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H_2S as a Physiologic Vasorelaxant: Hypertension in Mice with Deletion of Cystathionine γ-Lyase

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Abstract

Studies of nitric oxide over the past two decades have highlighted the fundamental importance of gaseous signaling molecules in biology and medicine. The physiological role of other gases such as carbon monoxide and hydrogen sulfide (H_2S) is now receiving increasing attention. Here we show that H_2S is physiologically generated by cystathionine γ-lyase (CSE) and that genetic deletion of this enzyme in mice markedly reduces H_2S levels in the serum, heart, aorta, and other tissues. Mutant mice lacking CSE display pronounced hypertension and diminished endothelium-dependent vasorelaxation. CSE is physiologically activated by calcium-calmodulin, which is a mechanism for H_2S formation in response to vascular activation. These findings provide direct evidence that H_2S is a physiologic vasodilator and regulator of blood pressure.

Nitric oxide (NO) and carbon monoxide (CO) are established physiologic messenger molecules, and NO has an important role as an endothelial cell–derived relaxing factor (EDRF) and regulator of blood pressure (1,2). Indirect evidence has implicated another endogenous gasotransmitter, hydrogen sulfide (H_2S), in similar functions (3–7). H_2S can be produced by cystathionine γ-lyase (CSE) or cystathionine β-synthase (CBS) (3,4), but definitive evidence for either of these enzymes in the physiologic formation of H_2S is lacking.

To investigate the role of H_2S as a physiologic vasorelaxant and determinant of blood pressure, we generated mice with a targeted deletion of the gene encoding CSE (8) (fig. S1, A to C). The homozygous ($CSE^{-/-}$) and heterozygous ($CSE^{-/+}$) mutant mice were viable, fertile, and indistinguishable from their control wild-type littermates ($CSE^{+/+}$) in terms of growth pattern.

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Materials and Methods
Figs. S1 to S6
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CSE mRNA and protein were absent in heart, aorta, mesenteric artery, liver, and kidneys of $CSE^{-/-}$ mice (fig. S1, D and E). Endogenous H_2S levels in aorta and heart of homozygous mutant male mice ($CSE^{-/-}$) were both decreased by about 80% (Fig. 1A), and H_2S levels in aorta and heart of heterozygous mutant male mice ($CSE^{-/+}$) were both decreased by about 50%. Serum H_2S levels in $CSE^{-/-}$ mice and $CSE^{-/+}$ mice were reduced by about 50 and 20%, respectively (Fig. 1B). Female $CSE^{-/-}$ mice showed a similar decline in H_2S levels (fig. S2, A and B). The residual H_2S in serum may reflect nonenzymatic reduction of elemental sulfur to H_2S or H_2S generated from other tissues that express CBS, another H_2S-generating enzyme (3,5,9).

CSE mutant mice developed age-dependent hypertension. Beginning at 7 weeks of age, both male (Fig. 1C) and female (fig. S2C) $CSE^{-/-}$ mice displayed a higher blood pressure than age-matched wild-type (WT) mice. Blood pressure in the mutant mice peaked at more than 135 mm Hg when the mice were 12 weeks of age; this was almost 18 mm Hg higher than that in control mice. Heterozygous $CSE^{-/+}$ mice also showed elevated blood pressure beginning at 7 weeks of age. The rise in blood pressure was similar in homozygous and heterozygous mice until the mice were 10 weeks of age; after this point, the blood pressure of $CSE^{-/-}$ mice was about 10 mm Hg higher than that of $CSE^{-/+}$ mice. Blood pressure levels assessed by the tail-cuff method were confirmed by direct monitoring of arterial blood pressure through intra-carotid artery catheterization (fig. S3A). Heart rates were similar in mutant and WT mice. In humans, CSE activity increases rapidly after birth, reaching adult levels when infants are about 3 months of age (10, 11). The age-dependent hypertension of the mutant mice paralleled the ontogeny of CSE in mice, increasing to peak adult levels 3 weeks after birth (12). Endogenous H_2S levels in brains from $CSE^{-/-}$ mice were similar to WT mouse values (fig. S3B), consistent with evidence that CSE is not the source of brain H_2S (3, 5, 11, 12), and this similarity suggests that the hypertension in the mutant mice is not due to alterations in the central nervous system. In addition, endothelial NO synthase (eNOS) protein was not decreased in $CSE^{-/-}$ mice, which indicated that the hypertension was not due to a loss in NO-mediated vasorelaxation. Kidney architecture was also preserved in the $CSE^{-/-}$ mice, which signifies that the elevation in blood pressure was not caused by renal damage (fig. S4).

H_2S relaxes blood vessels and lowers blood pressure by opening ATP-sensitive K^+ channels in vascular smooth muscle (4,13,14). We explored whether exogenous H_2S could influence the hypertension of $CSE^{-/-}$ animals. Intravenous bolus injections of NaHS, an H_2S donor (4, 13,15), elicited dose-dependent transient decreases in systolic blood pressure of both anesthetized $CSE^{-/-}$ and $CSE^{+/+}$ mice (Fig. 1D). The magnitude of decline was greater in mutant versus WT mice, which suggested that the former have a heightened sensitivity to H_2S. NaHS injections did not alter the heart rate of WT or mutant mice. Intravenous bolus injections of ammonium (39 μmol/kg) or pyruvate (39 μmol/kg), the two other products of CSE activity (3,16), did not influence blood pressure or heart rate. Plasma levels of oxobutanoate, an intermediate in the catabolism of cystathionine by CSE, were similar in $CSE^{-/-}$ and $CSE^{+/+}$ mice (fig. S3C).

CSE deficiency may elicit accumulation of homocysteine and diminished levels of L-cysteine (17). In 10-week-old male $CSE^{-/-}$ mice, plasma homocysteine and L-cysteine levels were, respectively, about 18 and 0.8 times the levels seen in age-matched WT mice, whereas the levels in $CSE^{-/+}$ mice were, respectively, about 2.0 and 0.8 times those in WT mice (Fig. 1, E and F). A similar decrease in L-cysteine levels was observed in female $CSE^{-/-}$ mice (fig. S2E). To ascertain whether the hypertension of $CSE^{-/-}$ mice reflects hyperhomocysteinemia (18), we administered L-methionine to WT mice in their drinking water for 6 weeks. This intervention augmented plasma homocysteine levels (fig. S3D), but did not alter blood pressure (fig. S3E). Also, while plasma homocysteine levels in male $CSE^{-/-}$ mice were nine times higher than those in male $CSE^{-/+}$ mice (Fig. 1E), blood pressure in the two genotypes was similar

(Fig. 1C). Moreover, female and male $CSE^{-/-}$ displayed similar blood pressures (fig. S5A), despite females having six times the plasma homocysteine levels and homocysteine/cysteine ratios seen in males (Fig. 1E and figs. S2D and S5B). Thus, homocysteine is unlikely to be the principal determinant of hypertension in the CSE mutant mice.

We next investigated whether hypertension in the $CSE^{-/-}$ mice reflected alterations in the vascular redox state. Analysis of vascular tissue indicated that the levels of superoxide anion, a reactive oxygen species (ROS) that regulates vascular tone, were not significantly different in $CSE^{-/-}$ versus WT mice (fig. S6A). Glutathione (GSH) levels were moderately decreased in the aorta and mesenteric artery beds of the mutant mice (fig. S6B), possibly as a result of the modest decreases seen in L-cysteine levels. As substantially greater decreases of GSH are not associated with hypertension, it is unlikely that GSH plays a major role in the hypertension of CSE mutant mice (19).

To investigate mechanisms underlying $CSE^{-/-}$ hypertension, we examined blood vessel responses of the mutant mice. Phenylephrine contracts blood vessels by activating α-adrenoceptors in vascular smooth muscle, whereas H_2S and NO directly relax the muscle (4, 20). By contrast, relaxation after cholinergic stimulation reflects influences on endothelium (21). Phenylephrine evoked contraction of mesenteric arteries to a similar extent in WT and $CSE^{-/-}$ mice (Fig. 2A), and the NO donor sodium nitroprusside produced a similar vasorelaxation response in mutant and WT mesenteric arteries (Fig. 2B). H_2S more potently relaxed mesenteric arteries of $CSE^{-/-}$ mice, with a median inhibitory concentration (IC_{50}) of 75 μM, as compared with WT mice (IC_{50} = 120 μM), consistent with supersensitivity associated with diminished formation of endogenous H_2S (Fig. 2C). By contrast, methacholine-induced relaxation of mesenteric arteries that had been constricted by phenylephrine was markedly impaired in mutant mice (Fig. 2D), and endothelial removal abolished methacholine relaxation of both WT and mutant arteries (Fig. 2E). Immunohistochemistry experiments revealed that CSE protein predominantly localized to the endothelium, with faint staining in smooth muscle (Fig. 2F). In an earlier study, we had shown that CSE mRNA is expressed in vascular smooth muscle (4). Our reexamination of these data revealed that CSE mRNA is also expressed in the endothelium. Thus, H_2S displays properties characteristic of an EDRF. It is formed in endothelium, and prevention of its synthesis impairs relaxation elicited by a neurotransmitter that acts via the endothelium, but does not impair effects of agents that act directly on smooth muscle. The extent to which H_2S, NO, or CO contribute to EDRF activity in different vascular beds is unclear.

How does endothelial stimulation generate H_2S? eNOS and heme oxygenase-2 (HO-2), the biosynthetic enzymes for NO and CO, respectively, are activated by calcium-calmodulin (1, 22). Thus, endothelial activation by substances such as acetylcholine or bradykinin elicits formation of inositol 1,4,5-trisphosphate, which releases intracellular calcium to stimulate formation of NO or CO. We found a similar mode of regulation for CSE. Using recombinant CSE, we demonstrated its direct binding to calmodulin, which was abolished by the calcium chelator EGTA and the calmodulin antagonist W7 (Fig. 3A). Catalytic activity of pure CSE was increased more than twofold by calcium and calmodulin, but not by either substance alone (Fig. 3B), a level of stimulation similar to the NO- and CO-generating enzymes eNOS (23) and HO-2 (22). Calcium-dependent regulation of CSE was also evident in endothelial cells, which contain abundant levels of CSE (Fig. 3C). H_2S formation by these cells was markedly augmented by the calcium ionophore A23187, with the increase blocked by the calcium chelator BAPTA [1,2-bis(2-aminophenoxy)ethane- *N*,*N*,*N′*,*N′*-tetraacetic acid] and by W7 (Fig. 3D). These agents markedly reduced basal levels of H_2S, which indicated that H_2S generation by CSE is physiologically regulated by calcium-calmodulin.

Endothelial-dependent vasorelaxation reflects muscarinic cholinergic activation of eNOS (24). We demonstrated similar regulation of H_2S formation. Thus methacholine treatment of endothelial cells tripled H_2S levels (Fig. 3E), an effect blocked by the anticholinergic drug atropine. Depletion of CSE by RNA interference markedly diminished the enhancement of H_2S formation by methacholine and A23187 and lowered basal levels of H_2S (Fig. 3F).

In summary, we have established CSE as the physiologic source of H_2S in multiple tissues, especially the vascular system. Mice genetically deficient in this enzyme display marked hypertension, comparable to that of $eNOS^{-/-}$ mice (24–26). Our findings are consistent with the previous observation that administration of the CSE inhibitor D,L-propargylglycine elevates blood pressure (27). H_2S has properties in common with physiologic EDRFs. Thus, blood vessel relaxation in response to muscarinic stimulation is profoundly reduced in CSE-deficient mice. Moreover, CSE is predominantly localized to the endothelial layer of blood vessels. The EDRF activity of H_2S reflects muscarinic activation of intracellular calcium release, with calcium-calmodulin physiologically stimulating CSE. NO-mediated EDRF activity arises through a similar mechanism.

Although NO is well established as an EDRF, in numerous vascular beds EDRF activity is only partially diminished by NO synthase inhibitors and in mice lacking the gene for eNOS (28,29). In our experiments, EDRF activity of murine mesenteric arteries from mutant mice lacking CSE was reduced by about 60%, which suggests that H_2S functions as an EDRF in this vascular bed. The similar elevation of blood pressure in mice with CSE and eNOS knockouts implies that H_2S influences vascular systems underlying peripheral resistance to an extent comparable to the action of NO. A physiologic role for H_2S in regulating blood pressure raises the possibility that pharmacologic enhancement of H_2S formation could be an alternative approach for treatment of hypertension.

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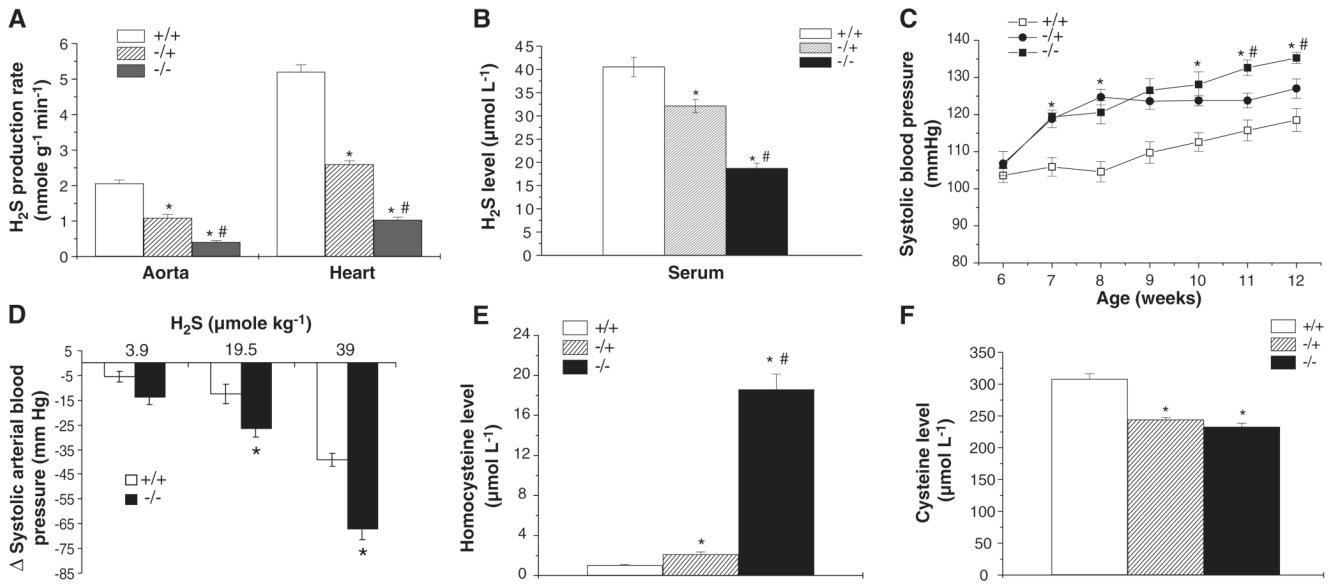

Fig. 1.

Phenotype of CSE male knockout mice. (**A**) Reduced H_2S production from aorta and heart tissues in $CSE^{-/-}$ mice and $CSE^{-/+}$ mice. Number of mice are given for each group; $n = 16$. (**B**) Reduced serum H_2S level in $CSE^{-/-}$ mice and $CSE^{-/+}$ mice ($n = 8$ to 10). (**C**) Age-dependent increase in blood pressure of $CSE^{-/-}$ mice and $CSE^{-/+}$ mice ($n = 12$). (**D**) H_2S administration lowers systolic arterial blood pressure in 10-week-old $CSE^{-/-}$ mice ($n = 13$ to 15). (**E**) Increased plasma homocysteine level in $CSE^{-/-}$ mice and $CSE^{-/+}$ mice ($n = 19$). (**F**) Decreased plasma L-cysteine level in $CSE^{-/-}$ mice and $CSE^{-/+}$ mice ($n = 15$). All results are means ± SEM. *$P < 0.05$ versus WT; #$P < 0.05$ versus heterozygote.

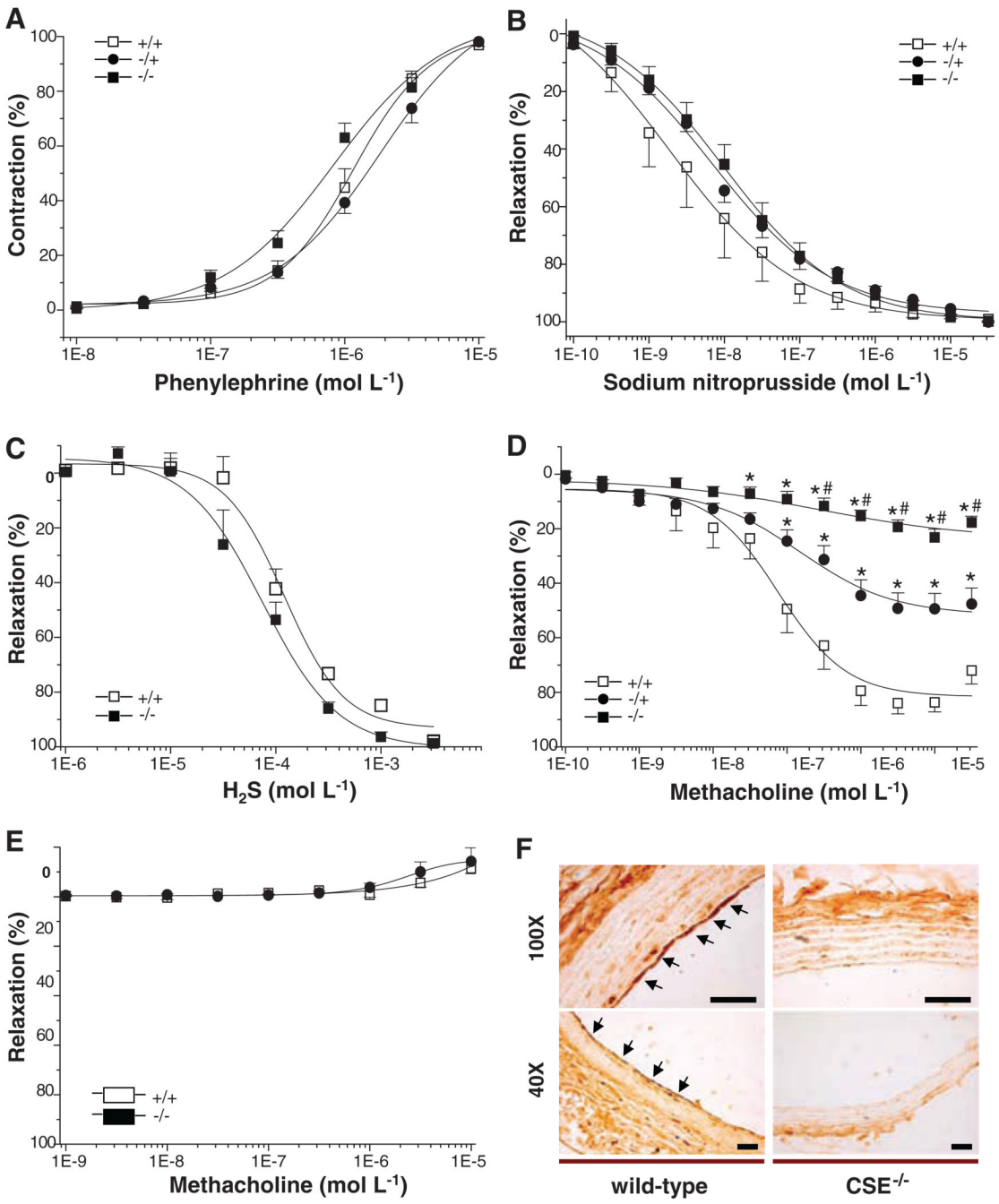

Fig. 2.

Impaired endothelial function in CSE mutant mice. Contraction of mesenteric artery evoked by phenylephrine (**A**) and relaxation of mesenteric artery by sodium nitroprusside (**B**), H_2S (**C**), and methacholine (**D**). $n = 15$ for each group. All results are means ± SEM. *$P < 0.05$ versus WT; #$P < 0.05$ versus heterozygote. (**E**) Endothelial removal abolishes methacholine-induced relaxation of mesenteric artery. No relaxation occurs in vessels of WT or mutant mice after stripping of the endothelium. For $CSE^{-/-}$ mice, $n = 8$; and for $CSE^{+/+}$ mice, $n = 9$. (**F**) Immunohistochemical localization of CSE to arterial endothelium (black arrows) is abolished in $CSE^{-/-}$ mice. Scale bars, 20 μm.

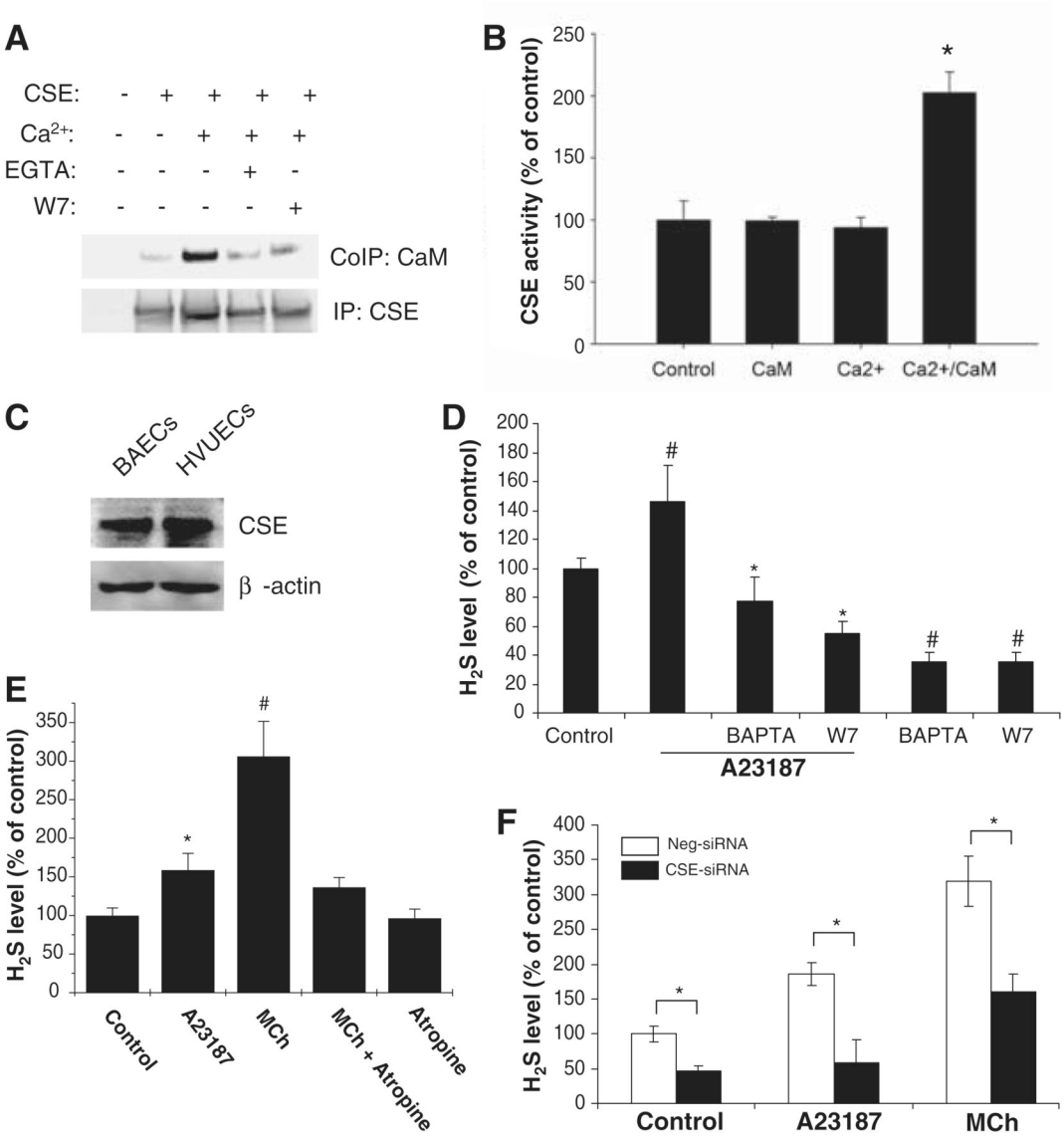

Fig. 3.

CSE is activated by calcium-calmodulin upon muscarinic cholinergic stimulation of vascular endothelial cells. (**A**) Calmodulin binds CSE in vitro in the presence of calcium (2 mM). The interaction is diminished by the calcium chelator, EGTA (1 mM), as well as the calmodulin antagonist W7 (100 μM). (**B**) Calcium-calmodulin activates purified CSE in vitro. Calcium (1 mM) or calmodulin (5 μM) separately has no effect on CSE activity. $n = 3$. *$P < 0.05$ versus the control. (**C**) CSE is endogenously expressed in bovine aortic endothelial cells (BAECs) and human umbilical vein endothelial cells (HUVECs). (**D**) CSE is activated in BAECs treated with the calcium ionophore A23187 (1 μM) for 10 min. Incubation beforehand with the acetoxy-methylester of the intracellular calcium chelator BAPTA (BAPTA-AM. 50 μM) or W7 (50μmM) for 30 min prevents CSE activation. $n = 3$. *$P < 0.05$ versus A23187 treatment; #$P < 0.05$ versus control. (**E**) CSE is strongly activated in BAECs treated with the muscarinic agonist methacholine (MCh, 1 μM) for 10 min. The activation is twice as much as with similar concentrations of A23187. The stimulatory effect of MCh is abolished by the muscarinic antagonist atropine (50 μM) ($n = 3$ or 4). *$P < 0.05$ versus control; #$P < 0.05$ versus all other groups. (**F**) CSE is the endogenousH_2S generator in BAECs. Transfecting cells with 100 nM

CSE-specific short interfering RNA (CSE-siRNA) for 48 hours markedly diminishes the enhanced H_2S production observed with A23187 (1 μM) or MCh (1 μM). Western blotting showed that CSE protein is decreased about 60 to 70% by CSE-siRNA ($n = 3$). *$P < 0.05$.