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Original Contribution

Microbial regulation of host hydrogen sulfide bioavailability and metabolism

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ABSTRACT

Hydrogen sulfide (H_2S), generated through various endogenous enzymatic and nonenzymatic pathways, is emerging as a regulator of physiological and pathological events throughout the body. Bacteria in the gastrointestinal tract also produce significant amounts of H_2S that regulates microflora growth and virulence responses. However, the impact of the microbiota on host global H_2S bioavailability and metabolism remains unknown. To address this question, we examined H_2S bioavailability in its various forms (free, acid labile, or bound sulfane sulfur), cystathionine γ-lyase (CSE) activity, and cysteine levels in tissues from germ-free versus conventionally housed mice. Free H_2S levels were significantly reduced in plasma and gastrointestinal tissues of germ-free mice. Bound sulfane sulfur levels were decreased by 50–80% in germ-free mouse plasma and adipose and lung tissues. Tissue CSE activity was significantly reduced in many organs from germ-free mice, whereas tissue cysteine levels were significantly elevated compared to conventional mice. These data reveal that the microbiota profoundly regulates systemic bioavailability and metabolism of H_2S.

Hydrogen sulfide has emerged as an important endogenous gasotransmitter in vivo that contributes to numerous physiological and pathological responses of various organs through its ability to modulate oxidative stress, signal transduction, and nitric oxide bioavailability [32]. Endogenous generation of hydrogen sulfide is complex, with enzymatic synthesis occurring through three proteins comprising cystathionine γ-lyase (CSE) and cystathionine β-synthase, which use cysteine as a substrate, and 3-mercaptosulfurtransferase, using 3-mercaptopyruvate as a substrate [25,32]. Once formed, hydrogen sulfide is very reactive, resulting in rapid metabolism into one of three major pools comprising free, acid-labile, and bound sulfane sulfur forms [28,29]. In this way, hydrogen sulfide bioequivalents may be regulated allowing for conversion and use for various cellular biochemical processes.

Hydrogen sulfide production has long been studied in prokaryotic cells, with its generation being important for antioxidant defense, energy production, and cell cycle regulation [1,11,17,20,21]. Various species of sulfate-reducing bacteria typically use thiosulfate to generate hydrogen sulfide, although disproportionation of $S_2O_3^-$ to hydrogen sulfide and SO_4^{2-} and decay of S-containing amino acids are alternative generation pathways [3,17]. Studies suggest that gastrointestinal hydrogen sulfide generation plays a critical role in regulating physiological responses such as motility, epithelial cell health, and inflammation [4,16,30]. Conversely, other reports suggest a pathological role of gastrointestinal hydrogen sulfide generation presumably due to differential microbial colonization contributing to various conditions such as inflammatory bowel disease, colonic nociception, and colorectal cancer [18,22]. However, the importance of microflora on host hydrogen sulfide formation, bioavailability, and metabolism remains unknown, as examination of tissue H_2S synthesis has been performed using only conventional mice [13,29]. Here we report that the normal microflora profoundly alters H_2S bioavailability along with alterations in synthesis enzyme activity and substrate availability.

Materials and methods

Animals and tissue collection

All animal experiments were approved by the ethics committee in Stockholm, Sweden. Eleven- to 12-week-old male germ-free C57BL/6 J mice ($n=10$) and specific-pathogen-free (conventional) C57BL/6 J mice ($n=10$) were used. All mice were maintained on autoclaved standard chow (R36; Lactamin, Stockholm, Sweden) and water ad libitum and kept under a controlled 12-h light–dark cycle. The germ-free status was checked weekly by culturing fecal

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samples, both aerobically and anaerobically, at +20 and +37 °C for up to 4 weeks [10].

On the day of the experiment, animals were anesthetized by inhalation of 2.2% isoflurane (Forane; Abbot Scandinavia AB, Kista, Sweden) in air. After blood sampling (inferior vena cava) the animals were sacrificed and tissues rapidly collected. Plasma and tissue samples were immediately homogenized in a stabilization buffer (degassed 100 mM Tris–HCl buffer, pH 9.5, 0.1 mM diethylenetriaminepentaacetic acid (DTPA)) to preserve the bioavailable pools of H_2S and metabolic proteins. Samples were snap-frozen and stored in liquid nitrogen until analyzed.

Detection of free sulfide, acid-labile sulfide, bound sulfide, and cysteine

Concentrations of sulfide and cysteine were measured by RP-HPLC after derivatization with excess monobromobimane (MBB) as stable sulfide-dibimane and cysteine-*S*-bimane as we have previously reported [23]. Briefly, 30 μl of samples was added to 70 μl of 100 mM Tris–HCl buffer (pH 9.5, 0.1 mM DTPA), followed by addition of 50 μl of 10 mM MBB and incubated for 30 min. The reaction was terminated with 50 μl of 200 mM 5-sulfosalicylic acid and the mixture centrifuged. The resulting supernatant was analyzed by RP-HPLC equipped with a fluorescence detector (λ_{ex} 390 nm and λ_{em} 475 nm) and an Eclipse XDB-C18 column (4.6 × 250 mm). Typical retention times for bimane adducts of hydrogen sulfide and cysteine were 15.75 and 10.12 min, respectively.

Hydrogen sulfide can exist in many biochemical forms as illustrated in Fig. 1. Acid-labile sulfide and bound sulfane sulfur were measured as we have previously reported [24]. Acid-labile sulfide was released by incubating samples in an acidic solution (pH 2.6, 100 mM phosphate buffer, 0.1 mM DTPA), in an enclosed system to contain volatilized hydrogen sulfide. Volatilized hydrogen sulfide was then trapped in 100 mM Tris–HCl buffer (pH 9.5, 0.1 mM DTPA). The bound sulfane sulfur pool was measured by incubating the sample with 1 mM Tris (2-carboxyethyl) phosphine (TCEP) in 100 mM phosphate buffer at pH 2.6 with 0.1 mM DTPA, and sulfide measurement was performed in a manner analogous to that described above. The acid-labile pool was determined by subtracting the free hydrogen sulfide value from the value obtained by the acid-liberation protocol. The bound sulfane sulfur pool was determined by subtracting the hydrogen sulfide measurement from the acid-liberation protocol alone from that of the TCEP plus acidic conditions.

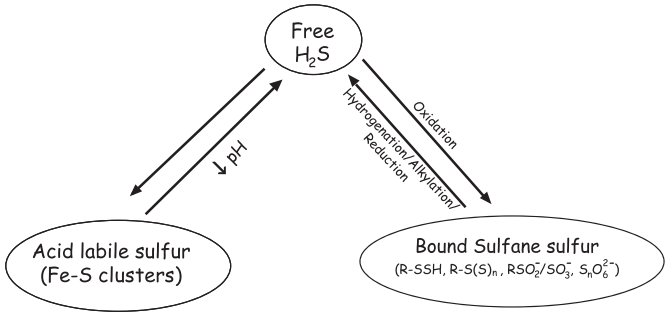

Fig. 1. Various biochemical forms of H_2S bioavailability. H_2S exists in various biochemical forms within biological systems that can be classified based on chemical properties and/or structure. Freely available H_2S represents gaseous H_2S and its HS^- anion, acid-labile sulfide represents iron–sulfur clusters and persulfides, and bound sulfane sulfur represents thiol sulfides, polysulfides, sulfate/sulfite, and bound elemental sulfur.

CSE activity measurement

CSE activity was measured as previously reported [12,36]. Tissue lysates were incubated with 2 mM cystathionine, 0.25 mM pyridoxal 5′-phosphate in 100 mM Tris–HCl buffer (pH 8.3) for 60 min at 37 °C. Trichloroacetic acid (10%) was added into the reaction mixture. After centrifugation, the supernatant was mixed completely with 1% ninhydrin reagent and incubated for 5 min in a boiling-water bath. After heating, the solution was cooled on ice for 2 min and the color reaction development measured 20 min at 455 nm using a SmartSpect Plus spectrophotometer (Bio-Rad). CSE activity was assessed by cystathionine consumption and enzyme activity expressed as nanomoles of cystathionine consumed per milligram of total protein per hour of incubation.

Statistical analysis

Resulting hydrogen sulfide species measurements and cysteine and CSE activity levels were statistically compared with Prism software (GraphPad, Inc.) using an unpaired Student *t* test between conventional and germ-free mice per organ examined. Distribution of tissue H_2S metabolite levels and CSE enzyme activity was also compared within each subject group using one-way ANOVA with the Newman–Keuls multiple comparison test to identify tissues with the most significant differences. A minimum $p < 0.05$ was necessary for significance.

Results

Plasma H_2S bioavailability in conventional versus germ-free mice

Bioavailable H_2S can be compartmentalized in various biochemical forms as illustrated in Fig. 1 [28]. Therefore, we employed specific analytical methods that we have developed to measure these various H_2S pools comparing conventional versus germ-free mice. Fig. 2 illustrates the amount of plasma free H_2S, acid-labile sulfide, and bound sulfane sulfur in conventional and germ-free mice (Fig. 2A–C, respectively). Germ-free mice had significantly reduced plasma free H_2S and bound sulfane sulfur levels compared to conventional mice.

Tissue free H_2S levels in conventional versus germ-free mice

Fig. 3 shows distinct differences regarding the amount of freely available H_2S in various organs. In conventional mice, the kidney, stomach, and heart showed the highest levels of free H_2S, whereas lung and fat tissues were found to have the lowest levels. Germ-free mice had significantly less free H_2S in cecum and colon compared to conventional mice. These data indicate that the presence of an intestinal microflora contributes significantly to plasma and gastrointestinal organ free H_2S levels.

Tissue acid-labile sulfide and bound sulfane sulfur levels in conventional versus germ-free mice

Experiments were performed to measure tissue levels of acid-labile sulfide and bound sulfane sulfur. Fig. 4A reports various tissue levels of acid-labile sulfide in conventional and germ-free mice. The absence of a microflora did not significantly affect acid-labile H_2S pools in any of the organs examined, although comparisons of tissue acid-labile H_2S levels in conventional and germ-free mice revealed significantly higher amounts in fat and aorta. Fig. 4B shows the various tissue levels of bound sulfane sulfur levels (i.e., polysulfides) in conventional and germ-free mice. Interestingly, fat and aorta tissues of conventional mice contained the greatest