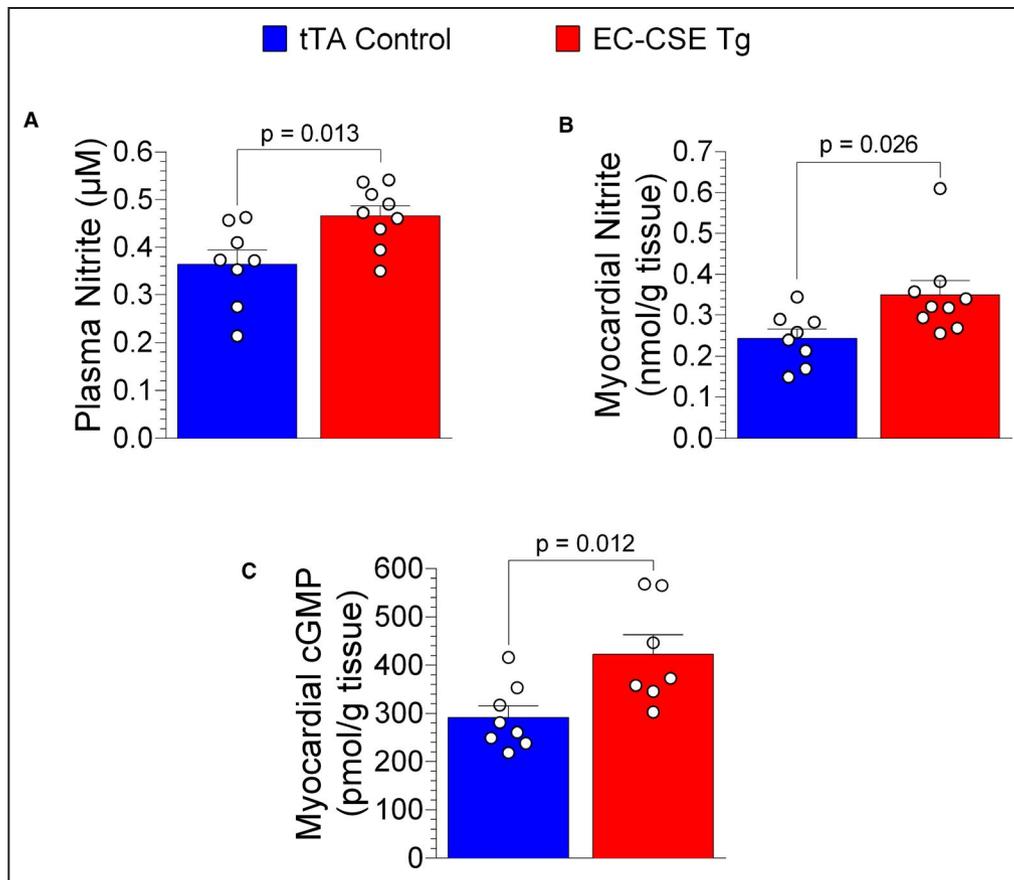

Figure 3. Overexpression of cystathionine γ-lyase in endothelial cells increased circulating and myocardial NO production.
Nitrite levels (μmol/L) were increased in both plasma (**A**) and cardiac tissue (**B**) of endothelial cell-specific cystathionine γ-lyase transgenic mice (n=9) compared with tTA (tetracycline trans-activator) controls (n=8). In addition, cardiac cyclic guanosine monophosphate levels (pmol/g) were higher in endothelial cell-specific cystathionine γ-lyase transgenic mice (n=7) than in tTA controls (n=8) (**C**). Data are presented as mean±SEM, Student t-test (**A**), Mann–Whitney U test (**B** and **C**). cGMP, cyclic guanosine monophosphate; EC-CSE Tg, endothelial cell-specific cystathionine γ-lyase transgenic mice; and tTA, tetracycline trans-activator.
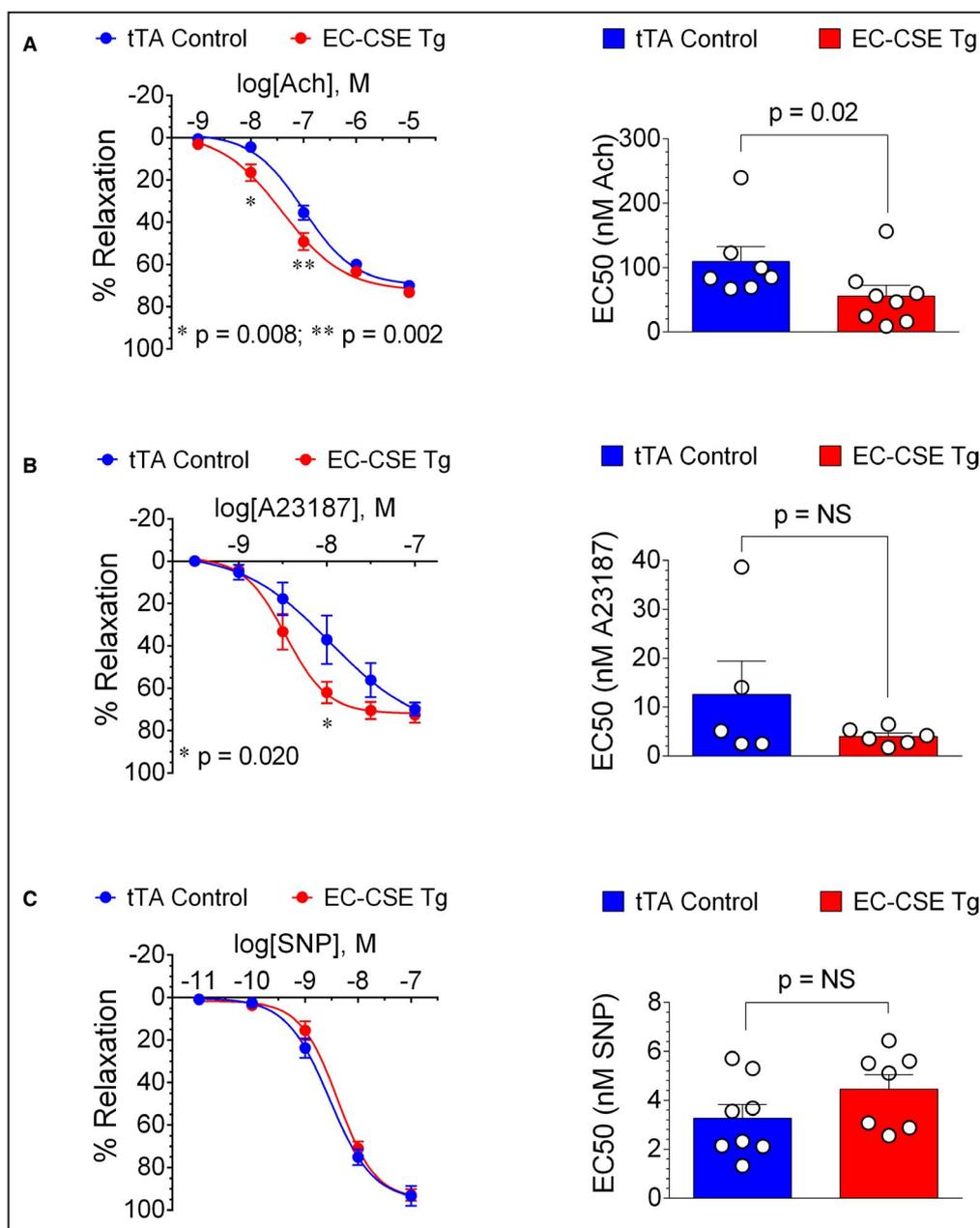
Figure 4. Overexpression of cystathionine γ-lyase in endothelial cells augmented endothelium-dependent vasorelaxation responses in isolated thoracic aortic rings.
A, Concentration-response curve shows that acetylcholine induced augmented relaxation in aortic rings from endothelial cell-specific cystathionine γ-lyase transgenic mice (EC-CSE Tg) (n=8) at concentration of 10^{-8} and 10^{-7} mol/L (LEFT). In addition, half maximal effective concentration value of acetylcholine was lower in aortic rings from EC-CSE Tg (n=8) compared with tTA (tetracycline trans-activator) controls (n=7) (RIGHT). **B**, Similarly, A23187-induced vasorelaxation was augmented in EC-CSE Tg mice (n=5) at concentration of 10^{-8} mol/L (LEFT), although half maximal effective concentration value of A23187 was not significantly different in EC-CSE Tg (n=5) compared with tTA controls (n=6) (RIGHT). **C**, However, neither concentration-response curve (LEFT) nor half maximal effective concentration values (RIGHT) showed statistically significant differences in vasorelaxation response to sodium nitroprusside between EC-CSE Tg (n=7) and tTA control (n=8) mice. Data are presented as mean±SEM, 2-way ANOVA analysis with Bonferroni multiple comparison test was used to compare the concentration—response curves, Mann–Whitney *U* test was used to compare half maximal effective concentration. EC50 indicates half maximal effective concentration; EC-CSE Tg, endothelial cell-specific cystathionine γ-lyase transgenic mice; SNP, sodium nitroprusside; and tTA, tetracycline trans-activator.
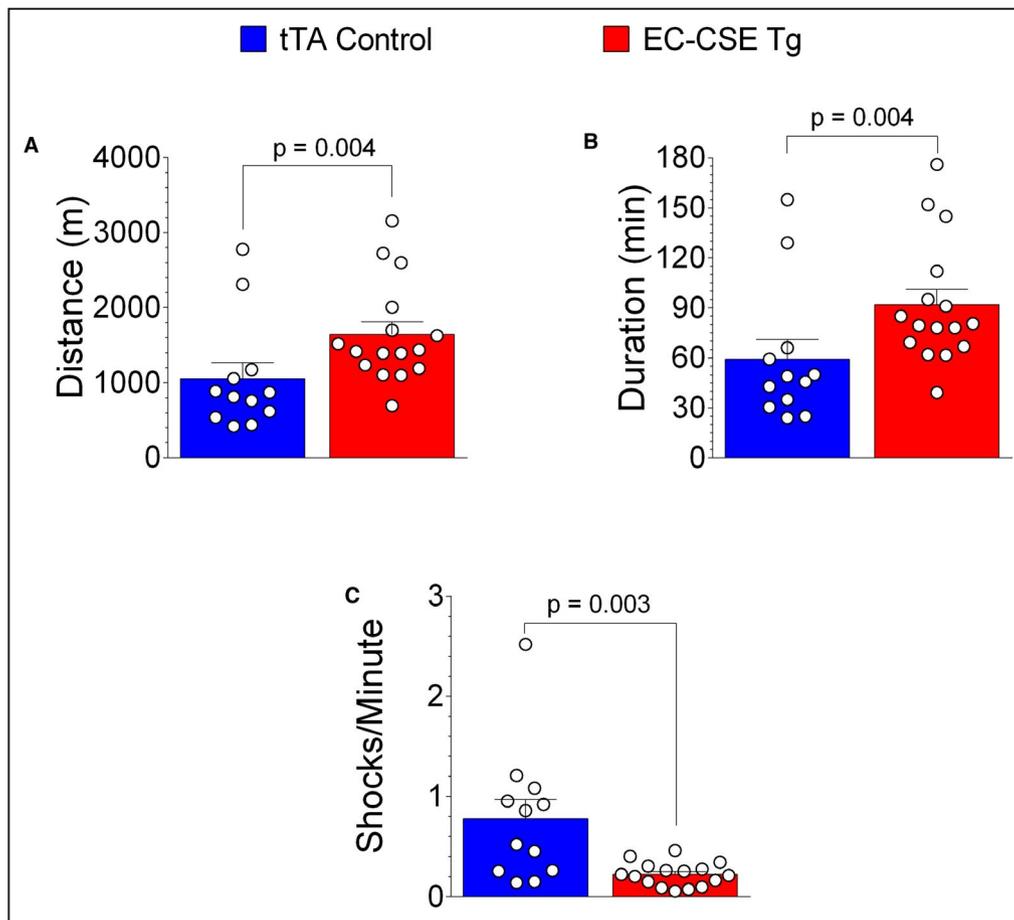
Figure 5. Overexpression of cystathionine γ-lyase in endothelial cells increased exercise capacity.
Treadmill exercise testing shows that the distance of running (meters) (**A**) and time to exhaustion (minutes) (**B**) were higher, while numbers of shocks per minute (**C**) were lower in endothelial cell-specific cystathionine γ-lyase transgenic mice (n=16) compared with tTA (tetracycline trans-activator) controls (n=12). Data are presented as mean±SEM, Mann–Whitney U test. EC-CSE Tg indicates endothelial cell-specific cystathionine γ-lyase transgenic mice; and tTA, tetracycline trans-activator.

quality-of-life under baseline conditions and during heart failure. We measured treadmill exercise capacity in EC-CSE Tg mice and tTA controls under baseline conditions and these data are presented in Figure 5. EC-CSE Tg mice displayed longer distance of running (P=0.004) in Figure 5A and time to exhaustion (P=0.0004) in Figure 5B. Furthermore, the EC-CSE Tg mice also exhibited lower numbers of shocks per minute (P=0.003) compared with controls (Figure 5C). Body weight was not significantly different between EC-CSE Tg and tTA controls (Figure S1A). Taken together, these data suggest augmented exercise capacity in the EC-CSE Tg mice.

Myocardial Ischemia-Reperfusion Injury is Attenuated in EC-CSE Tg Mice

We have previously reported that H_2S therapy with H_2S donors significantly attenuates myocardial and

hepatic ischemia-reperfusion injury.[9,13,24] To investigate whether selective genetic overexpression of CSE in endothelial cells protects the ischemic-reperfused myocardium, we subjected EC-CSE Tg mice to transient coronary artery ligation and reperfusion. Mice were subjected to 45 minutes of myocardial ischemia and 24 hours of reperfusion (Figure 6A). We evaluated the influence of the promoter cdh5-tTA with a tTA control group and also included a group of wild-type mice as another control. Neither myocardial infarct size (INF) per area-at-risk nor troponin-I levels were significantly different between cdh5-tTA and wild-type mice, suggesting the activator protein tTA has no influence on myocardial ischemia-reperfusion injury. However, the EC-CSE Tg mice displayed decrease in INF/area-at-risk (P<0.0001) compared with wild type or tTA controls (Figure 6B and 6C). Likewise, circulating troponin-I levels were reduced in EC-CSE Tg mice versus wild type (P=0.045 Figure 6D). The
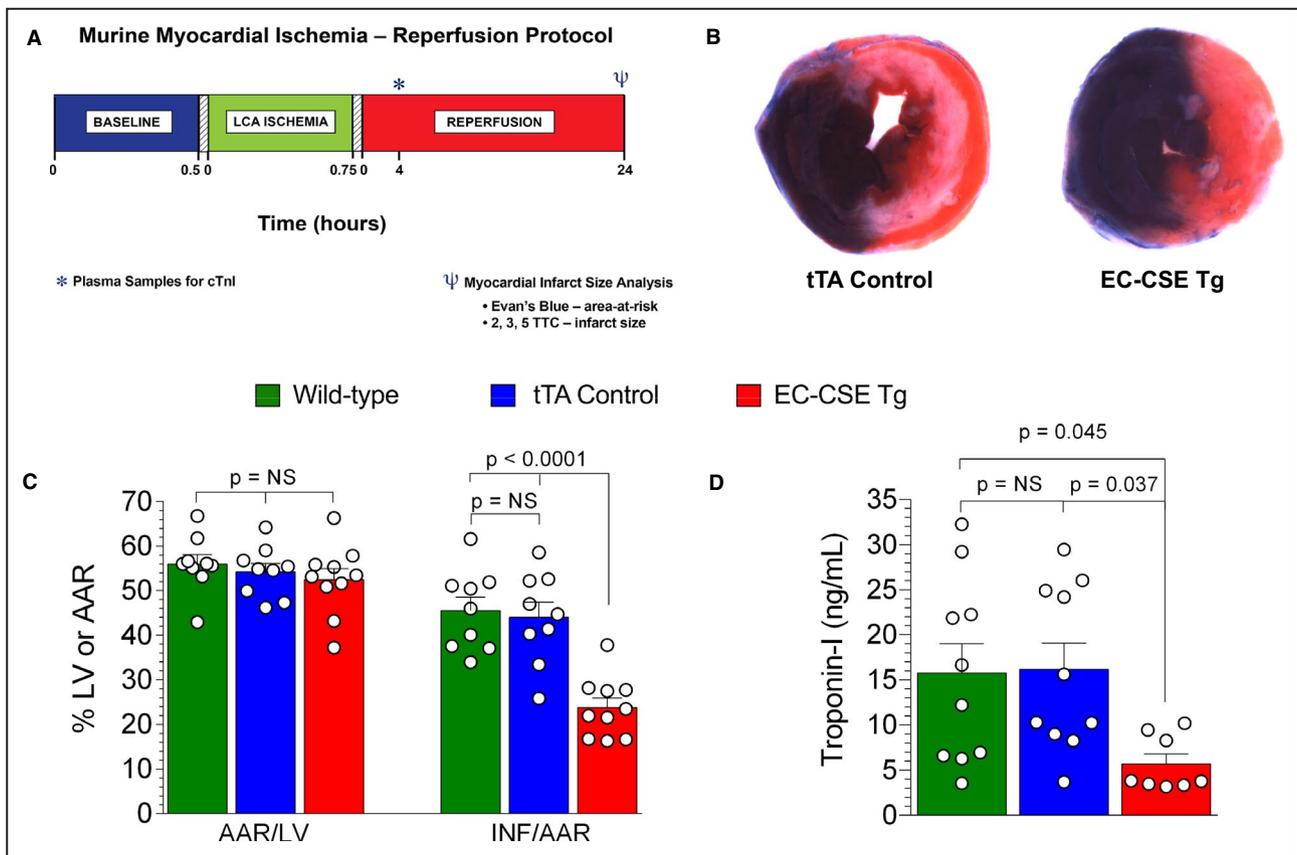
Figure 6. Overexpression of cystathionine γ-lyase in endothelial cells reduced myocardial ischemia-reperfusion injury.
A, Myocardial ischemia-reperfusion protocol. Mice were subjected to 45 minutes of left coronary artery (LCA) ischemia followed by 24 hours of reperfusion. Plasma samples were collected at 4 hours of reperfusion for troponin-I measurement. Evan's blue and 2,3,5-triphenyltetrazolium chloride staining were performed at 24 hours of reperfusion to determine the left ventricle area-at-risk (AAR) and infarct size. **B**, Representative images for left ventricle AAR and infarct size in tTA (tetracycline trans-activator) and endothelial cell-specific cystathionine γ-lyase transgenic mice following myocardial ischemia-reperfusion. **C**, AAR/left ventricle was not significantly different among all groups. Infarct size/AAR showed no statistically significant differences between cdh5-tTA (n=9) and wild-type mice (n=9), suggesting the activator protein tTA has no influence on myocardial ischemia-reperfusion injury. However, the endothelial cell-specific cystathionine γ-lyase transgenic mice (n=10) displayed a significant decrease in infarct size/AAR compared with wild type. **D**, Plasma troponin-1 level (a marker for myocardial injury) was not significantly changed in tTA (n=10) but reduced in endothelial cell-specific cystathionine γ-lyase transgenic mice (n=8) compared with wild type (n=10). Data are presented as mean±SEM, ordinary 1-way ANOVA with Tukey multiple comparison test. AAR indicates area-at-risk; EC-CSE Tg, endothelial cell-specific cystathionine γ-lyase transgenic mice; LCA, left coronary artery; LV, left ventricular; and tTA, tetracycline trans-activator.

area of left ventricle at risk was not significantly different among all groups (*P*=NS). These data suggest that increased CSE expression in vascular endothelial cells protects against myocardial ischemia-reperfusion injury, likely through increased endothelial H_2S and NO production.

Reduced Endothelium-Dependent Vasodilation Responses and Treadmill Exercise Capacity in EC-CSE KO Mice

To evaluate the role of constitutive endothelial cell CSE on cardiovascular physiology and pathology, we generated conditional knockout mice with endogenous CSE deleted specifically in endothelial cells. We measured H_2S, NO, vascular function, and exercise capacity in

EC-CSE KO mice and CSE[flox+/flxo+] controls. As shown in Figure 7A, plasma free H_2S levels were not significantly decreased in EC-CSE KO mice compared with CSE[flox+/flxo+] (*P*=0.15). In contrast, we observed decreased circulating sulfane sulfur levels (Figure 7B) in EC-CSE KO mice compared with control (*P*=0.01). Interestingly, we also observed a trend of decreased circulating nitrite level (*P*=0.06. Figure 7C) in the KO compared with CSE[flox+/flxo+] controls. In heart tissue, neither free H_2S (Figure 8A) nor sulfane sulfur levels (Figure 8B) were significantly changed in EC-CSE KO mice compared with control. However, cardiac nitrite levels were decreased in the knockout compared with CSE[flox+/flxo+] controls (*P*=0.002, Figure 8C).

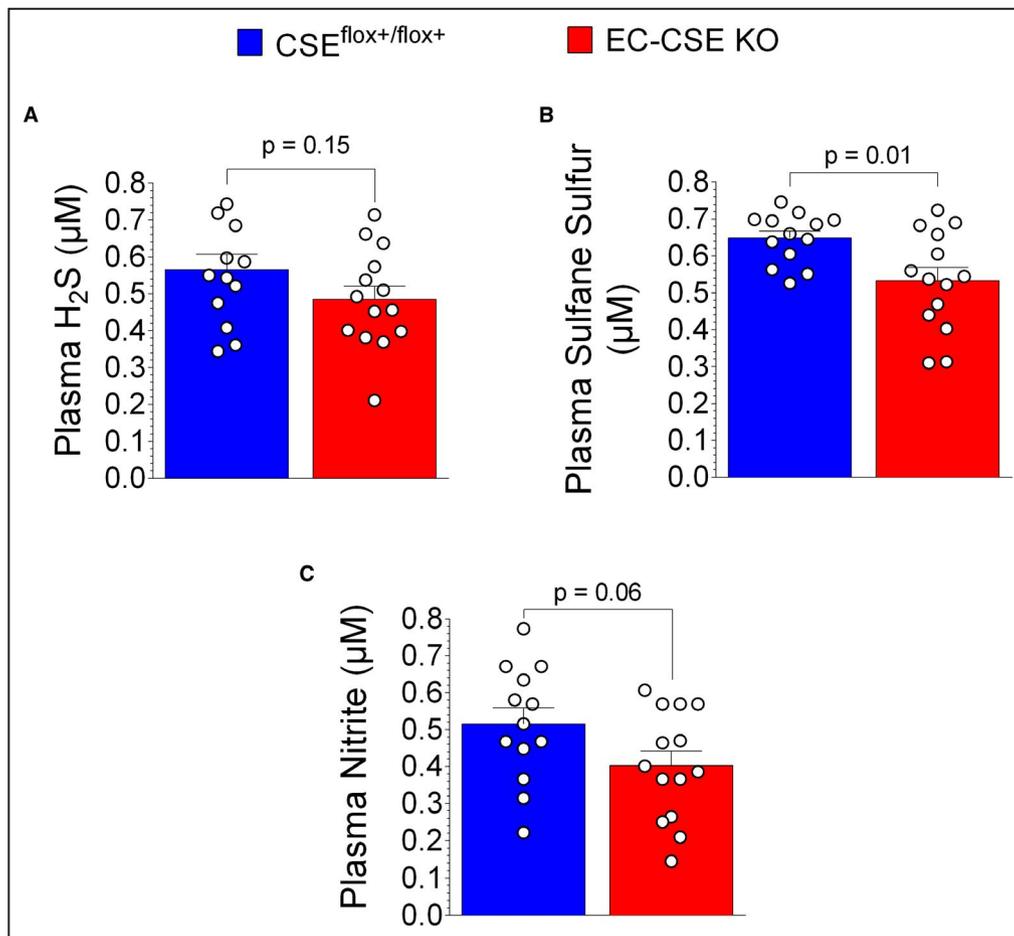Analysis of isolated aortic vascular rings harvested from EC-CSE KO mice revealed decreased relaxation

Figure 7. Genetic deletion of cystathionine γ-lyase in endothelial cells decreased circulating hydrogen sulfide storage and tended to decrease circulating NO production.
A, Plasma free hydrogen sulfide levels (μmol/L) were not significantly different between endothelial cell-specific cystathionine γ-lyase knockout (EC-CSE KO, n=14) and CSE[flox+/flox+] mice (n=13). **B**, Plasma sulfane sulfur levels (μmol/L) were decreased in EC-CSE KO mice (n=14) compared with CSE[flox+/flox+] (n=13). **C**, A trend of decreased circulating nitrite levels (μmol/L) were also observed in EC-CSE KO mice (n=14) compared with CSE[flox+/flox+] (n=13). Data are presented as mean±SEM, Student *t*-test. CSE indicates cystathionine γ-lyase; EC-CSE KO, endothelial cell-specific CSE knockout mice; and H$_2$S, hydrogen sulfide.

responses to acetylcholine and A23187 when compared with controls (Figure 9A and 9B). In contrast we failed to observe any statistically significant differences in the vascular relaxation response to SNP as shown in concentration-response curve and half maximal effective concentration values (Figure 9C).

Moreover, treadmill exercise capacity testing (Figure 10) revealed that EC-CSE KO mice displayed reduced total distance of running (P=0.012) as shown in Figure 10A and reduced time to exhaustion (P=0.012) in Figure 10B. We also observed higher numbers of shocks per minute (P=0.018) in EC CSE KO mice compared with control (Figure 10C). Body weights were not significantly different in EC-CSE KO and CSE[flox+/flox+] mice (Figure S1B). Our data suggest that deletion of CSE in endothelial cells results in endothelial dysfunction, decreased vascular reactivity, and decreased exercise capacity. This decreased exercise capacity is likely because of the impairment of H$_2$S production and downstream NO signaling.

Myocardial Ischemia-Reperfusion Injury is Unchanged in EC-CSE KO Mice

Global deletion of CSE has been shown to exacerbate cardiac ischemia-reperfusion injury.[9] To examine the impact of CSE deletion specifically in endothelial cells, EC-CSE KO mice were subjected to the same myocardial ischemia-reperfusion injury protocol. Interestingly, no statistically significant differences in myocardial INF/area-at-risk were observed between EC-CSE KO and CSE[flox+/flox+] mice, as shown in Figure 11A and 11B.
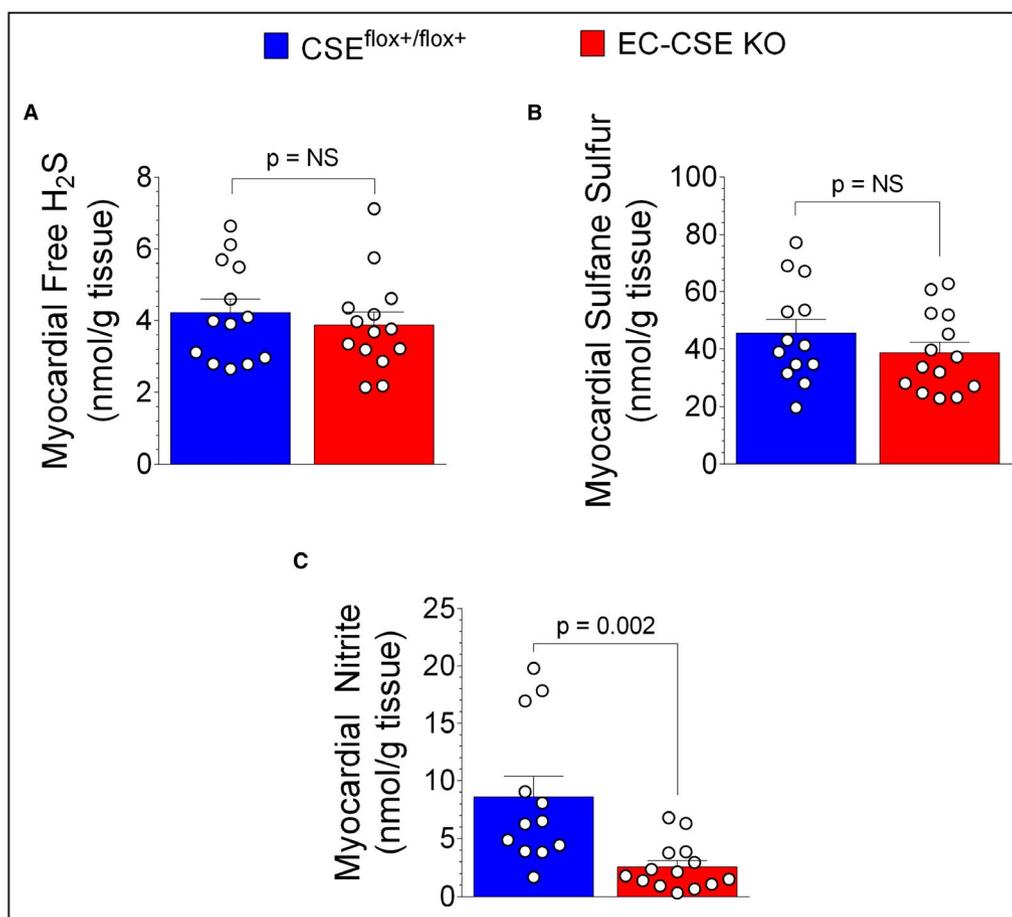
Figure 8. Genetic deletion of cystathionine γ-lyase in endothelial cells did not significantly change cardiac hydrogen sulfide levels but decreased cardiac NO production.
A, Cardiac free hydrogen sulfide levels (nmol/mg) were not significantly different between endothelial cell-specific cystathionine γ-lyase CSE knockout mice (n=14) and CSE$^{flox+/flox+}$ mice (n=13). **B**, Cardiac sulfane sulfur levels (nmol/mg) were not significantly changed in endothelial cell-specific CSE knockout (n=14) compared with CSE$^{flox+/flox+}$ (n=13). **C**, However, cardiac nitrite levels (nmol/mg) were decreased in endothelial cell-specific CSE knockout mice (n=14) compared with CSE$^{flox+/flox+}$ (n=13). Data are presented as mean±SEM, Student *t*-test. CSE indicates cystathionine γ-lyase; EC-CSE KO, endothelial cell-specific CSE knockout mice; and H$_2$S, hydrogen sulfide.

DISCUSSION

Since the discovery of endogenously produced H$_2$S in mammalian brain,[25–27] numerous studies have demonstrated that H$_2$S is an important mediator of diverse physiological processes.[28,29] H$_2$S is a potent gaseous signaling molecule that works in concert with NO and carbon monoxide.[30] In the cardiovascular system, H$_2$S modulates vascular tone, promotes angiogenesis, reduces inflammation, attenuates oxidative stress, downregulates apoptosis, protects against multiple cardiovascular disease states, and rescue compromised organs secondary to cardiovascular system such as kidney in a similar manner as NO.[16,31,32]

To date, the physiological functions of H$_2$S in the cardiovascular system have been investigated primarily by using H$_2$S donors and inhibitors. However, these studies have limitations because of the lack of cell selectivity of the compounds. Genetic modulation of H$_2$S producing enzyme levels in a tissue specific manner is an effective means to more precisely investigate the cardiovascular actions of endogenous H$_2$S. Using a global CSE deletion mouse model, studies from Yang et al and our group have demonstrated the critical role of endogenous H$_2$S derived from CSE for maintaining normal cardiovascular function and protecting against cardiac injury.[6,9] To evaluate the protective effects of cardiac specific H$_2$S, we later generated cardiomyocyte specific CSE overexpressed mice and demonstrated the cardioprotective role of endogenous H$_2$S produced in cardiomyocytes.[12,13] In the current study, we sought to identify the effects of endothelial cell-specific H$_2$S production on cardiovascular function and the response to ischemic injury.
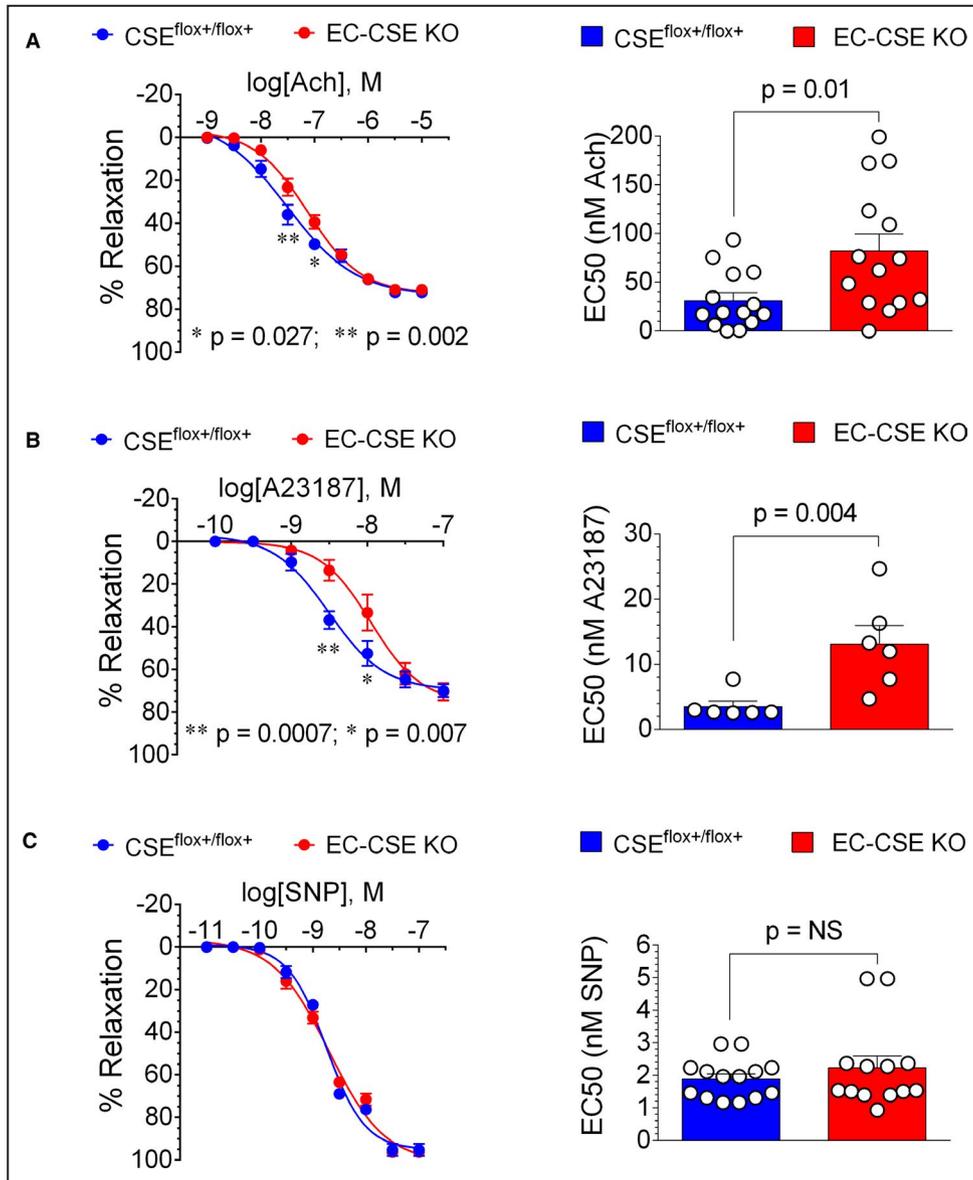
Figure 9. Genetic deletion of cystathionine γ-lyase in endothelial cells impaired endothelium-dependent vasorelaxation responses in isolated thoracic aortic rings.

A, Concentration-response curve shows that acetylcholine induced less relaxation (%) in aortic rings in endothelial cell-specific cystathionine γ-lyase knockout mice (EC-CSE KO) (n=13) than in $CSE^{flox+/flox+}$ (n=14) mice at concentration of $10^{-7.5}$ and 10^{-7} mol/L (LEFT). Half maximal effective concentration (EC50) value of acetylcholine was higher in aortic rings from EC-CSE KO (n=13) compared with $CSE^{flox+/flox+}$ (n=14) (RIGHT). **B**, A23187-induced vasorelaxation (%) was reduced in EC-CSE KO mice (n=6) compared with $CSE^{flox+/flox+}$ (n=6) at concentration of $10^{-8.5}$ and 10^{-8} mol/L (LEFT). The EC50 value of A23187 was higher in EC-CSE KO (n=6) compared with controls (n=6). **C**, Neither concentration-response curve (LEFT) nor EC50 values (RIGHT) showed statistically significant differences in vasorelaxation response to sodium nitroprusside between endothelial cell-specific cystathionine γ-lyase knockout mice (n=13) and $CSE^{flox+/flox+}$ control (n=14). Data are presented as mean±SEM, 2-way ANOVA analysis with Bonferroni multiple comparison test was used to compare the concentration—response curves, Student t-test was used to compare EC50 (**A** and **C**), Mann–Whitney U test was used to compare EC50 (**B**). CSE indicates cystathionine γ-lyase; EC50, half maximal effective concentration; EC-CSE KO, endothelial cell-specific CSE knockout mice; and SNP, sodium nitroprusside.

The adult mammalian heart is composed of several cell types, including cardiomyocytes, fibroblasts, endothelial cells, smooth muscle cells, and immune cells. In addition to cardiomyocytes, non-cardiomyocytes are critical for cardiac homeostasis, providing the extracellular matrix, intercellular network, and
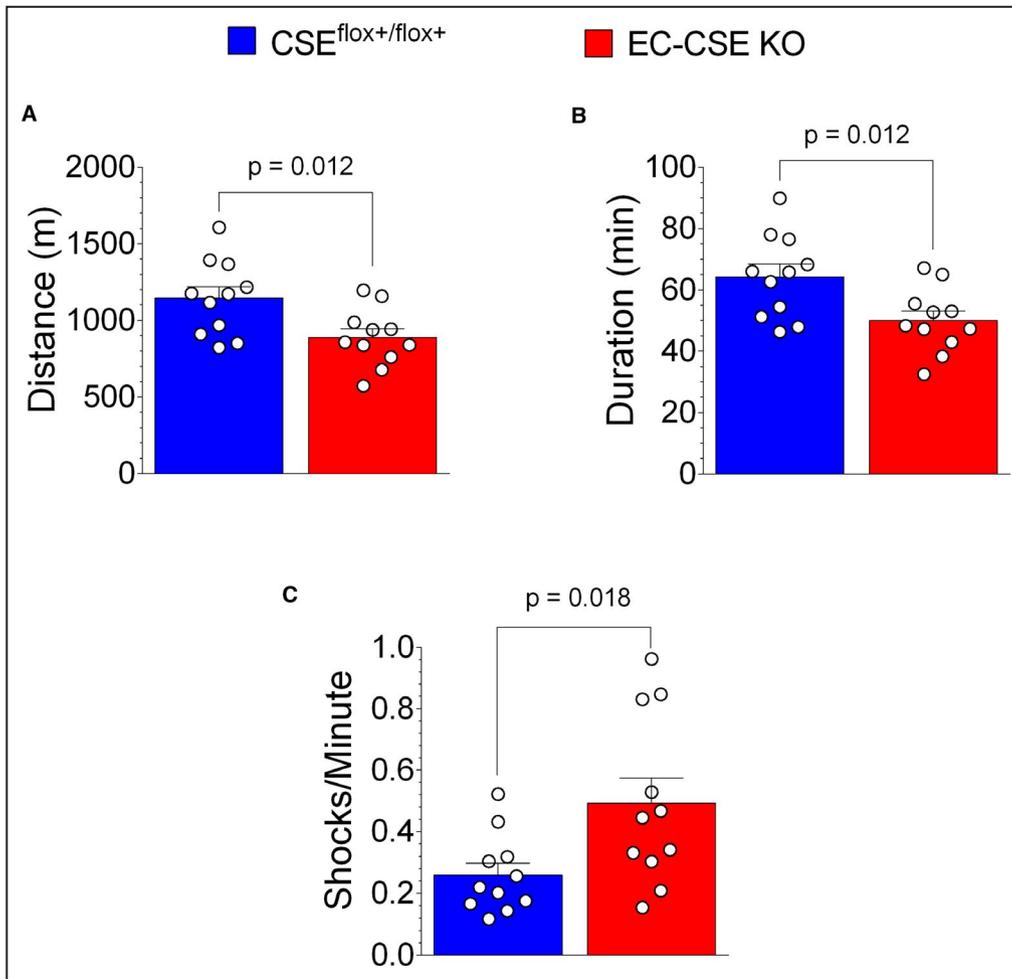
Figure 10. Genetic deletion of cystathionine γ-lyase in endothelial cells decreased exercise capacity.
Treadmill exercise testing showed that the distance of running (meters) (**A**) and time to exhaustion (minutes) (**B**) were lower, while numbers of shocks per minute (**C**) were higher in endothelial cell-specific cystathionine γ-lyase knockout mice (n=11) compared with CSE^flox+/flox+ control (n=11). Data are presented as mean±SEM, Student *t*-test. CSE indicates cystathionine γ-lyase; and EC-CSE KO, endothelial cell-specific CSE knockout mice.

other needs for efficient cardiomyocyte architecture and metabolic function. Cardiomyocytes occupy 70% to 85% of the volume of the mammalian heart and account for ≈25% to 35% of cardiac cells.[33,34] Using combined analyses, a recent study from Pinto et al demonstrated that endothelial cells are the most abundant cell population in human and mouse hearts (≈50% versus ≈30% cardiomyocytes).[35] The increase in CSE mRNA (≈500-fold) and protein levels (≈12-fold) in total heart tissue samples resulting from overexpression in endothelial cells in the current study confirm that endothelial cells comprise a significant proportion of total cardiac cells. Importantly, increased CSE protein expression resulted in increased H_2S production, as evidenced by increased free H_2S in the circulation and increased sulfane sulfur levels in the heart and circulation. Although CSE protein expression in isolated

vascular tissue was not measured, we can speculate that increased circulating H_2S levels in EC-CSE Tg mice are also attributed to increased CSE expression in the endothelium of peripheral vessels. Conversely, deletion of CSE in endothelial cells resulted in decreased circulating sulfane sulfur levels in EC-CSE mice, confirming that endothelial CSE is critical for endogenous H_2S production. However, cardiac H_2S and sulfane sulfur levels were not significantly affected by CSE deletion in endothelial cells, suggesting that the sources of cardiac H_2S generation are not limited to endothelial CSE. CSE expression in other cells, such as cardiomyocytes, and the other H_2S producing enzymes cystathionine β-synthase and 3-MST, also contribute to H_2S production in the heart.

We also observed increased NO bioavailability following CSE overexpression in endothelial cells. H_2S
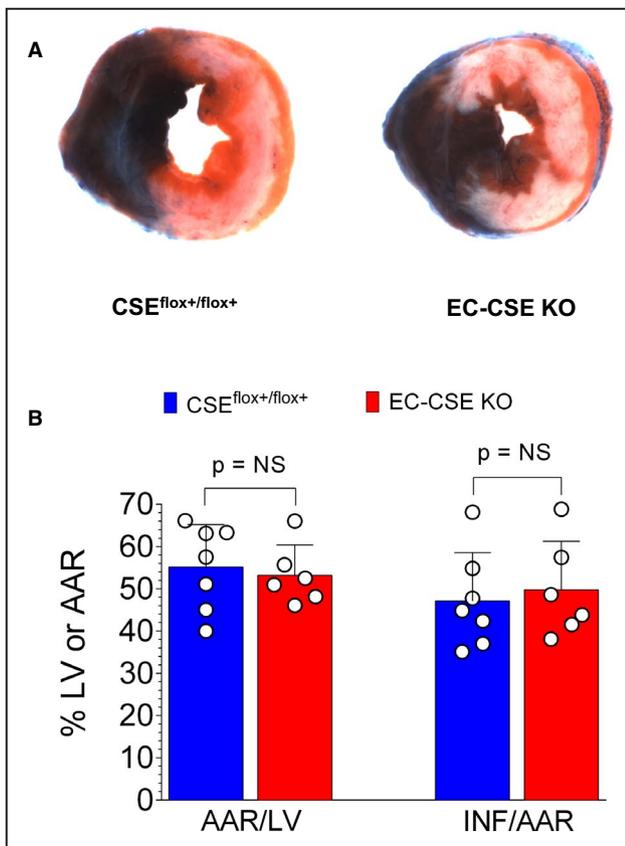
Figure 11. Genetic deletion of cystathionine γ-lyase in endothelial cells did not significantly affect myocardial ischemia-reperfusion injury.
Representative heart slice images taken at the mid-ventricular level (**A**) and quantified data (**B**) showed no statistically significant differences in area-at-risk/left ventricle (AAR/LV) or infarct size/area-at-risk (INF/AAR) between cystathionine γ-lyase knockout mice (n=6) and CSE^flox+/flox+ (n=7). Data are presented as mean±SEM, Mann–Whitney *U* test. AAR indicates area-at-risk; EC-CSE KO, endothelial cell-specific cystathionine γ-lyase knockout mice; INF, infarct size; and LV, left ventricular.

and NO share a wide range of physical properties and physiological functions. Growing evidence has shown that these 2 gaseous signaling molecules interact to regulate the biosynthesis of NO and H_2S as well cellular function. Both exogenously and endogenously derived H_2S have been shown to promote NO bioavailability and signaling.[9,23,36] On the other hand, NO donors increase CSE expression and H_2S production.[37] In endothelial cells, NO is synthesized from L-arginine by the endothelial isoform of NO synthase. NO diffuses through the endothelial cell membrane into smooth muscle cells where it activates soluble guanylyl cyclase and produces the second messenger cGMP, which regulates vascular tone, blood flow, and platelet function.[38] In the current study, overexpression of CSE in endothelial cells led to increased circulating and myocardial nitrite levels as well as myocardial cGMP in EC-CSE Tg mice. This finding

suggests that an increase in endogenous H_2S production, specifically in endothelial cells, augments NO bioavailability in the cardiovascular system. These results also suggest that H_2S generated in endothelial cells could provide beneficial effects in the cardiovascular system via NO signaling. However, deletion of CSE in endothelial cells only resulted in a trend of decreased circulating nitrite level. The mild change in circulating nitrite level could result from a mild decrease in circulating H_2S production (no significant change in free H_2S and 18% decrease in sulfane sulfur levels in EC-CSE KO mice). In addition, NO is not only generated in endothelium. Besides eNOS, other NO Synthases also contribute to NO production in circulation. Deletion of CSE in endothelial cells might impair eNOS function, but might not affect neuronal NO synthase function. As a result, the total circulating NO levels were not dramatically decreased following EC-CSE deletion. Interestingly, despite no significant changes in cardiac free H_2S and sulfane sulfur levels, cardiac nitrite levels were reduced by CSE deletion in endothelial cells. These data suggest that cardiac NO is mainly generated in endothelial cells. Deletion of CSE in endothelial cells impaired eNOS function in the heart therefore decreasing local NO production.

We also assessed the impact of endothelial generated H_2S on vascular function. It is postulated that the primary action of H_2S in the vasculature is vasodilation. It has been shown that global deletion of CSE resulted in decreased circulating H_2S levels and impaired endothelium dependent vasodilation.[6] However, it is not clear whether endothelial cell-specific production of H_2S is critical for regulating vascular function. It has also been suggested that the vasodilatory action of H_2S is mediated by eNOS generated NO.[39,40] By performing vascular reactivity measurements in isolated thoracic aorta of EC-CSE Tg and EC-CSE KO mice, we found enhanced vascular vasorelaxation responses to the endothelium-dependent vasodilator acetylcholine and eNOS-specific vasodilator A23187 in endothelial CSE transgenic mice. In contrast, endothelial-dependent vasorelaxation responses were impaired by the deletion of CSE in endothelial cells. These findings provide novel evidence that endothelial cell-specific H_2S production is critical for maintaining endothelial function and normal vascular tone. Although some reports have shown that H_2S mediates vasodilation responses via an endothelium-independent mechanism, such as inhibiting cGMP degradation,[41] we did not see significant changes in the direct vascular smooth muscle relaxation response to SNP in our endothelial cells specifically modified mice. In combination, our findings of increased NO production in EC-CSE Tg and decreased NO levels in EC-CSE KO mice suggest that CSE-derived H_2S in endothelial

cells protects vascular function, at least in part, through the eNOS—NO pathway.

Exercise capacity is influenced by cardiac contractile function, vascular function, oxygen-carrying capacity, and peripheral muscle mass and function.[42] Systemic administration of an H_2S donor has been shown to improve exercise capacity and vascular function in heart failure.[16] In the current study, although cardiac structure and function at rest were not significantly changed by CSE overexpression or deletion in endothelial cells (Figures S2 and S3), treadmill exercise capacity testing revealed that running distance, duration, and endurance were increased in mice with endothelial cell restricted overexpression of CSE and were all reduced by genetic deletion of constitutive endothelial cell CSE. In addition to endothelial function, the augmented exercise capacity in EC-CSE Tg mice could also reflect better cardiac function, improvement of mitochondrial function, or improved oxygen delivery to skeletal muscle. All of which are previously demonstrated actions of H_2S an NO.[2,38]

It is well established that H_2S exerts potent cytoprotective effects in the setting of cardiovascular diseases in various animal model systems.[31,43] Using transgenic mouse models, previous studies reported that systemic deficiency of CSE significantly attenuates H_2S bioavailability and results in exacerbated myocardial ischemia/reperfusion injury.[6,44] In contrast, cardiomyocyte-specific genetic overexpression of CSE increased endogenous cardiac H_2S production and resulted in cardiac protection against acute myocardial ischemia-reperfusion injury and ischemia-induced heart failure.[12,13] Here, we investigated the effects of endothelial cell-specific CSE modulation and H_2S production on acute myocardial ischemia-reperfusion injury. Similar to what was shown in cardiomyocyte-specific overexpression, endothelial cell-specific CSE overexpression significantly limited myocardial infarct size following acute myocardial ischemia reperfusion, suggesting that endothelial cell-derived H_2S production is critical in myocyte protection following ischemic insult. The mechanisms responsible for this cardiac protection, could involve, but are not limited to, the numerous mechanisms elucidated by previous studies. These include reduction in oxidative stress,[45] inflammation,[46] apoptosis[47] and mitochondrial dysfunction[13] which are associated with ischemia/reperfusion injury. Surprisingly, myocardial injury following ischemia-reperfusion was not significantly augmented by CSE deletion in endothelial cells. Combining with unchanged cardiac H_2S levels in EC-CSE KO mice, our findings suggest that H_2S from other sources also contribute to cardiac protection during ischemia-reperfusion injury.

In summary, to our knowledge, this is the first study using tissue/cell-specific gene modulation to address the role of endothelial cell-specific H_2S generation in the cardiovascular system. Our results provide evidence that CSE is a critical enzyme responsible for generating H_2S in endothelial cells. Moreover, we demonstrated that H_2S produced in endothelial cells is critical in maintaining endothelial function, exercise capacity, and protecting against myocardial ischemia/reperfusion injury. Modulation of endogenous endothelial H_2S production may be a potential therapeutic strategy in the treatment of cardiovascular disease.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Materials

Data S1
Figures S1–S3

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SUPPLEMENTAL MATERIAL

Data S1.

## Supplemental Methods

Echocardiography

Mice were anaesthetized with isoflurane (Henry Schein Animal Health) and Echocardiography was performed with an ultrasound system (Vevo-2100 ultrasonography system; Visual Sonic, Baltimore, Maryland) as previously described (16). Briefly, M-mode long-axis views were used to measure the thickness of interventricular septal walls at systole and diastole and left ventricle (LV) posterior wall at systole and diastole. LV chamber diameters at end systole and end diastole and LV ejection fraction (LVEF) were also measured using M-mode long-axis images.

Figure S1. Genetic modification of CSE in endothelial cells did not change body weight in mice.
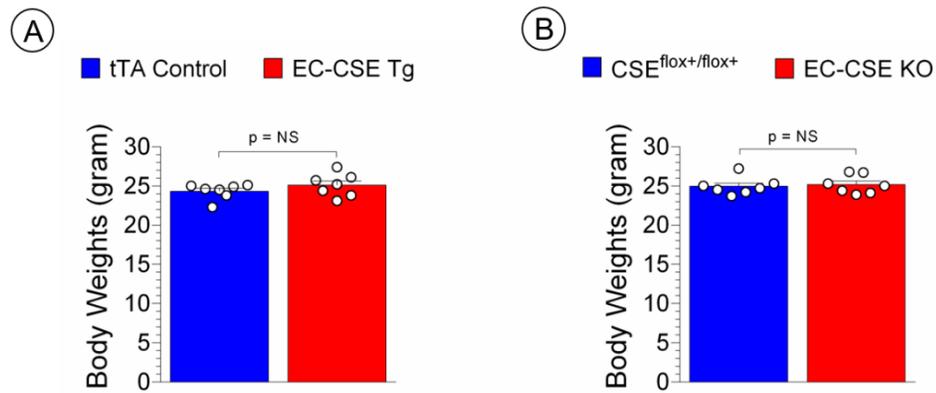

Baseline Body weights were not significantly different between EC-CSE Tg and tTA mice **(A)** or EC-CSE KO and CSE$^{flox+/flox+}$ mice **(B)**. Data are presented as mean ± SEM, n=7, Mann-Whitney U test.

Figure S2. Overexpression of CSE in endothelial cells did not change cardiac structure and function at rest.
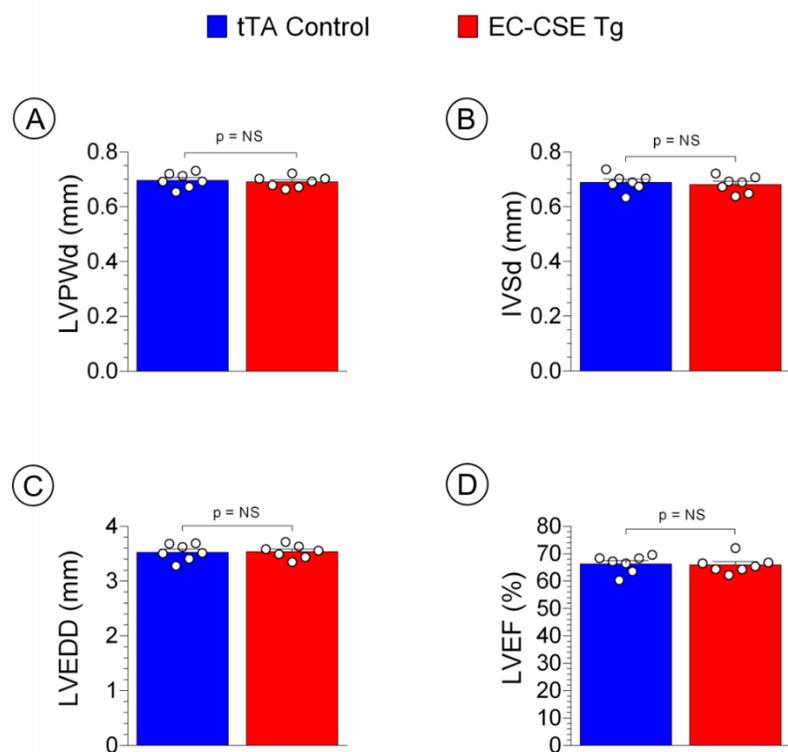
Echocardiography revealed that left ventricle posterior wall thickness at diastole (LVPWd), Interventricular septal thickness at diastole (IVSd), left ventricle end diastolic dimension (LVEDD), and left ventricle ejection fraction (LVEF) were not significantly different in EC-CSE Tg mice compared to tTA controls. Data are presented as mean ± SEM, n=7, Mann-Whitney U test.

Figure S3. Deletion of CSE in endothelial cells did not change cardiac structure and function at rest.
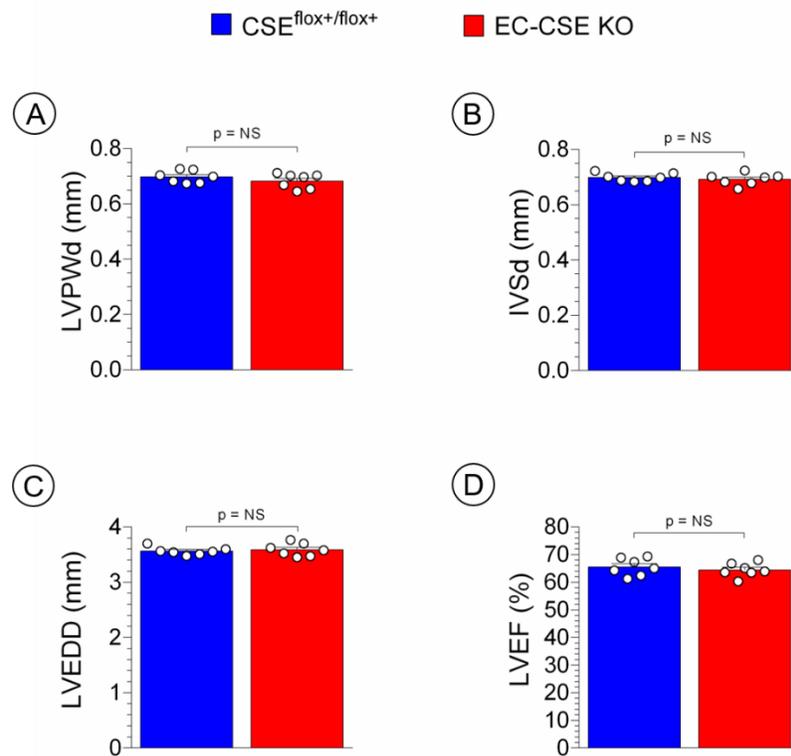
Echocardiography revealed that left ventricle posterior wall thickness at diastole (LVPWd), Interventricular septal thickness at diastole (IVSd), left ventricle end diastolic dimension (LVEDD), and left ventricle ejection fraction (LVEF) were not different in EC-CSE KO mice compared to CSE$^{flox+/flox+}$. Data are presented as mean ± SEM, n=7, Mann-Whitney U test.