H_2S mediates O_2 sensing in the carotid body

Ying-Jie Peng[a], Jayasri Nanduri[a], Gayatri Raghuraman[a], Dangjai Souvannakitti[a], Moataz M. Gadalla[b], Ganesh K. Kumar[a], Solomon H. Snyder[b,c,d,1], and Nanduri R. Prabhakar[a,1]

[a]Center for Systems Biology for O_2 sensing, Department of Medicine, University of Chicago, IL 60637; and [b]Department of Pharmacology and Molecular Sciences, [c]Solomon H. Snyder Department of Neuroscience; and [d]Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205-2105

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Gaseous messengers, nitric oxide and carbon monoxide, have been implicated in O_2 sensing by the carotid body, a sensory organ that monitors arterial blood O_2 levels and stimulates breathing in response to hypoxia. We now show that hydrogen sulfide (H_2S) is a physiologic gasotransmitter of the carotid body, enhancing its sensory response to hypoxia. Glomus cells, the site of O_2 sensing in the carotid body, express cystathionine γ-lyase (CSE), an H_2S-generating enzyme, with hypoxia increasing H_2S generation in a stimulus-dependent manner. Mice with genetic deletion of CSE display severely impaired carotid body response and ventilatory stimulation to hypoxia, as well as a loss of hypoxia-evoked H_2S generation. Pharmacologic inhibition of CSE elicits a similar phenotype in mice and rats. Hypoxia-evoked H_2S generation in the carotid body seems to require interaction of CSE with hemeoxygenase-2, which generates carbon monoxide. CSE is also expressed in neonatal adrenal medullary chromaffin cells of rats and mice whose hypoxia-evoked catecholamine secretion is greatly attenuated by CSE inhibitors and in CSE knockout mice.

cystathionine γ-lyase | hydrogen sulfide | hypoxia | hemeoxygenase-2

In adult mammals, carotid bodies are the sensory organs responsible for monitoring arterial blood O_2 concentrations and relay sensory information to the brainstem neurons associated with regulation of breathing and the cardiovascular system (1). Carotid bodies are comprised mainly of two cell types: glomus (also called type I) and sustanticular (or type II) cells. Glomus cells, of neuronal nature, are considered the main hypoxia-sensing cells. The gaseous messengers, carbon monoxide (CO) and nitric oxide (NO), generated by hemeoxygenase-2 (HO-2) and neuronal nitric oxide synthase (nNOS), respectively, physiologically inhibit carotid body activity (2–4). Because HO-2 and nNOS require molecular O_2 for their activity, stimulation of carotid body activity by hypoxia may reflect in part reduced formation of CO and NO (5).

Like NO and CO, hydrogen sulfide (H_2S) is a gasotransmitter physiologically regulating neuronal transmission (6) and vascular tone (7). Cystathionine γ-lyase (CSE) (EC 4.4.1.1) and cystathionine β-synthase (CBS) (4.2.1.22) are the major enzymes associated with generation of endogenous H_2S (8, 9). CBS is the predominant H_2S-synthesizing enzyme in the brain, CSE preponderates in the peripheral tissues whose H_2S levels are reduced 90% in $CSE^{-/-}$ mice (7–10). Given that carotid bodies are peripheral organs and that H_2S is redox active, we hypothesized that CSE-derived H_2S plays a role in hypoxic sensing by the carotid body. We examined carotid body response to hypoxia in wild-type ($CSE^{+/+}$) and $CSE^{-/-}$ mice as well as in rats treated with CSE inhibitor. Genetic deletion or pharmacologic inhibition of CSE dramatically impairs hypoxic sensing by the carotid body as well as in neonatal adrenal medullary chromaffin cells (AMC).

Results

Loss of Carotid Body Response to Hypoxia in $CSE^{-/-}$ Mice. CSE immunoreactivity was seen in glomus cells of carotid bodies from $CSE^{+/+}$ mice as evidenced by colocalization with tyrosine hydroxylase (TH), an established marker of glomus cells (11, 12). CSE expression was absent in carotid bodies from $CSE^{-/-}$ mice (Fig. 1*A*). To assess the impact of CSE disruption on carotid body response to hypoxia, carotid bodies were isolated from $CSE^{+/+}$ and mutant mice and "single" unit sensory discharges were recorded from the carotid sinus nerve. In $CSE^{+/+}$ mice, hypoxia augmented the sensory activity in a stimulus-dependent manner (Fig. 1 *B* and *C*). In striking contrast, carotid bodies from $CSE^{-/-}$ mice exhibited severely impaired sensory response to hypoxia (Fig. 1 *B* and *C*). Similar loss of the hypoxic response was also seen in $CSE^{+/+}$ mice treated systemically with DL-propargylglycine (PAG), an inhibitor of CSE (Fig. S1) (13, 14). On the other hand, acute application of PAG was ineffective in preventing the hypoxic response, a finding consistent with a recent report (15).

H_2S generation was monitored in carotid bodies from $CSE^{+/+}$ and $CSE^{-/-}$ mice under normoxia (P_{O_2} ~ 146 mmHg) and hypoxia (P_{O_2} ~ 40 mmHg). Basal H_2S generation under normoxia was significantly less in $CSE^{-/-}$ compared to $CSE^{+/+}$ mice ($CSE^{+/+}$ = 55 ± 3 vs. $CSE^{-/-}$ = 29 ± 1 nmol/h/mg protein; 53% less in mutant mice). Hypoxia increased H_2S generation in $CSE^{+/+}$ but not in mutant mice ($CSE^{+/+}$ mice 2.5-fold increase vs. $CSE^{-/-}$ mice 0.3-fold increase; $P < 0.001$; Fig. 1*D*).

To assess whether the attenuated hypoxic response in mutant mice was due to reduced excitability of the carotid body, sensory response to CO_2, another physiological stimulus, was determined. Carotid bodies from $CSE^{+/+}$ and $CSE^{-/-}$ mice responded to CO_2 with a comparable increase in sensory discharge (Fig. 1 *E* and *F*).

Impaired Ventilatory Response to Acute Hypoxia in $CSE^{-/-}$ Mice. Ventilatory responses to 21% O_2 (normoxia) and 12% O_2 (hypoxia) were determined by whole-body plethysmography in awake, nonsedated $CSE^{+/+}$ and $CSE^{-/-}$ mice. Baseline ventilation (V_E) was significantly lower in $CSE^{-/-}$ compared to $CSE^{+/+}$ mice (Fig. 2 *A* and *B*), which was due to significantly lower respiratory rates (RR) in mutant mice (Table S1). More importantly, the magnitude of hypoxic ventilatory response was significantly less in $CSE^{-/-}$ compared to $CSE^{+/+}$ mice (Fig. 2 *A* and *B*), which was due to decreased stimulation of RR and tidal volume (V_T) (Table S1). However, changes in O_2 consumption (V_{O_2}) and CO_2 production (V_{CO_2}) under hypoxia were comparable between mutant and $CSE^{+/+}$ mice (Table S1). In contrast, $CSE^{+/+}$ and $CSE^{-/-}$ mice responded to hypercapnia (5% CO_2) with comparable increases in V_E (Fig. 2 *A* and *C*), RR, and V_T (Table S2).

CSE and Hypoxic Sensitivity of the Rat Carotid Body. To assess whether CSE contributes to the carotid body hypoxic response in species other than mouse, CSE expression and the effect of its pharmacologic inhibition by PAG were determined in rat carotid bodies. CSE immunoreactivity was found in glomus cells of the

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[1]To whom correspondence may be addressed. E-mail: ssnyder@jhmi.edu or nanduri@uchicago.edu.

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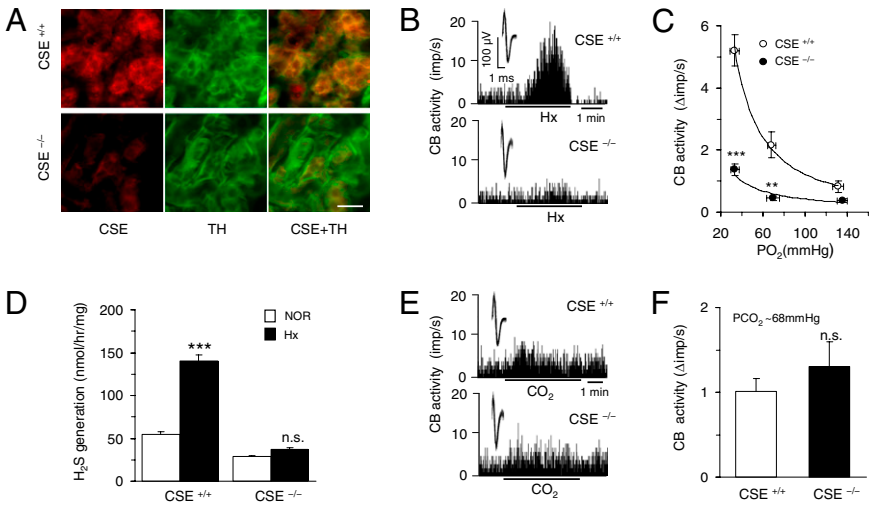

Fig. 1. CSE localization in the mouse carotid body and carotid body responses to hypoxia and hypercapnia in $CSE^{+/+}$ and $CSE^{-/-}$ mice. (*A*) CSE expression in carotid bodies from $CSE^{+/+}$ and $CSE^{-/-}$ mice. Carotid body sections were stained with antibodies specific for CSE or tyrosine hydroxylase (TH), a marker of glomus cells. (Scale bar: 20 μm.) (*B*) Sensory response of isolated carotid bodies to hypoxia (Hx) (P_{O_2} ~ 39 mmHg; at black bar) in $CSE^{+/+}$ and $CSE^{-/-}$ mice. Integrated carotid body sensory activity (CB activity) is presented as impulses per second (imp/s). Superimposed action potentials from the single fiber are presented in *Inset*. (*C*) Carotid body responses to graded hypoxia from $CSE^{+/+}$ and $CSE^{-/-}$ mice, measured as the difference in response between baseline and hypoxia (Δimp/s). Data are mean ± SEM of $n = 24$ ($CSE^{+/+}$) and $n = 23$ ($CSE^{-/-}$) fibers from eight mice each. (*D*) H_2S levels (mean ± SEM) in carotid bodies from $CSE^{+/+}$ and $CSE^{-/-}$ mice under normoxia (NOR) and hypoxia (Hx) (P_{O_2} ~ 40 mmHg) from four independent experiments. (*E*) Example illustrating carotid body responses to CO_2 (P_{CO_2} ~ 68mmHg; at black bar) in $CSE^{+/+}$ and $CSE^{-/-}$ mice. (*F*) Average data (mean ± SEM) of CO_2 response from $n = 24$ ($CSE^{+/+}$) and $n = 19$ ($CSE^{-/-}$) fibers from eight mice in each group. *** and **, $P < 0.001$ and $P < 0.01$, respectively; n.s. (not significant), $P > 0.05$.

rat carotid body (Fig. 3*A Left*). Rat carotid bodies being ~3 times bigger than those found in mouse (~80 μg in rat vs. 25 μg in mouse), we were able to determine the effects of graded hypoxia on H_2S generation. Basal H_2S generation in rat carotid body was 266 ± 61 nmol/h/mg protein which was significantly higher than in mouse carotid body (55 ± 3 nmol/h/mg protein; $P < 0.01$). Hypoxia increased H_2S levels in a stimulus-dependent manner (Fig. 3*A Right*). The magnitude of hypoxia-evoked H_2S production was comparable in rat and mouse (~5-fold increase in rats vs. ~3.5-fold in mice at P_{O_2} ~ 40 mmHg). In PAG-treated rats, basal H_2S levels were reduced by 55% (vehicle = 266 ± 61 vs. PAG = 147 ± 50 nmol/h/mg protein; $P < 0.05$) and hypoxia-evoked H_2S generation was nearly absent in the carotid bodies (Fig. 3*A Right*).

In vehicle-treated rats, hypoxia augmented sensory activity in a stimulus-dependent manner, and this response was significantly attenuated in PAG-treated rats (Fig. 3*B*). The attenuated hypoxic response in PAG-treated rats could be secondary to accumulation of cysteine resulting from CSE inhibition. However, 100 μM L-cysteine had no significant effect on the hypoxic response (Fig. S2). In sharp contrast, carotid body response to CO_2 was significantly augmented in PAG-treated rats (same rats tested for hypoxia; Fig. 3*C*).

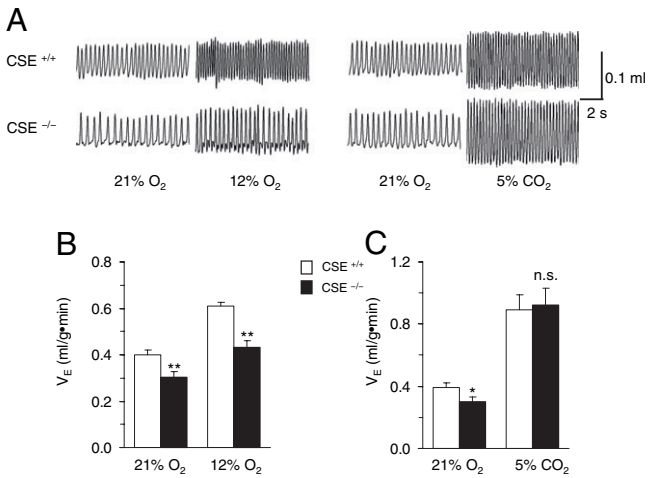

Fig. 2. Ventilatory responses to hypoxia and hypercapnia in $CSE^{+/+}$ and $CSE^{-/-}$ mice. Ventilation was measured in unsedated mice by whole body plethysmography under normoxia (21% O_2), hypoxia (12% O_2), and hypercapnia (5% CO_2). Hypoxia and hypercapnia lasted for 5 min. Representative tracings of breathing are shown in *A* and average data of minute ventilation (V_E) in response to 12% O_2, i.e., hypoxia (*B*) and 5% CO_2 i.e., hypercapnia (*C*). The data presented are mean ± SEM from eight $CSE^{+/+}$ and $CSE^{-/-}$ mice each. **, $P < 0.01$; n.s. (not significant), $P > 0.05$.

H_2S Stimulates Carotid Body Sensory Activity. The effects of exogenous administration of H_2S on the sensory activity of the carotid body were examined. NaHS, an H_2S donor, augmented carotid body activity in rats (Fig. 4*A*), and in $CSE^{+/+}$ and $CSE^{-/-}$ mice (Fig. S3). Like hypoxia, carotid body response to NaHS was stimulus-dependent, occurred within seconds after its application, and sensory activity promptly returned to baseline after termination of the stimulus (Fig. 4*A Center* and *Right*).

Ca^{2+} influx into glomus cells is critical for carotid body response to hypoxia (1). To assess whether Ca^{2+} is also important for sensory excitation by H_2S, carotid body responses to NaHS were examined in the presence of Ca^{2+}-free and EGTA-containing medium. Carotid body responses to NaHS as well as to hypoxia were abolished in the presence of Ca^{2+}-free and EGTA containing medium (Fig. 4*B*). On the other hand, glibenclamide, a potent inhibitor of ATP-sensitive potassium (K_{ATP}) channels which mediate H_2S responses in vasculature (16), failed to prevent carotid body response either to NaHS or hypoxia (Fig. S4).

HO-2 Modulates H_2S Generation by CSE. Glomus cells express HO-2, an enzyme that generates CO (3). To assess whether the HO-2-CO system affects CSE activity, H_2S generation was determined in $CSE^{+/+}$ and $CSE^{-/-}$ carotid bodies in the presence and absence of Cr (III) mesoporphyrin IX chloride [Cr(III)MP], an inhibitor

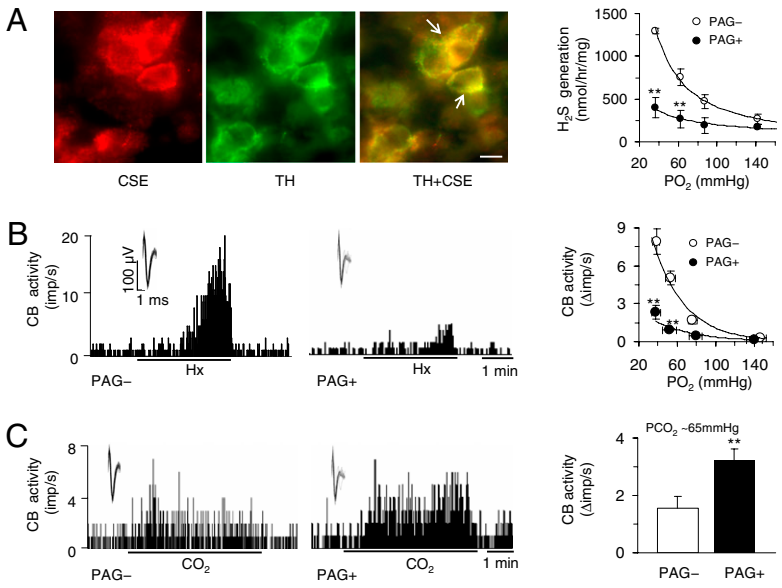

Fig. 3. CSE localization in the rat carotid body and rat carotid body responses to hypoxia and hypercapnia in the absence and presence of DL-propargylglycine. (*A*) Cystathionine γ-lyase (CSE) expression in rat carotid body. Carotid body sections were stained with antibodies specific for CSE and tyrosine hydroxylase (TH), a marker of glomus cells (*Left*). Effects of graded hypoxia on H_2S levels in vehicle-(PAG–) and DL-propargylglycine (PAG+)-treated carotid bodies. Data are mean ± SEM from five individual experiments (*Right*). (*B*) Examples of carotid body response to hypoxia (Hx) (P_{O_2} = 38 mmHg; at black bar) in vehicle- and PAG-treated rats (*Left*). Average (mean ± SEM) data of sensory response to graded hypoxia (*Right*), PAG– *n* = 12 fibers from six rats; PAG+ *n* = 10 fibers from six rats. (*C*) Example of carotid body response to CO_2 in the same rats as in *B* (P_{CO_2} ~ 68 mmHg; at black bar (*Left*)] and average (mean ± SEM) data of CO_2 response (*Right*). Data derived from *n* = 9 fibers (PAG–) and *n* = 10 (PAG+) fibers from six rats each. In *B* and *C*, integrated carotid body sensory activity (CB activity) is presented as impulses per second (imp/s). Superimposed action potentials from the single fiber are presented in *Inset*. **, $P < 0.01$.

of HO-2 (17). As little as 1 μM Cr(III)MP increased H_2S generation 6-fold in $CSE^{+/+}$ mice carotid bodies under normoxia, and this response was abolished in mutant mice (Fig. 5*A*). Systemic administration of Cr(III)MP significantly increased baseline carotid body activity and potentiated the hypoxic response in $CSE^{+/+}$ but not in mutant mice (Fig. 5 *B–D*). In rat carotid bodies, Cr(III)MP also enhanced H_2S generation under normoxia, and a CO donor (tricarbonyldichlororuthenium, $[Ru(CO)_3Cl_2]_2$) inhibited H_2S generation by hypoxia nearly to the levels seen under normoxia (Fig. S5).

CSE Disruption Impairs Hypoxic Sensing by Neonatal Adrenal Medullary Chromaffin Cells (AMC). Hypoxia stimulates catecholamine secretion from neonatal AMC (18, 19). To assess the role of CSE in AMC, studies were performed on neonatal mice and rats at age P10. CSE immunoreactivity was seen in mice and rat AMC (Fig. 6 *A* and *B*, *Top*) but was absent in AMC from $CSE^{-/-}$ mice. Hypoxia-evoked catecholamine secretion was monitored by amperometry from single AMC harvested from $CSE^{-/-}$ mice and PAG-treated rat pups. Control studies were performed on age matched $CSE^{+/+}$ mice and vehicle-treated rat pups, respectively. In $CSE^{+/+}$ mice, hypoxia elicited robust catecholamine secretion, which was greatly reduced or absent in AMC from mutant mice (Fig. 6*A Middle*). A similar reduction in hypoxia-evoked catecholamine secretion was also seen in PAG-treated rat pups (Fig. 6*B Middle*). In contrast, K^+-evoked (40 mM K^+; nonselective depolarizing stimulus) catecholamine secretion was unaffected in $CSE^{-/-}$ mice and PAG-treated rat pups (Fig. 6 *A* and *B*, *Middle* and *Bottom*). H_2S generation increased under hypoxia in adrenal glands from $CSE^{+/+}$ mice and vehicle-treated rat pups, and this

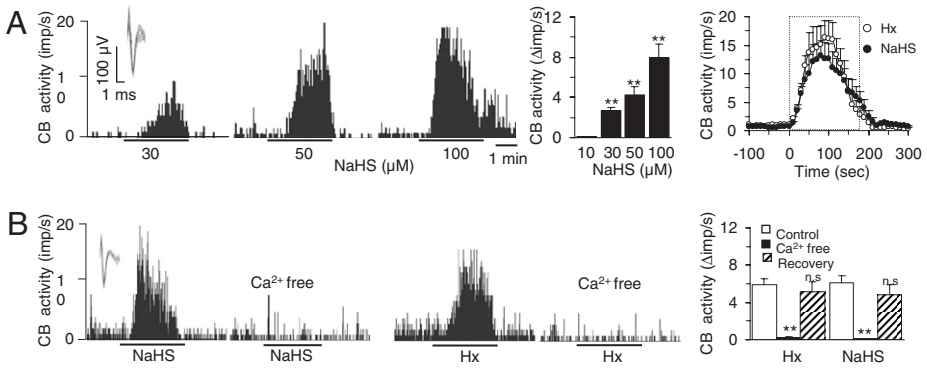

Fig. 4. Effect of NaHS on rat carotid body sensory activity. (*A*) Example of rat carotid body response to increasing concentrations of NaHS, an H_2S donor (at black bar; *Left*). Average (mean ± SEM) data of dose–response to NaHS (*Center*) and time course of sensory response to NaHS (100 μM) and hypoxia [P_{O_2} = 42 mmHg (*Right*)]. Data in middle and right panels were obtained from *n* = 13 fibers from six rats. (*B*) Effect of Ca^{2+} free medium on rat carotid body responses to 100 μM NaHS and hypoxia (Hx) (P_{O_2} = 42 mmHg; at black bar). $CaCl_2$ was replaced by 3 mM $MgCl_2$ and 5 mM EGTA was added to the medium. (*Left*) Example and *Right* average (mean ± SEM) data from five rats (*n* = 8 fibers). In *A* and *B*, Integrated carotid body sensory activity (CB activity) is presented as impulses per second (imp/s). Superimposed action potentials from the single fiber are presented in the inset. **, $P < 0.01$; n.s. (not significant), $P > 0.05$.

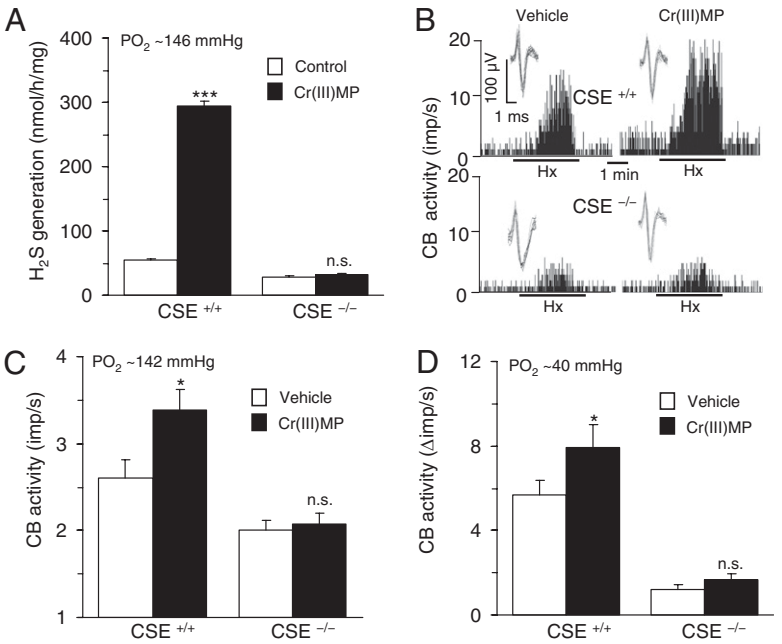

Fig. 5. Effect of HO-2 inhibition on carotid body H_2S generation and sensory activity in $CSE^{+/+}$ and $CSE^{-/-}$ mice. (*A*) Effect of Cr(III)MP (1 μM), an inhibitor of heme oxygenase 2 on H_2S generation in carotid bodies under normoxia (P_{O_2} ~ 146 mmHg) from $CSE^{+/+}$ and $CSE^{-/-}$ mice. Data presented are mean ± SEM from three experiments. Examples of baseline and hypoxic response (Hx) (P_{O_2} ~ 40 mmHg; at black bar) of carotid bodies from vehicle- and Cr(III)MP-treated $CSE^{+/+}$ and $CSE^{-/-}$ mice (*B*) and average data (mean ± SEM) from six mice in each group (n = 8–12 fibers) in *C* and *D*. In *B*, Integrated carotid body sensory activity (CB activity) is presented as impulses per second (imp/s). Superimposed action potentials from the single fiber are presented in *Inset*. *** and *, $P < 0.001$ and $P < 0.05$, respectively; n.s. (not significant), $P > 0.05$.

response was greatly reduced or absent in $CSE^{-/-}$ mice and PAG-treated rat pups, respectively.

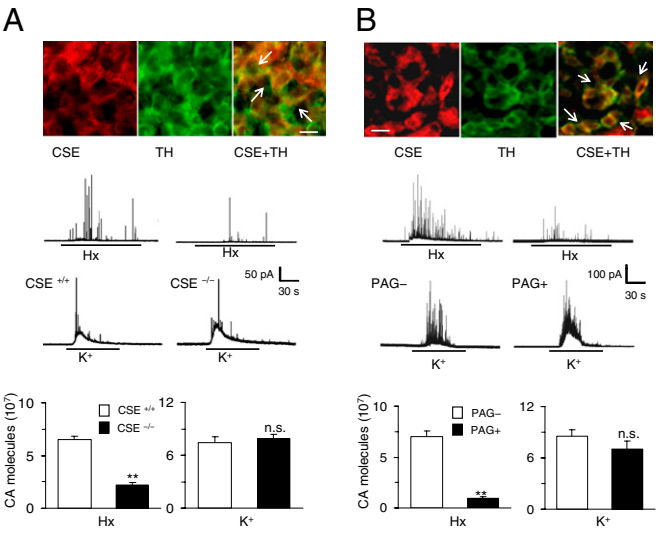

Fig. 6. CSE localization in the mouse and rat neonatal adrenal medullary chromaffin cells (AMC) and the effect of CSE disruption on mouse and rat AMC hypoxia sensing. (*Top*) Cystathionine γ-lyase (CSE) expression in AMC from mice (*A*) and rat pups (*B*). (*Middle*) Examples of catecholamine secretion from AMC from neonatal mice (*A*) and rats (*B*) in response to hypoxia (Hx) (P_{O_2} = 36 mmHg) or high K^+ (40 mM). Black bar represents the duration of hypoxia or K^+ application. (*Bottom*) Average data (mean ± SEM) of total catecholamine (CA) secreted during Hx or K^+ (CA molecules 10^7 i.e., number of secretory events multiplied by catecholamine molecules secreted per event). n = 9 cells each from $CSE^{+/+}$ and $CSE^{-/-}$ and n = 10–12 cells from rat pups. **, $P < 0.01$; n.s. (not significant), $P > 0.05$ compared with $CSE^{+/+}$ mice or vehicle-treated rat cells.

Discussion

The present study establishes a physiological role for H_2S generated by CSE in mediating hypoxic sensing by the carotid body. CSE is expressed in rat and mouse glomus cells, the main site of O_2 sensing in the carotid body. Moreover, chemoreceptor responses to acute hypoxia were markedly impaired in CSE knockout mice and following pharmacologic inhibition of CSE. The loss of hypoxic sensitivity does not likely reflect reduced carotid body excitability, because sensory response to CO_2 was intact in mutant mice and CSE inhibitor-treated rats. Carotid bodies are the primary mediators of ventilatory stimulation by acute hypoxia. $CSE^{-/-}$ mice exhibited selective loss of ventilatory response to hypoxia but not to CO_2, suggesting that CSE disruption profoundly impacts systemic responses to acute hypoxia by affecting the carotid body.

How might CSE contribute to hypoxic sensing? CSE catalyzes the formation of cysteine from cystathionine, and also generates H_2S from cyst(e)ine (8, 9). The following observations indicate that H_2S generated by CSE mediates carotid body hypoxic sensing. First, hypoxia increased H_2S generation in the carotid body in a stimulus-dependent manner, an effect that was lost in $CSE^{-/-}$ mice as well as in rats treated with a CSE inhibitor. Second, loss of hypoxia-evoked H_2S generation paralleled impaired hypoxic sensing by the carotid body. Third, an H_2S donor, but not L-cysteine, stimulated the carotid body with a time-course and magnitude comparable to that evoked by low O_2. An H_2S donor stimulated carotid body activity in CSE knockout mice, indicating that the loss of hypoxic sensitivity is due to absence of H_2S generation rather than impaired H_2S signaling. These findings demonstrate that during hypoxia, CSE is the main source of H_2S generation in the carotid body, and suggest that CSE contributes to hypoxic sensing by catalyzing H_2S generation.

Interestingly, like the sensory activity, basal H_2S generation in the carotid bodies was low under normoxia. Because HO-2 is an O_2 requiring enzyme, it has been proposed that the low nor-

moxemic sensory activity reflects tonic inhibition by HO-2 generated CO in the carotid body (3). We reasoned that a similar inhibitory influence by the HO-2-CO system might account for low normoxic levels of H_2S. Indeed, an HO-2 inhibitor not only markedly elevated basal H_2S levels but also augmented baseline sensory activity and potentiated the hypoxic response in $CSE^{+/+}$ mice. These effects were absent in $CSE^{-/-}$ mice, implying that HO-2 tonically inhibits CSE. CO generated by HO-2 presumably mediates the inhibition of H_2S generation from CSE, as a CO donor inhibited H_2S generation during hypoxia. How might CO inhibit H_2S generation by CSE? CO interacts with CBS, another enzyme that generates H_2S, by binding to its heme moiety (20). Given that CSE is not a heme containing enzyme; it is likely that CO inhibits CSE activity by interacting with histidine residues, as it does with other proteins (21). Thus, CO appears to physiologically inhibit the CSE-H_2S system with hypoxia reducing HO-2 activity to reverse the inhibition and augment H_2S formation.

Although K_{ATP} channels are targets of H_2S (8, 9, 16), glibenclamide, a potent inhibitor of K_{ATP} channels, was ineffective in preventing carotid body stimulation by NaHS or hypoxia. On the other hand, Ca^{2+} influx appears critical for carotid body stimulation by H_2S as well as hypoxia. Previous studies showed that Ca^{2+} influx via high voltage-gated Ca^{2+} channels, especially the L-type, is necessary for carotid body stimulation by hypoxia (1). L-type Ca^{2+} channels in glomus cells are redox sensitive, activated by hypoxia, and inhibited under normoxia, as well as by gaseous messengers such as NO (22, 23). A recent study demonstrated that H_2S signaling involves covalent modification of redox sensitive cysteine residues in proteins through *S*-sulfhydration (10). It is conceivable that H_2S generated by hypoxia activates L-type Ca^{2+} channels in glomus cells via *S*-sulfhydration. In addition, H_2S might affect Ca^{2+}-activated K^+ currents in glomus cells as suggested recently (15, 24).

Carotid bodies are the main organs for sensing acute hypoxia in adults but in neonates they are relatively insensitive to low O_2 (25, 26). On the other hand, AMC are extremely sensitive to hypoxia in neonates, and low O_2 stimulates catecholamine secretion (18, 19), which is critical for maintaining homeostasis in neonates under hypoxic stress. Like glomus cells, neonatal AMC expressed CSE, and hypoxia-evoked catecholamine secretion was severely impaired in $CSE^{-/-}$ mice and in rats treated with a CSE inhibitor. Because hypoxia also increased H_2S generation in adrenal glands, CSE-H_2S system appears to mediate acute hypoxic sensing by neonatal AMC. However, hypoxic sensitivity of AMC decline with age (19). Whether age-related loss of hypoxic sensing by AMC is associated with developmental decline in CSE expression remains to be investigated. It is interesting to note that H_2S has been demonstrated to mediate O_2 sensing by the trout gill chemoreceptors, indicating that it is an ancient well conserved system across phyla (27).

Reflexes arising from the carotid body have been implicated in pathological situations including sleep-disordered breathing with recurrent apnea i.e., periodic cessations of breathing (28). Patients with recurrent apnea experience periodic hypoxemia or intermittent hypoxia and are prone to autonomic morbidities including hypertension (29). In rodent models chronic intermittent hypoxia enhances carotid body responses to hypoxia, and the ensuing chemo-reflex mediates increases in sympathetic nerve activity resulting in elevated blood pressure (28). By reducing carotid body activity, CSE inhibitors or other interventions that reduce H_2S formation may afford therapeutic benefits for sleep apnea and related conditions.

Methods

Preparation of Animals. Experiments were approved by the Institutional Animal Care and Use Committee of the University of Chicago and were performed on age-matched, male $CSE^{+/+}$ and $CSE^{-/-}$ mice (2–3 mo old) and male Sprague–Dawley rats (2–3 mo old) except where otherwise noted. In the experiments requiring sedation, animals were anesthetized with i.p. injections of urethane (1.2 g/kg; Sigma). Supplemental doses, 10% of the initial dose, of urethane were given when corneal reflexes and responses to toe pinch persisted. Animals were allowed to breathe spontaneously. Core body temperature was monitored by a rectal thermistor probe and maintained at 37 ± 1 °C by a heating pad. At the end of the experiment, animals were euthanized by intracardiac injection (0.1 mL) of euthanasia solution (Beuthanasia-D Special; Schering-Plough).

Immunohistochemistry. Carotid bodies and adrenal glands were harvested from anesthetized rats or mice (urethane 1.2 g/kg, i.p.) perfused with heparinized saline followed by 4% paraformaldehyde. The protocols for fixation of carotid bodies and adrenal glands were essentially the same as described in refs. 3 and 11. For assessing CSE immunoreactivity, sections (8 μm thick) were incubated at room temperature for 2 h with polyclonal rabbit anti-CSE antibody (1:400), this antibody was raised using bacterially purified full-length His-tagged CSE as antigen, and monoclonal mouse anti-tyrosine hydroxylase (1:2,000; Sigma), an established marker of glomus and chromaffin cells (11, 12), followed by Texas red-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG (1:250; Molecular Probes) in PBS with 1% normal goat serum and 0.2% Triton X-100. After washing with PBS, sections were mounted in DAPI-containing media and visualized using a fluorescent microscope (Eclipse E600; Nikon).

Carotid Body Sensory Activity. Sensory activity from carotid bodies ex vivo was recorded as described in refs. 30 and 31. Briefly, carotid bodies along with the sinus nerves were harvested from anesthetized mice or rats, placed in a recording chamber (volume, 250 μL) and superfused with warm physiological saline (35 °C) at a rate of 2.5 mL/min having the composition 140 mM NaCl/5.4 mM KCl/2.5 mM $CaCl_2$/0.5 mM $MgCl_2$/5.5 mM Hepes/11 mM D-glucose/5 mM sucrose, and the solution was bubbled with 100% O_2 and the pH was adjusted to 7.35. Carotid bodies were challenged with graded hypoxia by switching the perfusate equilibrated with gas mixtures containing varying levels of O_2. For assessing the carotid body response to CO_2, bicarbonate-buffered medium equilibrated with either 90% O_2 + 5% CO_2 (baseline) or 90% O_2 + 10% CO_2 balance N_2 was used. Clearly identifiable action potentials (two to three active units) were recorded from one of the nerve bundles with a suction electrode and stored in a computer via a data acquisition system (PowerLab/8P; AD Instruments). "Single" units were selected based on the height and duration of the individual action potentials using a spike discrimination program (Spike Histogram Program, Power Laboratory; AD Instruments). In each carotid body, at least two chemoreceptor units were analyzed. The P_{O_2} and P_{CO_2} of the superfusion medium were determined by a blood gas analyzer (ABL 5; Radiometer).

Measurements of Respiratory Variables. Ventilation was monitored by whole-body plethysmograph, and O_2 consumption and CO_2 production were determined by the open-circuit method in unsedated mice as described in ref. 31. Ventilation was recorded while the mice breathed 21% or 12% O_2-balance N_2. Each gas challenge was given for 5 min. O_2 consumption and CO_2 production were measured at the end of each 5-min challenge. For determining ventilatory response to CO_2, baseline ventilation was determined while mice breathed 21% O_2 followed by hypercapnic challenge with 5% CO_2–21% O_2–balance N_2. Sighs, sniffs, and movement-induced changes in breathing were monitored and excluded in the analysis. All recordings were made at an ambient temperature of 25 ± 1 °C.

Measurements of H_2S Levels. H_2S levels in the carotid body were assayed as described in refs. 32 and 33 with few modifications. Briefly, carotid bodies were pooled (four carotid bodies per experiment in rats; six carotid bodies per experiment in mice) and tissue homogenates were prepared in 100 mM potassium phosphate buffer (pH 7.4). The enzyme reaction was carried out in sealed tubes flushed with either N_2 or different levels of O_2-N_2 gas mixtures. The P_{O_2} of the reaction medium was determined by blood gas analyzer (ABL5). The assay mixture in a total volume of 500 μL contained (in final concentration) 800 μM L-cysteine, 80 μM pyridoxal 5′-phosphate, 100 mM potassium phosphate buffer (pH 7.4), and tissue homogenate (2 μg of protein). The reaction mixture was incubated at 37 °C for 1 h and at the end of the reaction, alkaline zinc acetate (1% wt/vol; 250 μL) and trichloroacetic acid (10% vol/vol) was added sequentially to trap H_2S generated and to stop the reaction, respectively. The zinc sulfide formed was reacted sequentially with acidic *N*,*N*-dimethyl-*p*-phenylenediamine sulfate (20 μM) and ferric chloride (30 μM) and the absorbance was measured at 670 nm using a microplate reader. A standard curve relating the concentration of Na_2S

and absorbance was used to calculate H_2S concentration and expressed as nanomoles of H_2S formed per hour per milligram protein.

Measurements of Catecholamine Secretion from Chromaffin Cells. The protocols for preparation of chromaffin cells and measurements of catecholamine secretion by amperometry are essentially the same as described in ref. 18. Briefly, adrenal glands were harvested from anesthetized mice and rats aged P10. Chromaffin cells were enzymatically dissociated, plated on collagen (type VII; Sigma) coated coverslips, and maintained at 37°C in a 5% CO_2 incubator for 12–24 h. The growth medium consisted of F-12 K medium (Invitrogen) supplemented with 10% horse serum, 5% FBS, and 1% penicillin/streptomycin/glutamine mixture (Invitrogen). Catecholamine secretion from chromaffin cells was monitored by amperometry using carbon fiber electrodes as described in ref. 18. Amperometric recordings were made from adherent cells that were superfused (flow rate ~1.0 mL/min, chamber volume ~80 μL) with a medium having the composition 1.26 mM $CaCl_2$/0.49 mM $MgCl_2{\cdot}6H_2O$/0.4 mM $MgSO_4{\cdot}7H_2O$/5.33 mM KCl/0.441 mM KH_2PO_4/137.93 mM NaCl/0.34 mM $Na_2HPO_4{\cdot}7H2O$/5.56 mM dextrose/20 mM Hepes (pH 7.35) and 300 mOsmol. Normoxic solutions were equilibrated with room air (P_{O_2} ~ 146 mmHg), and hypoxic solutions were equilibrated with appropriate gas mixtures that yielded medium P_{O_2} of ~30–40 mmHg as measured by blood gas analyzer.

Drugs. Rats and mice were treated with DL-propargylglycine (Sigma; 200 mg/kg) or Cr (III) Mesoporphyrin IX chloride [1 mg/kg (CrM459; Frontier Scientific)] administered intraperitoneally 2–3 h before acute experiments on anesthetized animals. NaHS (Sigma) was given acutely to the ex vivo carotid body preparation. In the in vitro assays for H_2S measurements, known concentrations of HO-2 inhibitor and CO donor (tricarbonyldichlororuthenium [II] dimer, $[Ru(CO)_3Cl_2]_2$; Sigma) were added to cell lysates. All solutions with drugs were prepared fresh during the experiments.

Data Analysis. In unsedated mice RR (breaths/min), V_T (μL), minute ventilation [V_E (mL/min) = RR × V_T], O_2 consumption (V_{O_2}, mL/min), and CO_2 production (V_{CO_2}, mL/min) were analyzed. Respiratory variables (RR and V_T) were averaged for at least 20 consecutive breaths over 5 min of inspired O_2 and CO_2 challenge. V_T, V_E, V_{O_2}, and V_{CO_2} were normalized to body weight. Carotid body sensory activity (discharge from "single" units) was averaged during 3 min of baseline and during the 3 min of gas challenge and expressed as impulses per second unless otherwise stated. The number of secretory events and the amount of catecholamine secreted per secretory event were analyzed in each experiment and the data were expressed as total catecholamines secreted. Average data are presented as mean ± SEM. Statistical significance was assessed by either ANOVA or two-way ANOVA with repeated measures followed by Tukey's test. $P < 0.05$ was considered significant.

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