British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01681.x
www.brjpharmacol.org

RESEARCH PAPER

Sulphide quinone reductase contributes to hydrogen sulphide metabolism in murine peripheral tissues but not in the CNS

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Keywords
hydrogen sulfide (H_2S); sulphide quinone reductase; thiosulphate; gas chromatography; protein sequestration; gasotransmitter; sulphide metabolism; disulphide oxidoreductase (DiSR); sulphide oxidase; enteric nervous system

Received
26 May 2011
Revised
16 August 2011
Accepted
8 September 2011

BACKGROUND AND PURPOSE

Hydrogen sulphide (H_2S) is gaining acceptance as a gaseous signal molecule. However, mechanisms regarding signal termination are not understood. We used stigmatellin and antimycin A, inhibitors of sulphide quinone reductase (SQR), to test the hypothesis that the catabolism of H_2S involves SQR.

EXPERIMENTAL APPROACH

H_2S production and consumption were determined in living and intact mouse brain, liver and colonic muscularis externa using gas chromatography and HPLC. Expressions of SQR, ethylmalonic encephalopathy 1 (Ethe1) and thiosulphate transferase (TST; rhodanese) were determined by RT-PCR and immunohistochemistry.

KEY RESULTS

In the colonic muscularis externa, $H_2{}^{35}S$ was catabolized to [^{35}S]-thiosulphate and [^{35}S]-sulphate, and stigmatellin reduced both the consumption of $H_2{}^{35}S$ and formation of [^{35}S]-thiosulphate. Stigmatellin also enhanced H_2S release by the colonic muscularis externa. In the brain, catabolism of $H_2{}^{35}S$ to [^{35}S]-thiosulphate and [^{35}S]-sulphate, which was stigmatellin-insensitive, partially accounted for $H_2{}^{35}S$ consumption, while the remainder was captured as unidentified ^{35}S that was probably bound to proteins. Levels of mRNA encoding SQR were higher in the colonic muscularis externa and the liver than in the brain.

CONCLUSIONS AND IMPLICATIONS

These data support the concept that termination of endogenous H_2S signalling in the colonic muscularis externa occurs via catabolism to thiosulphate and sulphate partially via a mechanism involving SQR. In the brain, it appears that H_2S signal termination occurs partially through protein sequestration and partially through catabolism not involving SQR. As H_2S has beneficial effects in animal models of human disease, we suggest that selective inhibition of SQR is an attractive target for pharmaceutical development.

Abbreviations

DiSR, disulphide oxidoreductase flavoprotein; DTT, dithiothreitol; Ethe1, ethylmalonic encephalopathy 1; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; GOI, gene of interest; HKG, housekeeping genes; SQR, sulphide quinone reductase; Sqrdl, sulphide quinone reductase domain-like; TRP, transient receptor potential; TST, thiosulphate sulphur transferase

Introduction

In the past 15 years, H_2S has become recognized as an important endogenous gaseous signal molecule that functions as a neuromodulator in the brain (Abe and Kimura, 1996; Ishigami *et al.*, 2009), a smooth muscle relaxant (Hosoki *et al.*, 1997; Zhao *et al.*, 2001; Zhao and Wang, 2002; Cheng *et al.*, 2004; Yang *et al.*, 2008) and pro-angiogenic (Papapetropoulos *et al.*, 2009; Szabo and Papapetropoulos, 2011) in the vascular system, a smooth muscle relaxant (Hosoki *et al.*, 1997; Teague *et al.*, 2002; Gallego *et al.*, 2008; Gil *et al.*, 2011) and prosecretory molecule (Schicho *et al.*, 2006; Hennig and Diener, 2009; Krueger *et al.*, 2010; Pouokam and Diener, 2011) in the gastrointestinal tract, and as an anti-inflammatory (Wallace *et al.*, 2009, 2010; Ekundi-Valentim *et al.*, 2010) and cellular protective molecule (Esechie *et al.*, 2009; Suzuki *et al.*, 2011; Taniguchi *et al.*, 2011). Enzymes involved in transsulphuration are involved in the synthesis of H_2S, and biochemical alterations (e.g. increased glucose or decreased oxygen) regulate the expression or activity of the H_2S-releasing enzymes (Linden *et al.*, 2010). Although vascular K_{ATP} channels appear to be a major target of H_2S (Whiteman and Moore, 2009) putative cellular targets of H_2S in other systems, which include diverse proteins such as K_{ATP} channels, NMDA channels, transient receptor potential (TRP) channels, T-type voltage-gated calcium channels and sodium channels, remain controversial (Kimura, 2010; Linden *et al.*, 2010). The mechanism by which the H_2S-mediated signalling is terminated also remains controversial. While several mechanisms have been proposed, two are substantiated with relevant physiological evidence. First, because H_2S can be released from sulphane–sulphur pools via acidification (Ishigami *et al.*, 2009) and from bound stores via reducing agents such as dithiothreitol (DTT) (Ishigami *et al.*, 2009; Wallace *et al.*, 2009), it has been proposed that enzymatically synthesized H_2S can be removed via binding in these pools. Second, H_2S signalling may be terminated by rapid enzymatic catabolism of H_2S to thiosulphate and sulphate (Levitt *et al.*, 1999). The molecular identity of the enzyme responsible for this catabolism has been controversial with both thiosulphate sulphur transferase (TST; rhodanese) (Picton *et al.*, 2002) and an unknown sulphide oxidase (Levitt *et al.*, 1999; Wilson *et al.*, 2008) proposed as the rate-limiting step in the oxidation of H_2S to thiosulphate. A recent study using rat liver demonstrated that H_2S oxidation to thiosulphate involves the serial action of the enzymes sulphide quinone reductase (SQR), sulphur dioxygenase and TST (Hildebrandt and Grieshaber, 2008). Mice lacking ethylmalonic encephalopathy 1 (Ethe1) exhibit elevated sulphide levels, suggesting that Ethe1 is the sulphur dioxygenase involved in H_2S metabolism, and that sulphide toxicity is the cause of ethylmalonic encephalopathy in patients with truncated, non-functional mutations in ETHE1 (Tiranti *et al.*, 2009). Recombinant expression of human SQR enhances sulphide oxidation in a mammalian cell line (Lagoutte *et al.*, 2010).

The present study was designed to test the hypothesis that SQR plays a role in the catabolism of H_2S in the mouse colonic muscularis externa, liver and brain. In these experiments, living and intact tissues were used in order to determine the physiological relevance of the findings. These tissues were not amenable to gene knockdown approaches, so we used a pharmacological approach by utilizing two known inhibitors of SQR in H_2S production and consumption assays. Additionally, the expressions of SQR, Ethe1 and TST were investigated using RT-PCR and immunohistochemistry.

Methods

Animals

All methods used in this study were approved by the Mayo Clinic and VA Medical Center Animal Care and Use Committees. Adult C57Bl/6 (Jackson Laboratories, Bar Harbor, ME, USA), 129Sv (in house breeding at Mayo Clinic) and C57BL/6 (Harlan, Indianapolis, IN, USA) mice of either sex, aged 6–10 weeks, weighing 17 to 30 g, were housed five per cage in plastic cages with soft bedding. The animals had access to food and water *ad libitum* and were maintained at 23–24°C on a 12:12 h light–dark cycle. At the time of tissue collection, animals were killed by CO_2 asphyxiation.

Tissue preparation

A 5 cm segment of the muscularis externa of the mouse colon containing circular muscle, longitudinal muscle and the myenteric plexus was isolated from the mucosal and submucosal layers in a sterile manner such that the muscle layers were never exposed to the luminal contents of the colon, as previously described (Linden *et al.*, 2008). Briefly, the anus, intact with surrounding skin, was sutured and hung outside of a 100 × 15 mm plastic Petri dish. The colon and caecum were placed into a dish with iced normal Krebs solution (in mM: Na^+, 137.4; K^+, 5.9; Ca^{2+}, 2.5; Mg^{2+}, 1.2; Cl^-, 134; HCO_3^-, 15.5; $H_2PO_4^-$, 1.2; and glucose, 11.5). A small incision made around the circumference of the colon allowed the muscle to be dissected away from the mucosa, keeping the muscularis mucosa and the barrier to the lumen and luminal bacteria intact. These large segments of muscularis externa were cut into either six (for H_2S release assays) or eight (for tissue H_2S concentration assays and $H_2{}^{35}S$ consumption assays) evenly sized pieces (~0.5–0.75 cm in length).

After the mice had been killed their brain and liver tissue were immediately removed and homogenized in ice-cold RPMI at a 1:10 dilution. Homogenization was performed with a Duall grinder (Kontes, Vineland, NJ, USA) with a Teflon pestle, using 8–10 strokes.

Following their death, mice that were used to rapidly dissect tissue for RNA analysis or immunohistochemistry were transcardially perfused with 60 mL ice-cold PBS (0.1 M, pH 7.4). While the entire brain was used to extract RNA, liver samples were obtained only from distal lobes. The muscularis externa of the colon including the myenteric plexus were dissected free from the mucosal and submucosal layers.

H_2S release assays

Intact living colonic muscularis externa tissue was assayed for H_2S release using a previously described method (Linden *et al.*, 2008). Briefly, colonic muscularis externa tissue was incubated in 1 mL Krebs solution containing 10 mM L-cysteine. A gas mixture containing 97% O_2 and 3% CO_2 was bubbled through this solution, which maintained a pH of 7.4. The gas coming off this solution was subsequently

bubbled through a 1% solution of zinc acetate. Modifications were made to the previously described experimental setup to decrease variability and improve the detection of H_2S. The newly designed apparatus was constructed of polysulphone with sensitive rotometers to more precisely control gas flow (4 mL·min^{-1}). It contained a thin nozzle in the tissue chamber to ensure that gas bubbles formed a column in the centre of the chamber and tissue hooks to ensure the suspended tissue was within the column of gas bubbles. The connecting tubing was made of Teflon instead of silastic to reduce loss of H_2S via diffusion through the tubing during transit to a sealed solution of zinc acetate. The H_2S stored as stable zinc sulphide was released via acidification with 50% HCl into a sealed gas space that was analysed using a gas chromatograph (model 5890; Hewlett-Packard, Palo Alto, CA, USA) equipped with a Teflon column [8 ft × 0.125 in, packed with Chromosil 330 (Supelco), maintained at 80°C with a flow rate of 20 mL·m^{-1}] and a sulphur chemiluminescence detector (model 355; Sievers Instruments, Boulder, CO, USA), which is specific for sulphur-containing gases. Two small pieces of colon muscularis externa from each of two mice (four pieces total) were incubated in a single chamber to form a single data point. The data from six tissue chambers, three with vehicle (0.1% EtOH) and three with vehicle plus stigmatellin (3 μM), from each pair of mice were averaged to single paired observations for each condition.

Tissue H_2S concentration assay

The concentration of H_2S in colonic muscularis externa was determined using gas chromatography, with minor modifications to a previously described method (Furne *et al.*, 2008). Briefly, the colonic muscularis externa was rapidly homogenized in ice-cold glycine–NaOH buffer (pH 9.3). An aliquot of the homogenate was then transferred to a 3 mL polypropylene syringe, sealed with a plunger and a three-way stopcock attached to an empty, sealed 3 mL syringe and acidified to pH 5.8 to aid the conversion of HS^- to dissolved H_2S. The homogenate in solution was vigorously mixed between the two syringes for 1 min to speed the equilibriums between HS^- and dissolved H_2S and between dissolved H_2S and H_2S released into the gas space. The gas space was then assayed immediately using gas chromatography as described above. Control syringes containing the buffers without tissue were used to calculate background H_2S concentrations, which were subtracted from concentrations obtained in the presence of tissue. Concentrations of H_2S in the gas space (minus controls) were used to calculate total molar content of H_2S in the sample using known physical constants for H_2S including the water dissolved-H_2S to H_2S gas solubility ratio (2.2 at 4°C) and dissociation to HS^- at pH 5.8, normalized to the weight of the tissue to yield values in units of fmol·mg^{-1}.

H_2S consumption assays

H_2S consumption assays were done similar to previously described methods (Levitt *et al.*, 1999). Tissue was placed into 20 mL polypropylene syringes containing 36 μL Krebs solution and 4 μL of either 1% ethanol (in H_2O) or 30 μM stigmatellin (in 1% ethanol/H_2O) to achieve final concentrations of 0.1% ethanol and 3 μM stigmatellin, respectively. The syringes were then filled with 20 mL of gas containing 5 ppm $H_2{}^{35}S$, 97% O_2 and 3% CO_2, sealed with the plunger and a three-way stopcock, and incubated at 37°C for 30 min. When using liver, unlabelled H_2S was delivered to the gas space instead of $H_2{}^{35}S$ because further characterization of H_2S metabolites was not carried out. The gas space was analysed for H_2S (or $H_2{}^{35}S$) using gas chromatography as described above. The reduction in H_2S concentration, minus that observed in no tissue controls, and the volume of gas space were used to calculate the quantity of H_2S removed by the homogenate. Values were normalized for weight of the tissue sample and incubation time to yield H_2S consumption in units of pmol·mg^{-1}·min^{-1}.

For the brain and colonic muscularis externa tissues, further analyses of $H_2{}^{35}S$ metabolites were performed. After removing the remaining $H_2{}^{35}S$ by flushing the gas space with air several times, the samples were weakly acidified with HCl (to pH ~5.8) to convert HS^- to volatile H_2S. The gas space was again flushed with air several times before the addition of 20 mM sodium hydroxide, 2 mM sodium sulphate and 2 mM sodium thiosulphate. Samples were then sonicated (Misonix Sonicator 3000, Farmingdale, NY, USA) and analysed for protein, [^{35}S]-thiosulphate and [^{35}S]-sulphate content. Following sonication, the solution was passed over a filter that limits the passage of proteins greater than 30 kDa (Millipore, Billerica, MA, USA). The resulting solution was analysed by HPLC (model C-R3A Chromatopac; Shimadzu Corp., Kyoto, Japan), run at 2 mL·min^{-1} and 13.8 MPa using an anion–ion exchange column (IonPac AS16; Dionex Corp., Salt Lake City, UT, USA) and a conductivity monitor (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for mass measurements. The column eluate was collected in 2 mL fractions in individual scintillation vials, and the radioactivity of these fractions was determined by scintillation counting. The retention times of the unknowns were compared with retention times of authentic thiosulphate and sulphate standards.

Although the experiments were not initially designed as recovery assays, differences in the results obtained using colonic muscularis externa and brain prompted us to calculate the recovery of $H_2{}^{35}S$ metabolites in our studies. Percentage recovery of the ^{35}S that was added to the syringe was determined at various steps: (i) in the gas space after incubation, (ii) in the homogenate just before filtration, (iii) in the homogenate after filtration (30 kDa cut-off) and (iv) in [^{35}S]-sulphate and [^{35}S]-thiosulphate after HPLC analysis. The difference between the pre- and post-filter counts was used to calculate the percentage of [^{35}S] caught within the filter (molecules >30 kDa). The difference between the post-filter counts and sum of the [^{35}S]-sulphate and [^{35}S]-thiosulphate counts weas used to calculate the percentage of ^{35}S that was injected into the HPLC column but not recovered as an identified molecule (i.e. not sulphate or thiosulphate).

RT-qPCR

RNA was extracted and converted to cDNA as described previously (Linden *et al.*, 2008) using RNA-Bee (Tel-Test INC., Friendswood, TX, USA), RNAeasy Plus (Qiagen, Valencia, CA, USA) and GeneAmp Gold RNA PCR (Applied Biosystems, Foster City, CA, USA) kit systems according to the manufacturers' instructions. Real-time PCR reactions were completed using an Applied Biosystems Prism 7000 Sequence Detector. Forward and reverse primer sets for mouse sulphide quinone reductase domain-like (Sqrdl), Ethe1 and TST (rhodanese), as

well as the housekeeping genes (HKG) β-actin and GAPDH were purchased from SuperArray Bioscience (Frederick, MD, USA). A 1:5.55 dilution of the RT reaction and 0.4 nM of the appropriate primer set in 1× CyberGreen master mix (SuperArray) were first subjected to 10 min at 95°C to activate the Taq polymerase followed by 40 cycles of 15 s 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, USA) 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 $\log_{10}$ concentration of plasmid DNA versus Ct. These standard curves were linear between 30 and 3 million copies with slopes (r^2) of these lines for β-actin, GAPDH, SQR, TST and Ethe1-specific amplification of –3.32 ± 0.18 (r^2: 0.9826), –3.59 ± 0.37 (r^2: 0.9947), –3.45 ± 0.06 (r^2: 0.9937), –3.58 ± 0.16 (r^2: 0.9979) and –3.13 ± 0.18 (r^2: 0.9826), respectively, indicating amplification efficiency within the acceptable range for absolute quantification. The molar quantity of SQR, TST and Ethe1, calculated for each sample, was normalized to the average molar quantity of both β-actin and GAPDH and presented as pmol gene of interest (GOI) over nmol HKG.

Immunohistochemistry

Colon, liver and brain were obtained from mice transcardially perfused with 20 mL PBS and 6 mL 4% paraformaldehyde and immersion fixed in 4% paraformaldehyde for 2 h. Cryostat sections of tissue (15 µm) were blocked with PBS containing 4% normal donkey serum and 0.5% triton X-100 and stained with 1:100 dilutions of rabbit anti SQR antisera (Sigma, St. Louis, MO, USA; Proteintech, Chicago, IL, USA) or 1:100 dilutions of rabbit anti Ethe1 (Sigma). Following three 15 min washes in PBS, tissue sections were incubated with FITC-conjugated donkey anti-rabbit IgG (1:200; Millipore) or Cy3-conjugated donkey anti Rabbit IgG (1:800; Jackson Immunoresearch, West Grove, PA, USA). Immunostained tissues were viewed on an Olympus BXS1W1 epifluorescence microscope, and images were taken using MagnaFire software (Optronics, Goleta, CA, USA).

Chemicals

All drugs and molecular targets conform to BJP's *Guide to Receptors and Channels* (Alexander *et al.*, 2011). Stigmatellin, the myxobacteria-derived antibiotic (Kunze *et al.*, 1984), antimycin A, the *Streptomyces*-derived antibiotic (Dunshee *et al.*, 1949), both quinone substrate inhibitors, and carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), the protonophore that uncouples oxidative phosphorylation (Heytler and Prichard, 1962) were purchased from Sigma, and dissolved in 100% EtOH to form a stock concentration of 3 mM for stigmatellin and antimycin A and 10 mM for FCCP. Vehicle controls were 0.1% EtOH for stigmatellin and antimycin A and 0.01% EtOH for FCCP. Neither antibiotic-containing nor vehicle stock solutions changed the pH of buffer solutions in which they were used. $H_2{}^{35}S$ was synthesized by heating to 37°C 10 µL of a solution containing 1 mg [^{35}S]-cysteine (1075 Ci·mol^{-1}; Moravek Biological, Brea, CA) in glass tubing and capturing the liberated $H_2{}^{35}S$ gas in a 2% zinc acetate solution. Before the experiments, an aliquot of the zinc acetate was acidified, releasing $H_2{}^{35}S$ into a gas space that was collected in a Teflon gas storage bag equipped with a stopcock. The concentration of $H_2{}^{35}S$ was determined by gas chromatography (as described above) and diluted to the final concentration using 97% O_2 and 3% CO_2. In addition, the radioactivity of the $H_2{}^{35}S$ was determined by scintillation counting. All other chemicals were purchased from Sigma.

Statistical analyses

All statistical analyses were completed using GraphPad Prism software, using tests described in the results section. For all tests, *P*-values less than 0.05 were considered significant.

Results

Stigmatellin enhances the release of H_2S by the colonic muscularis externa

The muscularis externa of the mouse colon generated and released detectable levels of H_2S (Figure 1), in agreement with our previous study (Linden *et al.*, 2008). In the present experiments, the rate of H_2S production and release was greater than previously reported due to the improvements in the methodology. H_2S release increased in the presence of tissue by nearly sixfold over that of the control Krebs solution containing 10 mM cysteine. In the presence of cysteine and 3 µM stigmatellin, the rate of H_2S release increased twofold over tissue incubated with vehicle alone such that the release rate was 11-fold increased over the vehicle without tissue

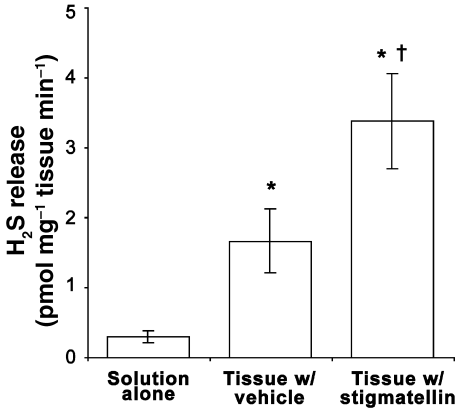

Figure 1

The rate of H_2S generation in normal Krebs solution containing 10 mM cysteine (solution alone), and the rate of H_2S release in the same solution containing colonic muscularis externa with vehicle (0.1% EtOH) alone or with 3 µM stigmatellin. H_2S release was greater in the presence of tissue, which was further increased by the presence of stigmatellin. Data are the mean ± SEM values for three independent experiments run in triplicate. *$P < 0.05$ compared with no tissue control; †$P < 0.05$ compared with vehicle treated control; repeated-measures ANOVA, Neuman–Keuls post test.

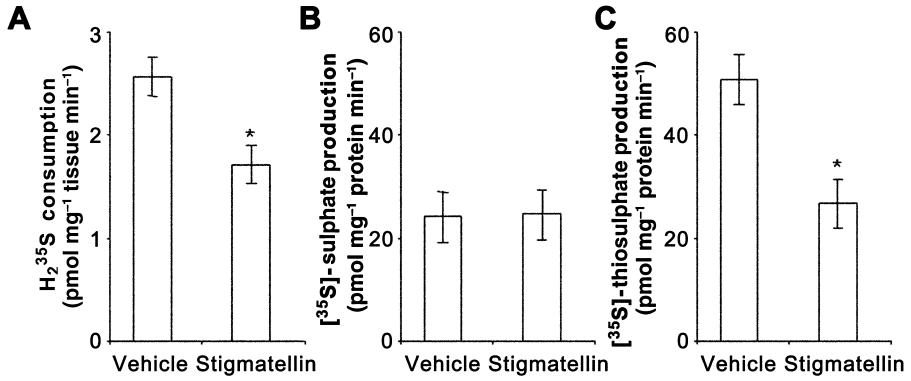

Figure 2

The rates of consumption of $H_2{}^{35}S$ (A) and conversion of $H_2{}^{35}S$ to [^{35}S]-sulphate (B) and [^{35}S]-thiosulphate (C) by colonic muscularis externa incubated with vehicle (0.1% EtOH) alone or with 3 μM stigmatellin. Stigmatellin reduced $H_2{}^{35}S$ consumption and the conversion of $H_2{}^{35}S$ to [^{35}S]-thiosulphate production but did not affect the conversion of $H_2{}^{35}S$ to [^{35}S]-sulphate. Data are the mean ± SEM values for six independent experiments run in duplicate. *$P < 0.05$ compared with vehicle-treated control; paired *t*-test.

(control Krebs solutions with cysteine). When colonic muscularis externa was assayed to measure the concentration of free H_2S using previously described techniques (Furne *et al.*, 2008), the tissue concentration was determined to be 76 ± 25 fmol·mg^{-1} tissue ($n = 4$). This concentration is higher than concentrations reported for brain and liver (Furne *et al.*, 2008) but less than the concentration reported for the aorta (Levitt *et al.*, 2011).

Stigmatellin reduces H_2S consumption and thiosulphate production but does not alter sulphate production in the colonic muscularis externa

Analysis of the gas space over muscularis externa of the mouse colon showed that the muscularis externa consumed $H_2{}^{35}S$, and that this consumption was reduced by 33% during incubation with stigmatellin (Figure 2A). Homogenates (tissue plus incubating solution) were analysed for the conversion of $H_2{}^{35}S$ to [^{35}S]-sulphate and [^{35}S]-thiosulphate (Figure 2B and C). Stigmatellin reduced the conversion of $H_2{}^{35}S$ to [^{35}S]-thiosulphate by 47% but did not affect the conversion of $H_2{}^{35}S$ to [^{35}S]-sulphate. Data from these experiments were analysed to determine the proportion of $H_2{}^{35}S$ that was converted to [^{35}S]-sulphate and [^{35}S]-thiosulphate or remained as $H_2{}^{35}S$ at the end of the experiment (Figure 3). The proportion of $H_2{}^{35}S$ converted to [^{35}S]-sulphate or [^{35}S]-thiosulphate was significantly higher in samples that contained tissue compared with samples that did not, and the proportion of $H_2{}^{35}S$ that remained as $H_2{}^{35}S$ was significantly lower in samples that contained tissue compared with samples that did not ($P < 0.05$, Kruskal–Wallis test followed by Dunn's test). The total proportion of $H_2{}^{35}S$ recovered as $H_2{}^{35}S$, [^{35}S]-sulphate or [^{35}S]-thiosulphate was not affected by the presence of tissue ($P > 0.05$, Kruskal–Wallis test). Stigmatellin significantly reduced the proportion of $H_2{}^{35}S$ converted by tissue to [^{35}S]-thiosulphate compared with vehicle-treated tissue ($P < 0.05$, Kruskal–Wallis test followed by Dunn's test). When tested with Kruskal–Wallis test (including the samples that did not contain tissue), the proportion of $H_2{}^{35}S$ that remained as $H_2{}^{35}S$ was not significantly affected by stigmatellin. However, when only the two tissue-containing experiments were compared, stigmatellin caused a significant increase in the proportion of $H_2{}^{35}S$ that remained $H_2{}^{35}S$ ($P < 0.05$, Mann–Whitney *U*-test). Both statistical tests showed that the proportion of $H_2{}^{35}S$ recovered as [^{35}S]-sulphate was unaffected by stigmatellin. There were no differences between groups in either the proportion of $H_2{}^{35}S$ recovered on the filter (>30 kDa) (vehicle no tissue: –0.8 ± 0.4%; vehicle plus tissue: –1.6 ± 1.2%; stigmatellin plus tissue: –1.6 ± 0.9%) or the proportion of $H_2{}^{35}S$ injected onto but not recovered from the HPLC column (vehicle no tissue: 0.7 ± 0.5%; vehicle plus tissue: 2.3 ± 0.7%; stigmatellin plus tissue: 2.3 ± 0.5%) ($P > 0.05$, Kruskal–Wallis test followed by Dunn's test).

The consumption of H_2S by the intact colonic muscularis externa was also sensitive to antimycin A, another inhibitor of SQR (Griesbeck *et al.*, 2000). The rate of H_2S consumption in the presence of 3 μM antimycin A (2.6 ± 0.2 pmol·min^{-1}·mg^{-1}) was significantly less than in tissue incubated with the vehicle control solution (4.3 ± 0.1 pmol·min^{-1}·mg^{-1}; $P < 0.05$, paired *t*-test). The consumption of H_2S by the intact colonic muscularis externa was insensitive to FCCP, a mitochondrial uncoupler. The rate of H_2S consumption in the presence of 1 μM FCCP (6.7 ± 1.1 pmol·min^{-1}·mg^{-1}) was not different from tissue incubated with the vehicle control solution (5.9 ± 1.0 pmol·min^{-1}·mg^{-1}; $P > 0.05$, paired *t*-test).

Stigmatellin reduced H_2S consumption in the liver but not the brain

The effect of stigmatellin on the consumption of $H_2{}^{35}S$ by colonic muscularis externa prompted us to test the effect of stigmatellin on liver and brain. Stigmatellin (3 μM) reduced the consumption of H_2S by mouse liver by approximately 50% (Figure 4A) but had little effect on $H_2{}^{35}S$ consumption in mouse brain (Figure 4B). Likewise, antimycin A, another inhibitor of SQR, did not significantly alter H_2S consumption by mouse brain homogenate at either 3 μM (13.8 ± 0.8 pmol·min^{-1}·mg^{-1}) or 70 μM (19.3 ± 1.6 pmol·min^{-1}·mg^{-1})

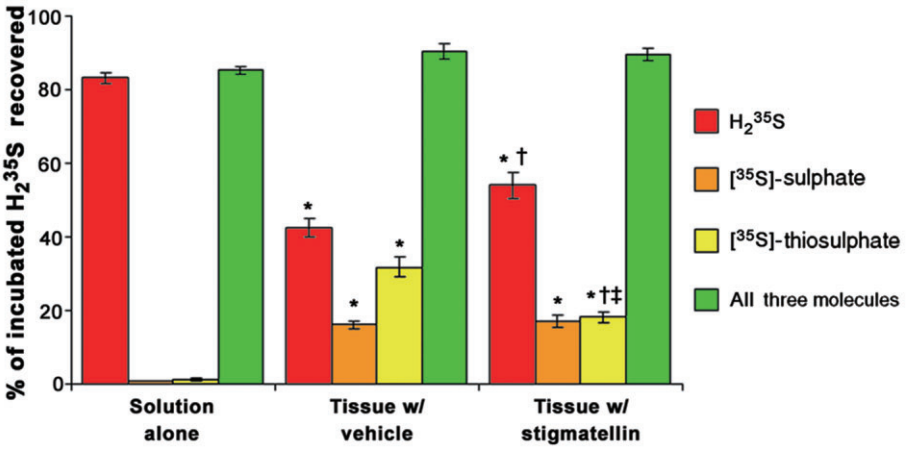

Figure 3

The proportion of $H_2{}^{35}S$ delivered to colonic muscularis externa incubated with vehicle (0.1% EtOH) or 3 μM stigmatellin that was recovered as either [^{35}S]-sulphate, [^{35}S]-thiosulphate or $H_2{}^{35}S$ or all three of these molecules. Nearly all of the supplied $H_2{}^{35}S$ was recovered as $H_2{}^{35}S$ when added to syringes containing the incubation solution without tissue (solution alone). The presence of tissue reduced the proportion of supplied $H_2{}^{35}S$ recovered as $H_2{}^{35}S$ and increased the proportion of $H_2{}^{35}S$ recovered as [^{35}S]-sulphate and [^{35}S]-thiosulphate (*$P < 0.05$ compared with solution alone, Kruskal–Wallis test followed by Dunn's test). Stigmatellin (3 μM) increased the proportion of $H_2{}^{35}S$ recovered as $H_2{}^{35}S$ (†$P < 0.05$ compared with tissue with vehicle, Kruskal–Wallis test followed by Dunn's test) and decreased the proportion of $H_2{}^{35}S$ recovered as [^{35}S]-thiosulphate (‡$P < 0.05$ compared with tissue with vehicle, Mann–Whitney *U*-test) but did not affect the proportion of $H_2{}^{35}S$ recovered as [^{35}S]-sulphate ($P > 0.05$ compared with tissue with vehicle, Kruskal–Wallis test and Mann–Whitney *U*-test). Data are the mean ± SEM proportions for 8–12 observations.

when compared with tissue incubated with the vehicle control solutions (16.0 ± 1.4 pmol·min^{-1}·mg^{-1}, 16.5 ± 0.8 pmol·min^{-1}·mg^{-1}, respectively; $P > 0.05$, paired *t*-test). Like the colonic muscularis externa, FCCP did not significantly alter H_2S consumption by either mouse liver homogenate (vehicle: 123 ± 3 pmol·min^{-1}·mg^{-1}; FCCP: 136 ± 11 pmol·min^{-1}·mg^{-1}; $P > 0.05$, paired *t*-test) or mouse brain homogenate (vehicle: 21 ± 1 pmol·min^{-1}·mg^{-1}; FCCP: 20 ± 2 pmol·min^{-1}·mg^{-1}; $P > 0.05$, paired *t*-test).

Brain homogenates (tissue plus incubating solution) were analysed for the conversion of $H_2{}^{35}S$ to [^{35}S]-sulphate and [^{35}S]-thiosulphate (Figure 4C and D). Consistent with a lack of effect of $H_2{}^{35}S$ consumption, stigmatellin had no effect on the conversion of $H_2{}^{35}S$ to either [^{35}S]-thiosulphate or [^{35}S]-sulphate. Data from these experiments were combined to determine the proportion of the supplied $H_2{}^{35}S$ that was converted to [^{35}S]-sulphate and [^{35}S]-thiosulphate or remained as $H_2{}^{35}S$ at the end of the experiment (Figure 5). The proportions of $H_2{}^{35}S$ converted to [^{35}S]-sulphate or [^{35}S]-thiosulphate were significantly higher in samples that contained tissue compared to samples that did not ($P < 0.05$, Kruskal–Wallis test followed by Dunn's test). Likewise, the proportion of $H_2{}^{35}S$ that remained as $H_2{}^{35}S$ was significantly lower in samples that contained tissue compared with samples that did not ($P < 0.05$, Kruskal–Wallis test followed by Dunn's test). Of particular interest, the total proportion of $H_2{}^{35}S$ recovered as $H_2{}^{35}S$, [^{35}S]-sulphate or [^{35}S]-thiosulphate was also lower in samples that contained tissue ($P < 0.05$, Kruskal–Wallis test followed by Dunn's test). The proportion of ^{35}S recovered on the filter (>30 kDa) (vehicle no tissue: −1.6 ± 0.4%; vehicle plus tissue: 7.4 ± 1.0%; stigmatellin plus tissue: 8.0 ± 1.3%) and the proportion of $H_2{}^{35}S$ injected onto but not recovered from the HPLC column (vehicle no tissue: 2.2 ± 0.5%; vehicle plus tissue: 9.0 ± 1.5%; stigmatellin plus tissue: 9.2 ± 1.4%) were greater in solutions containing tissue compared with samples that did not ($P < 0.05$, Kruskal–Wallis test followed by Dunn's test). The molar quantities of ^{35}S captured on the filter or unidentified on the HPLC column were combined and used to calculate the proportion of $H_2{}^{35}S$ (Figure 5). When these measurements were included in the calculation of total recovery (Figure 5), the proportion of $H_2{}^{35}S$ recovered was not affected by the presence of tissue ($P > 0.05$, Kruskal–Wallis test). A 50 μL sample of a 1:5 dilution of pre-filtered solutions was also subjected to chloroform extraction to assess elemental sulphur (and other lipid soluble metabolites) of H_2S. The proportion of $H_2{}^{35}S$ recovered as lipid soluble metabolites (vehicle no tissue: 1.6 ± 0.1%; vehicle plus tissue: 2.7 ± 0.4%; stigmatellin plus tissue: 2.9 ± 0.3%) was not different between groups ($P > 0.05$, Kruskal–Wallis test) and was not included in the calculations for Figure 5. In brain tissue, the proportion of $H_2{}^{35}S$ recovered as $H_2{}^{35}S$, [^{35}S]-sulphate, [^{35}S]-thiosulphate, [^{35}S]-sulphur or unidentified [^{35}S]-containing molecules in the filter or HPLC column was unaffected by stigmatellin.

Mouse tissues express various levels of the mRNA encoding SQR

RNA encoding SQR was expressed at relatively high levels, approximately 40% and 20% of the expression levels of the HKG Gapdh and Actb, in the liver and colonic muscularis externa, respectively (Figure 6), and at especially high levels in the colonic mucosa at 220% the levels of HKG. The brain contained relatively low levels of SQR (approximately 1% of HKG). RNA encoding Ethe1, the sulphur dioxygenase and TST, implicated in H_2S catabolism was also expressed by these tissues. In the colonic muscularis externa and brain, the

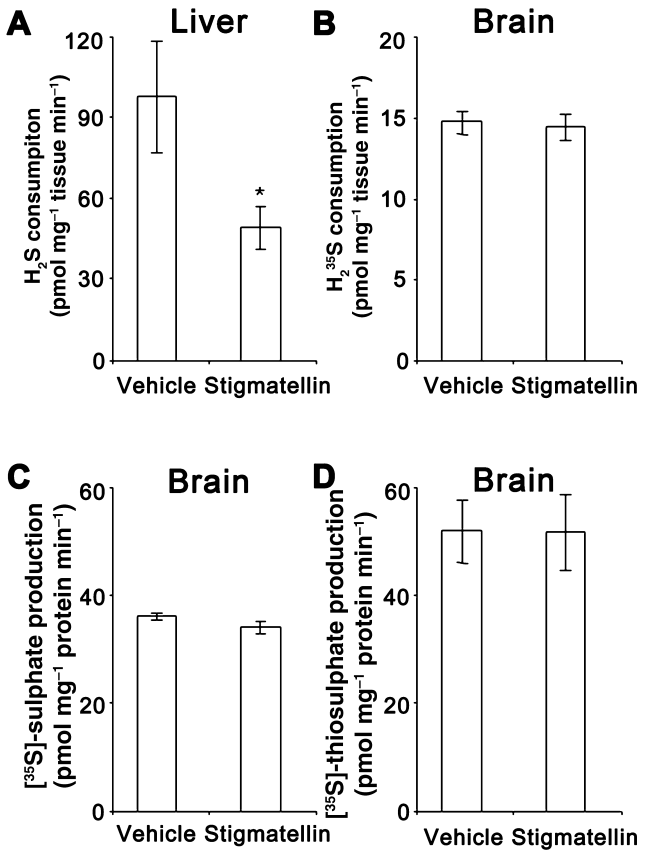

Figure 4

The rate of H_2S consumption in liver tissue (A) and $H_2{}^{35}S$ consumption in brain tissue (B) incubated with vehicle (0.1% EtOH) alone or with 3 μM stigmatellin. Stigmatellin reduced H_2S consumption by the liver but did not alter $H_2{}^{35}S$ consumption by brain tissue. Brain tissue was further analysed for the conversion of $H_2{}^{35}S$ to [^{35}S]-sulphate (C) and [^{35}S]-thiosulphate (D). Stigmatellin had no significant effect on the conversion of $H_2{}^{35}S$ to [^{35}S]-sulphate or [^{35}S]-thiosulphate. Data are the mean ± SEM values for three (liver) or five (brain) independent experiments run in duplicate. *$P < 0.05$ compared with vehicle-treated control; paired *t*-test.

expression levels of Ethe1 were at relatively low levels (0.009% and 6% of HKG, respectively) as were levels of Tst (0.2% and 3% of HKG, respectively). In the liver, Ethe1 and Tst expression were relatively high (approximately 130% and 50% of HKG, respectively) as were expression of these genes in the colonic mucosa (approximately 11% and 40% of HKG, respectively).

Mouse liver and colon demonstrate SQR and Ethe1 immunoreactivity

Using two distinct antisera for SQR resulted in different immunoreactive structures in the mouse brain. The polyclonal antiserum available from Sigma identified immunoreactivity in epithelial cells of the choroid plexus and the subfornical organ. In addition, this antiserum identified diffuse immunoreactivity in the molecular layer of the cerebellum and glomeruli in the olfactory bulb. The polyclonal antiserum available from Proteintech identified diffuse immunoreactivity in the subfornical organ, but distinct immunoreactivity in the cell bodies of cortical neurons, cell bodies, dendrites and axons of Purkinji cells of the cerebellum, and cell bodies of mitrial cells, but not glomeruli in the olfactory bulb. The mostly non-overlapping immunoreactivity for SQR in the brain by these two antisera raises doubt regarding specificity of each antiserum and, together with the mRNA data, suggests that SQR expression might be quite limited in the brain. Of interest is that the subfornical organ, which exhibited overlapping immunoreactivity by both antisera, is a region of the brain outside the blood–brain barrier. In contrast to the CNS, both antisera identified similar immunoreactive structures in the liver and in the colon. Both antisera stained hepatocytes in the liver (Figure 7A and B). In the colon, both antisera-stained epithelial cells in the mucosa as well as numerous fine structures throughout the musculature and cells within the myenteric plexus (Figure 7C and D). Immunoreactivity for Ethe1 was not detected in the mouse brain. Conversely, Ethe1 immunoreactivity was detected in the epithelium of the mouse colonic mucosa (Figure 8A) as well as hepatocytes in the liver (Figure 8B).

Discussion and conclusions

The results of this study support the concept that SQR contributes to the catabolism of H_2S and suggest that robust catabolic pathways contribute to low resting tissue levels of H_2S, thereby increasing the signalling capacity of H_2S synthesized on demand. This study demonstrates for the first time that stigmatellin, an inhibitor of SQR, enhanced H_2S release in the colonic muscularis externa and inhibited H_2S catabolism in the colonic muscularis externa and liver. A related SQR inhibitor, antimycin A, also inhibited H_2S catabolism in the colonic muscularis externa. These findings extend previous reports that SQR contributes to H_2S catabolism in the rat liver (Hildebrandt and Grieshaber, 2008), and that recombinant expression of the human SQR gene oxidizes sulphide (Lagoutte *et al.*, 2010). Furthermore, our findings in the present study suggest that catabolism of endogenous H_2S through SQR contributes to H_2S signal termination in the colonic muscularis externa and liver. The lack of effect of stigmatellin and antimycin A on H_2S catabolism in brain tissue, which may be a reflection of the observed low levels of SQR mRNA and protein expression compared with colonic muscularis externa and liver tissues, suggest that SQR does not contribute to H_2S signal termination in the brain. These results confirm a previous finding that mitochondria isolated from mouse brain lack SQR-like enzymatic activity (Lagoutte *et al.*, 2010). In the brain, other unidentified enzymatic pathways of H_2S catabolism to thiosulphate and sulphate and H_2S sequestration probably contribute to H_2S signal termination. This difference in H_2S catabolism is not unlike different mechanisms of H_2S production in the brain and periphery (Ishigami *et al.*, 2009).

SQR is a member of the disulphide oxidoreductase flavoprotein (DiSR) superfamily and is responsible for H_2S oxidation by phototrophic and chemolithoautotrophic bacteria where H_2S acts as an electron donor to the respiratory chain (Griesbeck *et al.*, 2000). SQR was recently identified as the

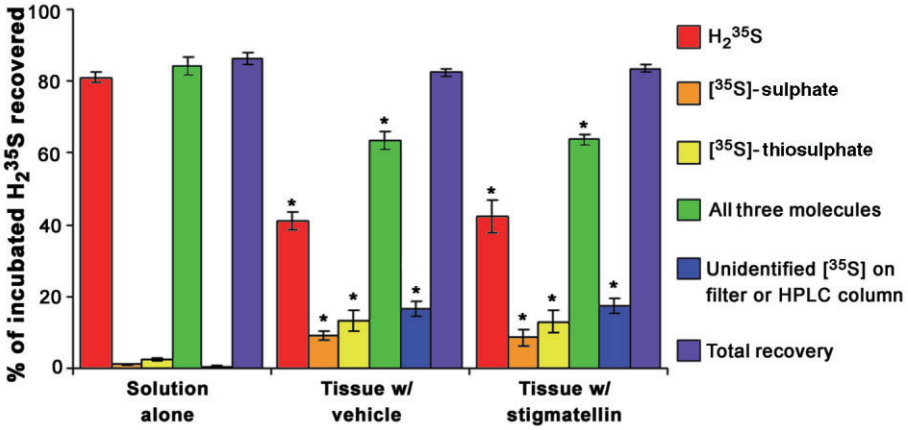

Figure 5

The proportion of $H_2^{35}S$ delivered to brain tissue with vehicle (0.1% EtOH) or 3 μM stigmatellin that was recovered as either [^{35}S]-sulphate, [^{35}S]-thiosulphate or $H_2^{35}S$, all three of these molecules, unidentified ^{35}S recovered from the filter (>30 kDa) or HPLC column or a combination of the three identified molecules, and the ^{35}S that was not identified. Nearly all of the supplied $H_2^{35}S$ was recovered as $H_2^{35}S$ when added to syringes containing the incubation solution without tissue (solution alone). Brain tissue reduced the proportion of $H_2^{35}S$ recovered as $H_2^{35}S$ and increased the proportions of $H_2^{35}S$ recovered as [^{35}S]-sulphate, [^{35}S]-thiosulphate, and unidentified [^{35}S] (*$P < 0.05$ compared with solution alone, Kruskal–Wallis test followed by Dunn's test). Stigmatellin (3 μM) had no effect on any of these measurements. Data are the mean ± SEM proportions for 4–10 observations.

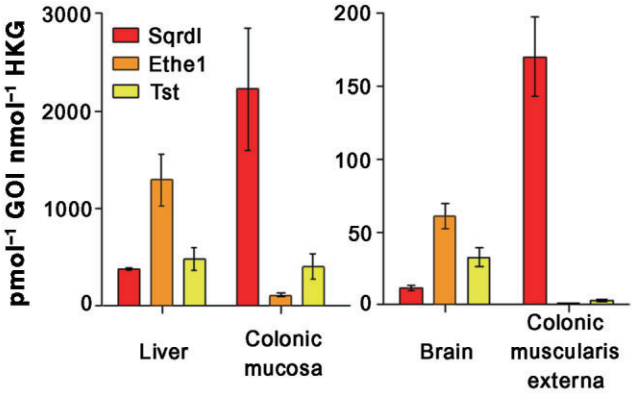

Figure 6

Expression of mRNA encoding SQR in the mouse liver, colonic mucosa and colonic muscularis externa, mRNA encoding Ethe1 in the mouse liver, colonic mucosa and brain, and mRNA encoding Tst in the mouse liver and brain. SQR, Ethe1 and Tst were expressed at low levels in brain tissue. Likewise, Ethe1 and Tst mRNA levels were at the detection limit in the colonic muscularis externa. Data are the mean ± SEM 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 HKG in nmol for n = 3–4 animals.

initial protein responsible for H_2S oxidation in the rat liver (Hildebrandt and Grieshaber, 2008), and recombinant expression of the human SQR gene enhances sulphide oxidation of a mammalian cell line (Lagoutte *et al.*, 2010). Stigmatellin and antimycin A are inhibitors of the cytochrome bc complex (complex III) involved in mitochondrial respiration (Potter and Reif, 1952; Kunze *et al.*, 1984; Thierbach *et al.*, 1984; von Jagow and Ohnishi, 1985; Zhang *et al.*, 1998) as well as inhibitors of bacterial SQR (Griesbeck *et al.*, 2000). It is possible that the actions of stigmatellin and antimycin A on H_2S metabolism observed in the present study were through inhibition of complex III. However, several studies have been able to effectively separate sulphide oxidation activity from cytochrome bc oxidoreductase activity (Nubel *et al.*, 2000; Kawamukai, 2002; Hildebrandt and Grieshaber, 2008; Lagoutte *et al.*, 2010), suggesting that the enzymes are distinct proteins that share sensitivity to stigmatellin and antimycin A just as they share dependence on quinone as a cofactor. These enzymes are linked, however, as electrons donated to ubiquinone by SQR enter the electron transport chain through complex III and are eventually donated to oxygen (Griesbeck *et al.*, 2000; Nubel *et al.*, 2000; Hildebrandt and Grieshaber, 2008; Lagoutte *et al.*, 2010). This biochemical process, without direct implication of SQR, has been proposed as the mechanism by which inhibition of H_2S metabolism serves as a cellular oxygen sensor (Olson *et al.*, 2008). While it is possible that stigmatellin and antimycin A inhibit H_2S consumption via nonspecific effects downstream of blocking mitochondrial respiration (Thierbach *et al.*, 1984; von Jagow and Ohnishi, 1985), some effects of inhibiting respiration, including the loss of mitochondrial voltage potential and reduced cell viability, do not occur with stigmatellin as it does with antimycin A (Armstrong *et al.*, 2004). Furthermore, inhibition of cytochrome bc oxidoreductase by stigmatellin is not associated with an increase in reactive oxygen species often associated with other mitochondrial respiration inhibitors like antimycin A (Lai *et al.*, 2005; Panee *et al.*, 2007). In fact, stigmatellin has been shown to protect against the loss of mitochondrial voltage potential, mitochondrial damage and reduced cell viability caused by cellular models of oxidative stress (Armstrong *et al.*, 2004; Belyaeva *et al.*, 2006). These latter effects are probably due to the fact that stigmatellin binds to the Q_o binding site of cytochrome b rather than the Q_i site (site of action of anti-

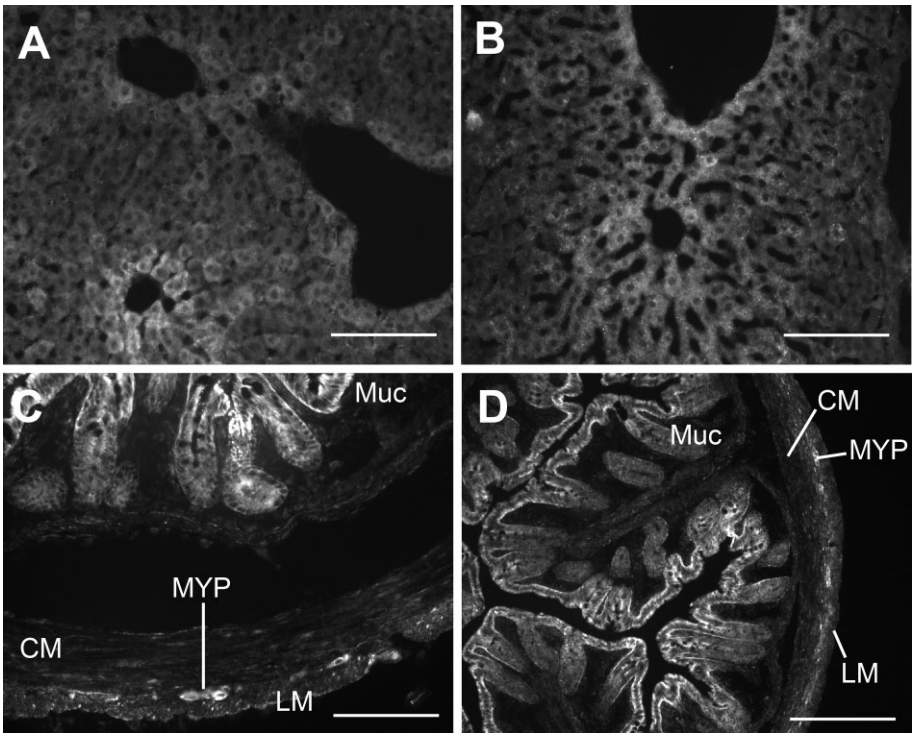

Figure 7

Representative micrographs illustrating immunoreactivity for SQR in the mouse liver (A and B) and colon (C and D). In the liver, both the Sigma antiserum (A) and Proteintech antiserum (B) produced immunoreactivity in hepatocytes. Likewise staining for both antisera were similar in the colon with strong immunoreactivity in epithelial cells and cells within the muscle layers and myenteric plexus (C and D) (Sigma: C; Proteintech: D). (Muc: colonic mucosa; CM: circular muscle layer of the colon; LM: longitudinal muscle layer of the colon; MYP: myenteric plexus of the colon.) Scale bars illustrate 100 μm.

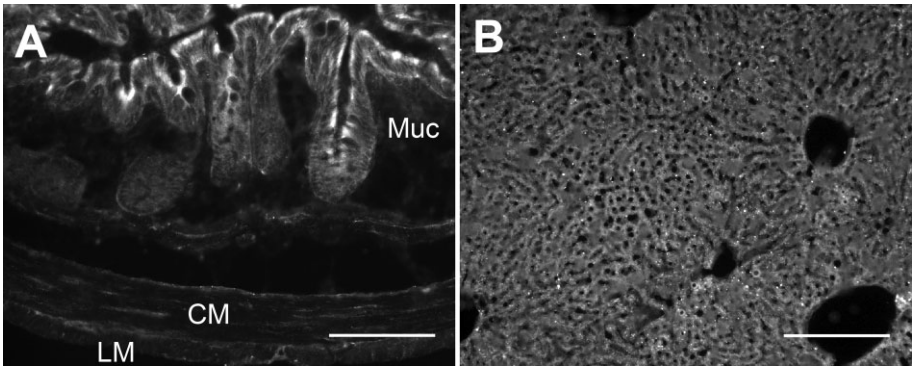

Figure 8

Representative micrographs illustrating immunoreactivity for Ethe1 in the mouse colon (A) and liver (B). Immunoreactivity was strong in the epithelial cells of the colonic mucosa while weak in the colonic muscularis externa. In the liver, there was strong immunoreactivity in hepatocytes. Scale bars illustrate 100 μm.

mycin A and related inhibitors), disabling the ability of the Rieske iron-sulphur protein (ISP) to move within the membrane and transfer electrons to the Q_i site (von Jagow and Ohnishi, 1985; Zhang *et al.*, 1998), so that electron transfer cannot take place. This type of respiratory chain inhibition is thus less damaging to the cell as ATP production can proceed, albeit at a lower efficiency, without the damaging effects of increased production of reactive oxygen species. We also used FCCP, an uncoupler of oxidative phosphorylation, and found that it did not inhibit H_2S consumption in any tissues tested. These data provide the strongest support for our hypothesis that the action of stigmatellin- and antimycin A-mediated inhibition of H_2S metabolism is distinct from inhibition of mitochondrial respiration. Therefore, we suggest that the

effects of stigmatellin and antimycin A observed in our study were due to inhibition of SQR.

It has recently been proposed that protein cysteine residues act as a sink to remove free H_2S by the formation of protein persulphides (Warenycia *et al.*, 1990; Ishigami *et al.*, 2009). While H_2S can certainly be released from protein persulphides upon reduction, there is little existing direct evidence to suggest that appreciable free H_2S, as supplied exogenously, or perhaps synthesized endogenously (Abe and Kimura, 1996; Wang, 2002; Furne *et al.*, 2008; Linden *et al.*, 2008), adds to the pool of persulphides or is retained as bound H_2S. Ishigami *et al.* (2009) have suggested that the rapid disappearance of H_2S exogenously applied to liver, brain and heart tissues resulted from absorption of sulphide because DTT-releasable H_2S was enhanced following the exposure of tissue to 3.3 μM Na_2S. However, they were not able to determine whether DTT-released H_2S were the same molecules as the supplied Na_2S. This limitation was circumvented in the present study by the use of $H_2{}^{35}S$. Approximately 90% of $H_2{}^{35}S$ delivered to the mouse colon muscularis externa was recovered as either [^{35}S]-sulphate, [^{35}S]-thiosulphate or $H_2{}^{35}S$. While it is possible that the unrecoverable label was bound to proteins in the tissue, the finding that samples without tissue had 15% loss of ^{35}S suggests that ^{35}S was lost in the experimental setup rather than being bound to tissue. The finding that the colonic muscularis externa catabolized $H_2{}^{35}S$ to only [^{35}S]-sulphate or [^{35}S]-thiosulphate is consistent with previous reports that nearly all $H_2{}^{35}S$ vascularly perfused or incubated with colonic mucosa, lung, kidney or liver tissues is recovered as [^{35}S]-sulphate or [^{35}S]-thiosulphate (Curtis *et al.*, 1972; Bartholomew *et al.*, 1980; Levitt *et al.*, 1999). Our findings are also compatible with earlier and with more recent literature using non-radioactive technologies that found that systemically applied H_2S is excreted as sulphate and does not remain as sulphide in the blood (Haggard, 1921; Denis and Reed, 1927; Whitfield *et al.*, 2008). In the present study, only 65% of $H_2{}^{35}S$ delivered to mouse brain homogenates was recovered as either [^{35}S]-sulphate, [^{35}S]-thiosulphate or $H_2{}^{35}S$. Samples without tissue had a 15% loss of ^{35}S, similar to the results from experiments using colonic muscularis externa, suggesting that 20% of $H_2{}^{35}S$ was converted to a form that we did not assay. There was no evidence that the unidentified ^{35}S-containing molecules were lipid soluble elemental sulphur. Rather, approximately half of the unidentified ^{35}S-containing molecules were captured by a 30 kDa filter, while the other half remained unidentified on the HPLC column, that is not recovered as [^{35}S]-sulphate or [^{35}S]-thiosulphate. Although further studies are required to determine the molecular fate of the unrecovered $H_2{}^{35}S$ incubated with brain tissue, we cannot at the present time exclude the possibility that H_2S becomes bound to protein sulphur pools in brain tissue as previously suggested (Ishigami *et al.*, 2009). The brain may be unique in this regard.

It is difficult to compare the rates of H_2S synthesis and catabolism in the current study given the differences in experimental conditions. When we normalized the rate of synthesis (pmol·min^{-1}) to the weight of the tissue (mg), we found that the rate of H_2S catabolism in colonic muscularis externa (2.4 pmol·min^{-1}·mg^{-1}) exceeded the rate of H_2S synthesis (1.7 pmol·min^{-1}·mg^{-1}). It should be noted that 10 mM of substrate (cysteine) was supplied in the H_2S release experiments, while only 0.2 μM of substrate ($H_2{}^{35}S$) was supplied in H_2S consumption experiments, such that the latter might be an underestimate of physiological catabolism and the former may be an overestimate of physiological H_2S production. Regardless, the difference in these rates may explain why the concentration of free H_2S in the colonic muscularis externa is just above the lower detection limit (ambient H_2S in room air) for the gas chromatograph. In the present study, the concentration of free H_2S measured in the colonic muscularis externa was 76 fmol·mg^{-1}. Previous studies indicate that the tissue levels of free H_2S in the brain and liver are approximately 20 fmol·mg^{-1} (Furne *et al.*, 2008) and <15 nM in the blood (Whitfield *et al.*, 2008). Thus, the results of these previous studies, as well as the present study, support the concept that the level of free H_2S in biological tissues is normally maintained very low by a robust catabolic system. Previous reports suggesting that tissue and blood levels of H_2S are in the micromolar range (Abe and Kimura, 1996; Wang, 2002; Wallace *et al.*, 2009) have recently been called into question (Furne *et al.*, 2008; Whitfield *et al.*, 2008; Linden *et al.*, 2010). The high levels of H_2S may have been due to the addition of cysteine, which can non-enzymatically produce H_2S (Figure 1), tissue acidification with trichloroacetic acid and errors in standard curves constructed with impure NaHS used to quantify H_2S using the methylene blue spectrophotometric assay (Ishigami *et al.*, 2009).

It has generally been assumed that H_2S conversion to sulphate occurs subsequent to thiosulphate (Levitt *et al.*, 1999). In the present study, stigmatellin reduced the conversion of $H_2{}^{35}S$ to [^{35}S]-thiosulphate by the colon muscularis externa but did not affect the conversion of $H_2{}^{35}S$ to [^{35}S]-sulphate. One possible explanation may be that the enzymes responsible for the conversion of thiosulphate to sulphate are saturated such that reduced thiosulphate levels may still exceed the capacity of the enzymes and thus still have similar sulphate levels. While H_2S has a low K_m value of 2.9 μM for SQR (Hildebrandt and Grieshaber, 2008), thiosulphate has a K_m value of 2.6 mM for thiosulphate:quinone oxidoreductase (Muller *et al.*, 2004), the putative enzyme for thiosulphate oxidation, suggesting that saturation of thiosulphate catabolism is not likely to explain this observation. An alternative explanation is that another pathway for the production of sulphate from H_2S exists in mouse colonic muscularis externa and liver. This idea is supported by the observation that stigmatellin did not inhibit H_2S catabolism in the brain where sulphate and thiosulphate were still produced, and that it reduced but did not block H_2S metabolism in the liver and colonic muscularis externa. A surprising finding of these studies was that while SQR is highly expressed in the liver, colonic mucosa and colonic muscularis externa, Ethe1, the putative sulphur dioxygenase responsible for SQR-persulphide oxidation and TST, the thiosulphate transferase responsible for thiosulphate production are highly expressed in the liver and colonic mucosa but negligible in the colonic muscularis externa. These results suggest that while the SQR-Ethe1-TST catabolism of H_2S to thiosulphate previously described in the rat liver (Hildebrandt and Grieshaber, 2008) may be responsible for the catabolism of H_2S in the mouse liver and colonic mucosa, other downstream acceptors of the

SQR-persulphides (i.e. not Ethe1 and TST) may exist in colonic muscularis externa.

H_2S is gaining acceptance as a gaseous signal molecule involved in diverse cellular effects (Wang, 2002; Whiteman and Moore, 2009; Kimura, 2010; Linden *et al.*, 2010). An important aspect of the biology of signal molecules is the termination of the signal. The results of this study support the concept that the mitochondrial protein SQR contributes to the rapid oxidation of H_2S. As H_2S appears to be anti-inflammatory (Wallace, 2010) and is beneficial in animal models of myocardial infarction (Ririe *et al.*, 1997; Elrod *et al.*, 2007; Zhu *et al.*, 2007), it is reasonable to suggest that an inhibitor of a catabolic pathway for endogenous H_2S may be an important target. New compounds that can inhibit H_2S catabolic pathways will have to distinguish between SQR and cytochrome bc complex. Because crystal structures for bacterial SQR have recently been solved (Brito *et al.*, 2009; Marcia *et al.*, 2009), it is expected this development will be forthcoming.

Acknowledgements

We gratefully acknowledge the secretarial support of Ms Janice Applequist and Mr James Craighead and Mr Tyler King of the Mayo Clinic Division of Engineering for the design and fabrication of the apparatus used to capture released H_2S. This work was supported by NIH grants DK17238 and DK76665 and a grant from the Minnesota Partnership for Biotechnology and Medical Genomics.

Conflicts of interest

No competing financial interests exist for the authors, DRL, JF, GJS, MA-R, MDL or JHS.

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