Production of the gaseous signal molecule hydrogen sulfide in mouse tissues

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Abstract

The gaseous molecule hydrogen sulfide (H$_2$S) has been proposed as an endogenous signal molecule and neuromodulator in mammals. Using a newly developed method, we report here for the first time the ability of intact and living brain and colonic tissue in the mouse to generate and release H$_2$S. This production occurs through the activity of two enzymes, cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS). The quantitative expression of messenger RNA and protein localization for both enzymes are described in the liver, brain and colon. Expression levels of the enzymes vary between tissues and are differentially distributed. The observation that tissues that respond to exogenously applied H$_2$S can endogenously generate the gas strongly supports its role as an endogenous signal molecule.

Keywords

immunohistochemistry; transsulferation; aseptic dissection; gastrointestinal; cysteine catabolism

Introduction

Hydrogen sulfide (H$_2$S), like its gaseous molecular cousins nitric oxide and carbon monoxide, is becoming recognized as a regulator of physiological function. Exogenous NaHS, which liberates H$_2$S, produces physiological responses in many systems. In the cardiovascular system, H$_2$S relaxes vascular smooth muscle (Hosoki et al. 1997; Zhao et al. 2001), inhibits platelet aggregation (Zagli et al. 2007) and reduces the output force of the left ventricle of the heart (Geng et al. 2004). In the gastrointestinal system, H$_2$S relaxes ileal smooth muscle (Teague et al. 2002; Hosoki et al. 1997), increases colonic secretion (Schicho et al. 2006), and reduces gastric injury to nonsteroidal anti-inflammatory drugs (Fiorucci et al. 2005). In the central nervous system, H$_2$S increases the sensitivity of NMDA receptors to glutamate in hippocampal neurons to enhance synaptic transmission (Abe and Kimura 1996; Kimura 2000). Inhaled H$_2$S induces a reversible suspended animation-like state in mice (Blackstone et al. 2005). H$_2$S exerts both nociceptive (Patacchini et al. 2004; Lee et al. 2008) and antinociceptive effects (Distrutti et al. 2006).

H$_2$S exists at the lowest oxidative state of sulfur along with thiols like cysteine or sulfides like methionine. In biological systems, H$_2$S is generated by the reduction of sulfate or

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elemental sulfur, which occurs in sulfur-reducing bacteria and archaea, or by its liberation from thiols and sulfides. Because mammals lack the ability to reduce elemental sulfur, sulfite or sulfate, they rely on the catabolism of the essential amino acids methionine and cysteine from the diet as a source of all reduced sulfur. Cysteine is also synthesized from methionine in a process called transsulfuration. H$_2$S has long been recognized as a product of cysteine degradation. Interestingly, the enzymes that catabolize cysteine, cystathionine-$\beta$-synthase (CBS; 4.2.1.22) and cystathionine-$\gamma$-lyase (CSE; EC 4.4.1.1) are the same as those involved in transsulfuration (Stipanuk 2004). The activities of these two H$_2$S-producing enzymes have been studied in homogenized tissues under hypoxic conditions (Stipanuk and Beck 1982). To date, however, the production of endogenous H$_2$S under physiological conditions in intact and living tissue has not been demonstrated. Our objectives were to determine whether H$_2$S is produced and released in intact and living brain, liver and gut tissue in the mouse, to quantitate the expression levels of CBS and CSE and to localize CBS and CSE protein in tissues using fluorescence immunohistochemistry.

**Methods**

**Tissue dissection**

All animal procedures were approved by the Mayo Clinic Animal Care and Use Committee. Mice (C57/Bl6 strain; 6-10 weeks of age; 20-25g; Jackson Laboratories, Bar Harbor, ME) were euthanized by CO$_2$ asphyxiation, and transcardially perfused with ice-cold phosphate buffered saline (PBS; 0.1M; pH 7.4). The liver, brain and the external muscle layers of the colon including the myenteric plexus located between the longitudinal and circular muscle layers were rapidly dissected from the animals. Because colonic bacteria produce copious H$_2$S (Suarez et al. 1998), we developed a microdissection technique to obtain the external muscle layers of the intact gastrointestinal tract without perforation into the lumen. Briefly, the colon was exteriorized keeping the anus intact by cutting the mesentery. While keeping the colon contiguous with the entire gastrointestinal tract, the segment of colon was held to the bottom of a Sylgard-coated Petri dish, which was filled with ice cold normal Krebs solution (NKS) and bubbled with 97% O$_2$ and 3% CO$_2$, via U-shaped pins. The NKS consisted of (in mM): Na$^+$, 137.4; K$^+$, 5.9; Ca$^{2+}$, 2.5; Mg$^{2+}$, 1.2; Cl$^-$, 134; HCO$_3^-$, 15.5; H$_2$PO$_4^-$, 1.2; and glucose, 11.5. The anus and cecum hung over the rim on opposite sides of the dish. Under a dissection microscope, the external muscle layers were removed from the intestinal tube while maintaining the mucosal barrier. Because of the high basal tension of the muscularis mucosa, penetration into the lumen was immediately apparent when the epithelium protruded through the puncture. If this occurred, the dissection was stopped and the tools sterilized before continuing on a new animal. Petri dishes were used only once. An example result of the dissection is shown in Figs. 1A and 1B.

**Measurement of H$_2$S**

We developed a novel system to trap physiologically produced H$_2$S that allowed the tissue to survive during gas collection. Coronal hemisections of the midbrain (0.5 mm), slices of distal lobes of the liver (0.5 cm x 0.5 cm x 0.5 mm) and intact strips of external muscle layers of the colon (10 x 60 mm) were used. They were incubated in NKS containing 10 mM L-cysteine and 2 mM pyridoxal 5’-phosphate. The vials containing the tissues were connected by silastic tubing and hypodermic tubing connectors to a 2 ml vial containing 0.5 ml of 1% (w/v) zinc acetate (Fig. 1C). The zinc acetate solution was not adjusted for pH and did not come in contact with the tissue. A gas mixture of 97% O$_2$ and 3% of CO$_2$ was delivered by silastic tubing into the first vial where it was bubbled at a rate of 1-4 ml • min$^{-1}$ through the solution containing the tissue. The build-up of gas in the first vial increased the pressure thereby forcing gas through silastic tubing to the second vial where it bubbled through the zinc acetate solution. To determine if the O$_2$ required to maintain tissue survival
could contribute to altered H\textsubscript{2}S oxidation and thus affect the measurements of H\textsubscript{2}S production from intact living tissue, we measured the spontaneous degradation of H\textsubscript{2}S in gas mixtures of differing concentrations of O\textsubscript{2}. A mixture of H\textsubscript{2}S (125 ppb) with air degrades at a rate of 0.4% / min while a mixture of H\textsubscript{2}S (125 ppb) with 97% O\textsubscript{2} and 3% CO\textsubscript{2} degrades at a rate of 0.6% / min. With a gas flow rate of 1-4 ml • min\textsuperscript{-1} and an apparatus volume of 6 ml, the spontaneous degradation of H\textsubscript{2}S by 97% O\textsubscript{2} in our system is negligible. The incubation mixture was prepared on ice, all tubing was connected and the reaction was started by transferring the vials to a water bath at 37°C. The reaction was stopped at 30 min by inserting a stainless steel needle into the first vial and injecting 50 μl of 500% (w/v) trichloroacetic acid into the incubation solution. Gas flow was allowed to continue for an additional 30 min to ensure complete trapping of H\textsubscript{2}S in the zinc acetate.

For H\textsubscript{2}S measurements in tissue homogenates, tissues were homogenized with a polytron for 5 min at a ratio of 1 ml of ice-cold 50 mM potassium phosphate (pH 6.8) per 40 mg of tissue. The incubation solution contained 10 mM L-cysteine, 2 mM pyridoxal 5\textsuperscript{'}-phosphate, 100 mM potassium phosphate buffer (pH 7.4) and either 1.2% (w/v; liver or colon) or 0.6% (w/v; brain) tissue homogenate. The reduced concentration of brain tissue was used as higher concentrations created too many bubbles that blocked gas flow during the hour-long incubation.

For both assay setups, following the 1 h incubation period, the zinc acetate solutions were capped and stored at room temperature until measured using gas chromatography (Levitt \textit{et al.} 1999). Briefly, the sulfide content of the zinc acetate solution was determined via acidification of the solution and measurement of the H\textsubscript{2}S released into a gas space. After 10 min incubation, the H\textsubscript{2}S content of the gas space was determined using a gas chromatograph (model 5890 Hewlett-Packard Company, Palo Alto, CA) equipped with a Teflon column (8 feet × 1/8 inch) packed with Chromosil 330 (Supelco, Bellefonte, PA). The column was maintained at 80° with a nitrogen flow rate of 20 ml • min\textsuperscript{-1}. A 0.3 ml aliquot of the gas space was injected into the chromatograph, and the H\textsubscript{2}S peak was identified and quantified with a sulfur chemiluminescence detector (model 355, Sievers, Boulder, CO) via comparison of the area under the curve of the unknown with that of authentic H\textsubscript{2}S standards of known concentration. The detection limit of this technique (peak area twice that of background) is roughly 40 parts per billion (ppb). Experimental and control samples were run in triplicate for six to nine mice. Data are expressed as molar amount of H\textsubscript{2}S min\textsuperscript{-1} mg\textsuperscript{-1} tissue.

\textbf{RT-qPCR}

Tissue was rapidly dissected from mice transcardially perfused with ~60 ml of ice-cold PBS. Liver samples were obtained from distal lobes. Brain samples included the entire brain. The external muscle layers of the colon including the myenteric plexus were dissected free from the mucosal and submucosal layers. Total RNA was extracted from flash-frozen tissue using RNA-Bee (Tel-Test INC., Friendswood, TX, USA), following the manufacturer’s instructions. The RNA pellets obtained were dissolved in nuclease-free water by incubating at 55-60°C for 10 min and the quality and quantity of the extracted RNA were determined spectrophotometrically by checking the 260/280 absorbance ratios. Approximately 10 μg of this RNA was subjected to DNase digestion using the RNAeasy centrifugation protocols according to the manufacturer’s instructions (Qiagen, Valencia, CA). The resulting RNA was quantified spectrophotometrically in triplicate and 1 μg of RNA was used to generate cDNA via reverse transcription (RT) reactions using GeneAmp Gold RNA PCR kit systems according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA, USA).

An Applied Biosystems Prism 7000 Sequence Detector was used for real-time PCR. Forward and reverse primer sets for mouse CSE and CBS, as well as the housekeeping
genes β-actin and GAPDH were purchased from SuperArray Bioscience (Frederick, MD). A 1:5.55 dilution of the RT reaction and 0.4 nM of the appropriate primer set in 1× CyberGreen master mix (SuperArray) was first subjected to 10 min at 95°C to activate the Taq polymerase followed by 40 cycles of 15 sec at 95°C and 2 min at 60°C. Melting curves generated with a dissociation protocol were used to ensure the specificity of primers for a single product for each reaction (Ririe et al. 1997). Plasmid DNA (TOPO2.1 vector, Invitrogen, Carlsbad, CA) containing the inserted PCR products were constructed to verify the sequence of the products and used to generate standard curves for each gene product. The cycle number at which the fluorescence intensity crosses a standard threshold value (Ct) for each sample was converted to transcript copy number using the standard curves of concentration of plasmid DNA versus Ct. These standard curves were linear between 30 and 3 million copies with R^2 values of 0.9826, 0.9947, 0.9989 and 0.9968 for β-actin, GAPDH, CSE and CBS amplification, respectively. The slopes of these lines for β-actin, GAPDH, CSE and CBS specific amplification were −3.32 ± 0.18, −3.59 ± 0.37, −3.34 ± 0.06 and −3.04 ± 0.09, respectively, indicating amplification efficiency within the acceptable range for absolute quantification. The molar quantity of CSE and CBS calculated for each sample is normalized to the average molar quantity of both β-actin and GAPDH and presented as pmol gene of interest (GOI) over nmol housekeeping genes (HKG).

**Immunohistochemistry**

The localizations of CSE and CBS proteins were examined in mouse tissues using immunohistochemistry. Liver, brain and colons were removed from mice transcardially perfused with ~60 ml of ice-cold PBS and 12 ml PBS containing 4% paraformaldehyde and immersion-fixed in 4% paraformaldehyde for 2 h, dehydrated in 30% sucrose overnight, embedded in OCT, cryosectioned to 5 or 15 μm-thick sections and thaw mounted on gelatin-coated slides. Whole mount preparations of the external muscle layers of the mouse colon were prepared by opening the intestine along the mesenteric border, pinning the segment flat on a Sylgard-coated dish and fixing for 2 h. After fixation, the mucosa and submucosa were removed by fine forceps. Preparations of the external muscle layers containing the myenteric plexus were then washed with 0.1 M PBS. Tissue sections and whole-mount colonic tissue were incubated for 5 min in Universal Mouse Blocking Solution (ID Labs, London, ON) to bind all native mouse IgG, followed by 2 h at room temperature with PBS containing 0.5% Triton X-100 and 4% normal goat or normal donkey sera. This solution was removed and the tissue or sections were incubated overnight at room temperature with this solution containing one of four primary antisera: polyclonal rabbit anti-CSE (1:500; a gift from Dr. Solomon Snyder, Johns Hopkins University, Baltimore, MD); monoclonal mouse anti-CSE (1:300; Abnova, Taiwan); polyclonal mouse anti-CBS (1:500; Abnova); or monoclonal mouse anti-CBS (1:1000; Abnova). Following this incubation, sections or tissue were washed with PBS (3 × 15 min), and incubated with either Cy3-conjugated goat anti-mouse IgG, donkey anti-mouse IgG, goat anti-rabbit IgG, or donkey anti-rabbit IgG (Jackson ImmunoResearch, West grove, PA) in PBS for 2 h. Finally, the tissue was washed with PBS (3 × 15 min) and dipped briefly in H2O to remove salts. Whole-mount tissue was mounted on glass slides and both sections and tissue were coverslipped with a solution to prevent fluorophore bleaching (CitiFluor AF1). The immunostained tissue was viewed on an Olympus BX51W1 epifluorescence microscope and images were taken using MagnaFire software (Optronics, Goleta, CA). Several controls ensured that immunoreactivity was specific for the protein of interest. Immunoreactivity obtained by incubation of tissue with antisera respectively preabsorbed with either 1 μM recombinant CSE or 0.5 μM recombinant CBS (Abnova) was significantly reduced or blocked (Figs. 4H and 5H). The staining patterns were similar for each of the two secondary antisera used for each primary antisera. Likewise, similar staining patterns were obtained for both primary antisera used to detect each protein of interest. The neuronal cytoplasm staining was more diffuse using the rabbit
anti-CSE antiserum compared to the mouse anti-CSE antiserum, but was present and specific nonetheless.

Results

To determine if living tissue is capable of producing $H_2S$, we used brain slices, intact sections of the liver, and freshly dissected external muscle layers of the colon. Intact coronal hemisections of the mouse midbrain, as well as the intact external muscle layers of the colon containing the myenteric plexus, generated detectable levels of $H_2S$. This production of $H_2S$ was enzymatic because incubation of the tissues with the CSE and CBS inhibitors DL-propargylalanine (2 mM) and hydroxylamine (2 mM), respectively, significantly reduced $H_2S$ production (Fig. 2A). Intact sections of the mouse liver did not generate detectable levels of $H_2S$ that were greater than tissues in the presence of the enzyme inhibitors. These data indicate that the brain and the external layers of the colon, but not the liver, with native subcellular organization are capable of generating and releasing $H_2S$. Using this system, we also measured $H_2S$ production in homogenized tissues. Homogenates of the brain, colonic muscle tissue and liver generated $H_2S$ that were all significantly reduced by the presence of CSE and CBS inhibitors (Fig. 2B).

The result that $H_2S$ is continuously synthesized in intact and living tissue and reduced with inhibitors of CBS and CSE led us to quantify the expression levels of these enzymes using real-time PCR. The transcripts for both CSE and CBS were identified in total RNA isolated from the brain, the external muscle layers of the colon, which includes the myenteric plexus, and the liver (Fig. 3). The expression levels of these enzymes varied between regions with expression being highest in the liver by 10-1000-fold compared to the brain and colon. CSE was expressed at similar levels in the brain and the colonic external muscle layers, while CBS was expressed at nearly 300-fold higher levels in the brain compared to the colon. Within each region, the relative levels of CSE and CBS varied. CSE levels were nearly two-fold more abundant than CBS levels in the liver, and 4- to 5-fold more abundant than CBS in the colonic muscle layers. In the brain, the inverse relationship was observed where CBS was expressed at levels approximately 6-fold more than CSE.

Because the tissues that were studied are cellularly heterogeneous, we next localized CSE and CBS protein expression using fluorescence immunohistochemistry. Specificity of the immunostaining was determined by using different primary and secondary antisera and preabsorption of the immunosera with recombinant proteins. Specific immunoreactivity for CSE was identified in the liver, brain and colon (Fig. 4). In the liver, the immunoreactivity was localized on what appeared to be the plasma membranes, and, with lesser intensity, in the cytoplasm of hepatocytes. While it is also likely that immunoreactivity for CSE was present in cells of the Space of Disse or in the extracellular matrix, the staining pattern was not discrete enough to identify a morphological class of cells such as Kupffer cells. In the brain, we found CSE-immunoreactivity in the cytoplasm of many neuronal cell bodies, including cortical neurons, hippocampal neurons and Purkinje cells of the cerebellum. Immunoreactivity was not present in all neurons. For example, only a few cells in the granular layer of the cerebellum displayed positive staining. CSE-immunoreactivity was not as prevalent in the processes of neurons as compared to the cell bodies with only a few clear examples of neurite staining (Fig. 4E). Intense immunoreactivity for CSE was present in major fiber tracts throughout the brain including the corpus callosum, internal capsule and the white matter of the cerebellum. These staining patterns are suggestive of positive CSE-immunoreactivity in the glial networks of the brain. In the colon, intense immunoreactivity for CSE was present in myenteric neurons. When examined in en face preparations, CSE-immunoreactivity was clearly identified in the cell bodies of myenteric and submucosal neurons (Fig. 4C). Like in the central nervous system (CNS), there was little evidence for specific staining in the processes of enteric neurons. Unlike the CNS, however, there was
little evidence for CSE-immunoreactivity in glial cells within ganglia of the enteric nervous system. Diffuse immunoreactivity for CSE was present in the lamina propria and some discrete cells in this sub-epithelial region.

Specific immunoreactivity for CBS was present in the liver, brain and colon (Fig. 5). Similar to the staining pattern of CSE in the liver, immunoreactivity for CBS was present in hepatocytes and structures within the Space of Disse. In the brain, the most intensely stained CBS-immunoreactive structures were major commissural fiber tracts including the corpus callosum, the ventral hippocampal commissure, the commissure of the inferior colliculus, and the anterior and posterior commissures. Other fiber tracts including the optic tract (Fig. 5C), the internal capsule (Fig. 5D), the white matter of the cerebellum (Fig. 5E), the fornix, the fasciculus retroflexus, and the optic nerve layer as well as the intermediate and deep white layers of the superior colliculus. These heavily myelinated structures suggest that CBS-immunoreactivity was localized primarily to glial cells and perhaps more specifically to oligodendrocytes. In thin sections with sparse immunoreactivity, stellate-shaped glial cells displayed specific staining for CBS (Fig. 5F). In the colon, like the pattern of CSE staining, CBS-immunoreactivity was present in the lamina propria (Fig. 5A). Unlike the CSE staining pattern, however, specific immunoreactivity for CBS was not typically detected in the external muscle layers and was not in myenteric neurons.

Discussion

This is the first report to demonstrate H$_2$S production in living intact tissue. Using a potentiometric assay, Benavides and colleagues (Benavides et al. 2007) recently demonstrated the ability of isolated human red blood cells to convert the polysulfides of garlic into H$_2$S. This methodology has not been applied to tissues where H$_2$S production has been demonstrated in homogenized tissue samples under hypoxic conditions using passive diffusion of the gas into solutions of heavy metals to chelate sulfides (Stipanuk and Beck 1982). We used a similar approach to these studies by using a zinc acetate solution to trap labile H$_2$S, but in contrast to previous procedures, we utilized a cascading gas flow system to facilitate transfer of the gas. Using this system, the rate of H$_2$S production in homogenized brain and liver tissues of the present study were similar to previous reports (Abe and Kimura 1996; Stipanuk and Beck 1982). Likewise, homogenized samples of the external layers of the colon including the myenteric plexus generated large amounts of H$_2$S. The relative rates of H$_2$S production in homogenized tissue were consistent with the relative levels of mRNA expression for the enzymes in each of the three tissues. In order to directly test our hypothesis that living tissue is capable of generating H$_2$S, we utilized our gas-flow system to deliver oxygen and carbon dioxide to a carbonate-based physiological buffer containing living intact tissue and subsequently trap the labile H$_2$S in a zinc acetate solution. Using this approach we demonstrate that both brain slices, and the intact external muscle layers of colon containing the myenteric plexus are capable of generating H$_2$S from cysteine. The rate of H$_2$S production by living brain and colonic tissue was less than their homogenates, and the intact liver did not produce detectable levels of H$_2$S despite the high rate of production of the gas from homogenized liver.

There are several possible mechanisms for the difference in rates of H$_2$S production between homogenized and intact tissue. The disruption of the tissue into the entire volume of the incubation solution likely facilitates the diffusion of the H$_2$S gas and thus the apparently higher rates of production. The difference in rates may also be due to the disruption in homogenized tissues of the normal proximity of the enzymes that produce the gas to the enzymes responsible for its degradation. Intact cells have a robust enzymatic degradation pathway for H$_2$S (Furne et al. 2001; Levitt et al. 1999; Picton et al. 2002; Weisiger et al. 1980). Thus, the amount of H$_2$S we measured is the H$_2$S that escaped the degradation...
pathway. In classical studies in which acetylcholine (Loewi and Navratil 1926) and
noradrenaline (Rosell et al. 1963) were established as neurotransmitters, specific inhibitors
of their degradative and reuptake pathways, respectively, were used thereby increasing the
amount of transmitter available for assay in the bathing medium. We were unable to block
H$_2$S degradative pathways because there are no known specific inhibitors of these pathways.
Likewise, we took no steps to induce or enhance release of H$_2$S. We suggest, therefore, that
the production of H$_2$S in intact and living tissue might in fact be much higher than that
measured here and that the H$_2$S synthetic enzymes, or stimulators of enzyme activity, were
constitutively active in these tissues. H$_2$S production is regulated by the expression levels of
CSE and CBS. To date, no study has determined whether enzymatic H$_2$S production can be
regulated in an acute manner. A benefit of the methodology described here to measure H$_2$S
production from intact tissue, is that acute regulation of enzyme activity, be it by neural
activity or other biochemical process, can now be assessed in real time.

While both CSE and CBS, the enzymes that catalyze the reaction of cysteine to H$_2$S, are
highly expressed in the liver, there is a differential expression of the two enzymes in the
brain and colon. In the brain, CBS expression is highest in cells within major fiber tracts
such as the corpus callosum, internal capsule, or the white matter of the cerebellum rather
than throughout the neuropil. This is most evident in the molecular layer of the cerebellum
and in the hippocampus, where there was little evidence for specific immunoreactivity.
Cellular processes extend from the major fiber tracts, especially in the cerebellum and
neocortex, in a radial fashion. Cells with stellate morphologies with long processes were
observed in thin sections containing sparsely populated immunoreactive cells. The
localization to major myelin-containing fiber tracts and the stellate morphology is consistent
with these cells being oligodendrocytes (Baumann and Pham-Dinh 2001). Astrocytes and
radial glial cells may also express CBS, which has been suggested previously (Enokido et al.
2005). The localization of CBS to glial cells is consistent with one previous report of CBS
immunoreactivity in the rodent brain (Enokido et al. 2005), but contradicts another report
that observed neuronal staining in the mouse brain (Robert et al. 2003). While CSE
immunoreactivity is contained in the same major fiber tracts as CBS, the intensity is reduced
compared to CBS. This is consistent with a lower level of mRNA encoding CSE compared
to CBS in the brain. The readily identifiable structure that displayed immunoreactivity for
CSE was neuronal cell bodies. The staining was consistent with most large neurons
containing immunoreactivity. Many small neurons, such as those in the granular layer of the
cerebellum, and throughout the brain stem, did not contain immunoreactivity for CSE. To
our knowledge, this is the first report of immunoreactivity for CSE in the central nervous
system. Others have identified the presence of CSE in the brain previously using enzymatic
assays that measure the conversion of cystathionine to cysteine or glutathione (Griffiths and
Tudball 1976; Rassin and Gaull 1975; Vitvitsky et al. 2006), by northern blot (Ishii et al.
2004), by RT-PCR (Geng et al. 2004), or by observing increased brain levels of
cystathionine following inhibition of CSE (Kodama et al. 1985; Yu et al. 2000). Others,
however, have reported that the brain lacks CSE (Abe and Kimura 1996). In the colon, both
CSE and CBS are highly expressed in the mucosa, but only CSE appears to be expressed in the
external muscle layers including the myenteric plexus. The absence of CBS
immunoreactivity in the enteric nervous system is not consistent with a previous report from
the guinea pig and human (Schicho et al. 2006), which may reflect species differences. Our
finding, however, is consistent with the very low expression levels of mRNA encoding CBS
in the external muscle layers of the colon including the myenteric plexus. The differential
expression of CSE and CBS in different tissues tends to support the concept that each
enzyme has a more prominent role in each of the various tissues. Either enzyme has the
capability to generate H$_2$S from cysteine. Using single enzyme inhibitors or gene knock-out
approaches, previous studies suggest that CBS activity predominates in the brain (Abe and
Kimura 1996) while CSE activity predominates in the vascular system (Hosoki et al. 1997; Zhao et al. 2001).

The data presented in the current study regarding transcript levels of the two enzymes support the concept of differential contribution of each enzyme to H\textsubscript{2}S production, however, this study raises the possibility that expression of an enzyme may not directly correspond to its activity. It is possible that localized concentrations of endogenous inhibitors of CSE or CBS could affect the relative activities of these enzymes. Both enzymes catalyze multiple reactions involved in the metabolic pathways of the amino acids serine, cysteine and methionine involving transsulfuration reactions (Stipanuk 2004). Competitive substrate inhibition for differing catalytic activities could contribute to overall activity levels of the H\textsubscript{2}S producing moiety of these enzymes. This potential mechanism for endogenous enzyme regulation deserves further study.

Several studies have demonstrated physiological effects of exogenously applied NaHS on brain and colonic tissues (Schicho et al. 2006; Abe and Kimura 1996; Kimura 2000). The results of this study show that tissues that respond to exogenously applied NaHS also generate and release H\textsubscript{2}S. These data provide key evidence that H\textsubscript{2}S is an endogenous gaseous signal molecule. With this evidence, the distinction of H\textsubscript{2}S as the third endogenous gaseous signal molecule is now established.

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Abbreviations

\begin{itemize}
  \item \textbf{H\textsubscript{2}S} \quad \text{hydrogen sulfide}
  \item \textbf{CBS} \quad \text{cysthionine-\textbeta-synthase}
  \item \textbf{CSE} \quad \text{cysthionine-\textgamma-lyase}
  \item \textbf{PBS} \quad \text{phosphate buffered saline}
  \item \textbf{NKS} \quad \text{normal Krebs solution}
  \item \textbf{RT} \quad \text{reverse transcription}
  \item \textbf{PCR} \quad \text{polymerase chain reaction}
  \item \textbf{HKG} \quad \text{housekeeping genes}
  \item \textbf{GOI} \quad \text{gene of interest}
\end{itemize}

References


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Fig. 1.
Photomicrographs of hematoxylin and eosin stained frozen sections of mouse colon before (A) and after (B) removal of the external muscle layers using the procedure described in the text. Note that although some muscle tissue was left behind, the muscularis mucosa layer remained intact. Thus, dissected external muscle layers were never exposed to luminal contents. (C) Schematic illustration of the experimental setup used to measure H$_2$S production in intact tissue. NKS = normal Krebs solution; ZnAc = zinc acetate.
Fig 2.
A) Segments of intact external muscle layers of the colon containing the myenteric plexus and intact brain slices incubated in normal Krebs solution generated detectable levels of H$_2$S that was blocked by incubation of tissue in propargylalanine (2 mM) and hydroxylamine (2 mM), inhibitors of CSE and CBS, respectively (*P<0.05 compared to basal samples; two-way ANOVA with Bonferroni post-hoc test). Intact sections of liver did not generate levels of H$_2$S greater than liver incubated with CSE and CBS inhibitors. Data are the mean (±SEM) rate of H$_2$S production for N=6 (brain and liver) or 9 (colon) animals with triplicate values for each animal. The weight of the tissue samples was between 14 and 29 mg. B) Samples of homogenized external colon muscle including the myenteric plexus, brain, and liver produced significant amounts of H$_2$S that was blocked by incubation of the homogenates in propargylalanine (2 mM) and hydroxylamine (2 mM) (*P<0.05 compared to basal sample, two-way ANOVA for repeated measures with Bonferroni post-hoc test). Data are the mean (±SEM) rate of H$_2$S production for N=6 animals with triplicate values for each animal under each condition.
Fig. 3. Quantitative RT-PCR demonstrates expression of mRNA encoding H$_2$S synthetic enzymes in the mouse liver, brain and external muscle layers of the colon. Two-way ANOVA analysis revealed that the tissue provided a significant contribution to the variance of the data (P < 0.05) with post-test analysis determining that liver samples were significantly more abundant in enzyme specific mRNA than the other tissues. This two-way ANOVA, however, determined that the transcript did not contribute to the overall variance of the data. Because the tissue-specific expression of each transcript was likely independent of each other, these data were also analyzed with T-tests within each tissue group to compare CSE to CBS expression levels and with one-way ANOVA with Newman-Keuls Post Test to compare each transcript between tissues. The former analysis revealed that CSE expression was significantly more abundant in the liver and colonic muscle layer compared to CBS (*P < 0.05). CBS expression was significantly more abundant in brain compared to CSE (*P < 0.05). The latter analysis revealed that the brain and colon were significantly less abundant in both CSE and CBS when compared to the liver (†P < 0.05) and the colon was significantly less abundant in CBS when compared to the brain (‡P < 0.05). Data are the mean (± SEM) logarithm of the molar content of RNA encoding each gene of interest (GOI) in pmol, normalized to the average molar expression of RNA encoding β-actin and GAPDH as housekeeping genes (HKG) in nmol for N=3-4 animals.
Fig 4.
Fluorescent micrographs illustrating CSE-immunoreactivity (ir) in mouse colon (A-C), liver (D), and brain (E-I). In the colon, specific staining was present in myenteric neurons (arrows) revealed by both the rabbit anti-CSE (A) and mouse anti-CSE antisera (B and C). B) Cross section of the mouse colon revealed CSE-ir in the mucosa and myenteric neurons. C) An en face preparation of the colonic muscle layers demonstrate CSE-ir neuronal cell bodies. D) CSE-ir in the liver was localized to hepatocytes and within the Space of Disse. E) Coronal section of the striatum revealed CSE-ir in neuronal cell bodies and within the fibers of passage in the internal capsule. Some neurons expressed CSE-ir in proximal processes. F) Coronal section of the hippocampus revealed CSE-ir in neuronal cell bodies. G) Neuronal cell bodies in the mouse neocortex revealed CSE-ir. H) A section adjacent to that in G was stained with an antibody solution that was incubated for 2 h with recombinant CSE (Abnova). This preabsorption reduced or blocked specific immunoreactivity demonstrating the specificity of the antibody in this study. Matched micrographs (G and H) were taken with identical camera settings. I) Montage of a parasaggital section of the mouse brain with CSE-ir in major fiber tracts. Neuronal cell bodies in neocortex and Purkinje cells in the cerebellum can be observed in this section. Scale bars: A, E, G, and H = 50 μm; B, C and D = 100 μm; F = 250 μm; I = 2 mm.
Fig. 5.
Representative fluorescent micrographs illustrating immunoreactivity (ir) for CBS in mouse colon (A), liver (B), and brain (C-I). A) Cross section of the mouse colon revealed CBS-ir structures in the lamina propria of the mucosa. There was little evidence for CBS-ir in the external muscle layers of the colon. B) CBS-ir was localized to hepatocytes and within the Space of Disse in the mouse liver. C) Coronal section of the hippocampus contained little CBS-ir. CBS-ir is readily observable in the optic nerve as well as fiber tracts in the thalamus in the lower half of this section. D) Coronal section of the striatum revealed CBS-ir in the internal capsule. E) Parasagittal section of cerebellum revealed CBS-ir in fibers extending from the white matter nearing the Purkinji layer and diminishing in the molecular layer. F) Some cells immunoreactive for CBS had the stellate morphology of astrocytes or oligodendrocytes. This cell was located in the neocortex. G) Coronal section of neocortex revealed a morphology of immunoreactive structures extending from the white matter that is similar to that observed in the cerebellum. H) A section adjacent to that in G was stained with an antibody solution that was incubated for 2 h with recombinant CBS (Abnova). This preabsorption reduced or blocked specific immunoreactivity demonstrating the specificity of the antibody in this study. Matched micrographs (G and H) were taken with identical camera settings. I) Montage of a parasagittal section of the mouse brain with CBS-ir in major fiber tracts. Scale bars: A, G and H = 100 μm; B and E = 150 μm; C and D = 250 μm; F = 30 μm; I = 2 mm.