BJP British Journal of Pharmacology

DOI:10.1111/bph.12084
www.brjpharmacol.org

RESEARCH PAPER

Hydrogen sulphide-induced relaxation of porcine peripheral bronchioles

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Keywords
hydrogen sulphide; airway smooth muscle; relaxation; K^+ channels

Received
6 July 2012
Revised
29 October 2012
Accepted
26 November 2012

BACKGROUND AND PURPOSE

Hydrogen sulphide (H_2S) is an endogenous gasotransmitter. Although it has been shown to elicit responses in vascular and other smooth muscle preparations, a role for endogenously produced H_2S in mediating airway tone has yet to be demonstrated. Therefore, the aim of this study was to determine whether H_2S is produced within the airways and to determine the functional effect on airway tone.

EXPERIMENTAL APPROACH

Small peripheral airways (<5 mm in diameter) from porcine lungs were set up in isolated tissue baths, pre-contracted with the muscarinic agonist carbachol, and then exposed to either the H_2S donor sodium hydrosulphide (NaHS), or the precursor L-cysteine. H_2S production from L-cysteine or 3-mercaptopyruvate in tissue homogenates was measured by the methylene blue assay. Expression of the H_2S-synthesizing enzymes cystathionine β-synthase (CBS), cystathionine γ lyase (CSE) and 3-mercaptopyruvate sulphurtransferase (3-MST) were measured by Western blotting.

KEY RESULTS

NaHS caused a large relaxation of the airways, which was inhibited partially by pre-contraction with KCl or exposure to tetraethylammonium, but not glibenclamide, paxilline or 4-aminopyridine. L-cysteine also caused a relaxation of the airways which was inhibited by the CBS inhibitor aminooxyacetic acid. Tissue homogenates from airways exposed to L-cysteine or 3-mercaptopyruvate *in vitro* showed a significant production of H_2S. Western blotting demonstrated immunoreactivity to CBS, CSE and 3-MST enzymes in the airways.

CONCLUSIONS AND IMPLICATIONS

These data demonstrate that H_2S can be produced endogenously within porcine airways causing relaxation. The mechanism of relaxation depends, in part, on K^+ channel activity.

Abbreviations

3-MST, 3-mercaptopyruvate sulphurtransferase; AOAA, aminooxyacetic acid; CBS, cystathionine β-synthase; CSE, cystathionine γ lyase; H_2S, hydrogen sulphide; PPG, D,L-propargylglycine

Introduction

Until relatively recently, hydrogen sulphide (H_2S) was considered as an environmental, toxic gas. However, recent studies have identified mammalian enzymes capable of synthesizing H_2S. The two main enzymes, cystathionine β-synthase (CBS) and cystathionine γ lyase (CSE), utilize L-cysteine to produce H_2S (Li *et al.*, 2011). A third pathway by which H_2S can be produced involves the enzyme 3-mercaptopyruvate sulphurtransferase (3-MST; Li *et al.*, 2011). Expression of these enzymes is widespread throughout the body, but particularly in the liver and brain (Kabil *et al.*, 2011; Kimura, 2011). Pharmacological inhibitors of CBS and CSE are available, which enable the investigation of the role of endogenously produced H_2S.

Much interest in the role of H_2S in the body has focused on its role in the vasculature, particularly due to the similarities with nitric oxide (see Li *et al.*, 2009 for review). In isolated

blood vessels, H_2S can produce both a contraction and a relaxation, depending on the concentration, with contractile responses predominating at lower concentrations (Kubo *et al.*, 2007b). In rat aorta, the relaxation response is partially blocked by glibenclamide, suggesting an involvement of K_{ATP} channels (Kubo *et al.*, 2007b), whereas in rat coronary arteries Kv channels are thought to mediate the relaxation to H_2S (Cheang *et al.*, 2010), and in porcine cerebral arterioles BKCa channels have been implicated (Liang *et al.*, 2012). Inhibition of K^+ channels also inhibited the relaxation to H_2S in mouse aorta, although again there was an incomplete inhibition (Al-Magableh and Hart, 2011).

Of the few studies which have investigated the effect of H_2S in the airways, investigations have only looked at the effects of exogenously applied H_2S in isolated airways. No study has yet determined whether H_2S is synthesized within the airways and whether this endogenously produced H_2S alters airway tone. Within the studies published, there are also contradictory responses. Addition of sodium hydrosulphide (NaHS), an H_2S donor, caused a concentration-dependent relaxation in mouse bronchi and a modest relaxation of guinea pig bronchi (Kubo *et al.*, 2007a). By contrast, Tevisani *et al*. reported that NaHS caused a contraction in guinea pig bronchi, which they ascribed to activation of TRPV1 receptors and release of sensory neuropeptides (Trevisani *et al.*, 2005). The differences between mouse and guinea pig airways may represent species differences, particularly in relation to differences in the sensory nervous system in the airways as guinea pig airways are extremely sensitive to sensory nerve stimulation. Indeed, when the sensory nerves were desensitized by capsaicin treatment, the H_2S-induced contraction turned into a slight relaxation (Trevisani *et al.*, 2005). On the other hand, Kubo *et al*. found that NaHS did not cause a contraction in guinea pig bronchi, but caused a slight relaxation (Kubo *et al.*, 2007a). The reasons for these opposing effects are not known.

H_2S is thought to have an anti-inflammatory role within the body (Li *et al.*, 2005); although some studies have also indicated that it has a pro-inflammatory role (Li *et al.*, 2005). A recent study in rats exposed to cigarette smoke for 4 months showed an increase in the expression of CSE in whole lungs, and an increase in H_2S levels in the plasma (Chen *et al.*, 2011). On the other hand, there was no change in H_2S levels in whole lung, although this study did not investigate the production of H_2S in the airways specifically. Inhibition of CSE with propargylglycine (PPG) enhanced the lung pathology caused by cigarette smoke suggesting that H_2S may have a protective effect in the lungs (Chen *et al.*, 2011). This is supported by the observation that NaHS treatment of rats with ovalbumin-induced inflammation of the lungs reduced the airway inflammation (Chen *et al.*, 2009).

From the studies carried out to date, it is unclear what the effects of H_2S are on airways and, indeed, whether H_2S can be produced endogenously within the airways themselves or whether the effects described in the literature are just the effects of exogenous H_2S. The aim of this study was to investigate the effects of H_2S on tone in isolated peripheral bronchioles from the pig and to determine whether H_2S can be produced endogenously within the airways.

Materials and methods

Tissue preparation

Porcine lungs were obtained from a local abattoir and transported to the laboratory on ice. Small peripheral bronchioles were dissected from one of the lobes of the lung and placed in ice-cold Krebs-Henseleit buffer containing 2% Ficoll which had been pre-gassed with 95% O_2/5% CO_2, and stored overnight at 4°C (Lelliott *et al.*, 2012). The following day, bronchioles (<5 mm in diameter) were dissected into 5 mm ring segments and suspended in an isolated tissue bath containing Krebs-Henseleit buffer maintained at 37°C and constantly gassed with 95% O_2/5% CO_2. The lower support was fixed and the upper support was connected to a force transducer (AD Instruments Ltd, Hastings, UK) linked to a PowerLab data acquisition system (AD Instruments Ltd) via an amplifier. After a 20-min equilibration period, tension was applied to the tissue, which was allowed to relax to a final resting tension of between 0.5 and 1 g wt. Before each experiment the tissues were contracted three times with 60 mM KCl.

Effect of NaHS on porcine bronchioles

After the tissues have been set up as above, the tissues were contracted with the muscarinic receptor agonist carbachol to give a contraction that was around 100% of the response to the final addition of 60 mM KCl. Previous studies demonstrated that this is approximately 50% of the maximum response to carbachol (Lelliott *et al.*, 2012) and that contractions are well-maintained over time. After the carbachol-induced tone had reached a plateau, a single concentration of the H_2S donor NaHS was added to the tissues. Responses to the tissues were then observed. As the relaxations to NaHS were transient, tissues were allowed to recover their original tone and then left for a further 20 min prior to a second addition of NaHS. Relaxation responses were expressed as a percentage of the contractile response to carbachol. Alternatively, tissues were contracted with KCl or endothelin-1 to a similar level of tone prior to addition of NaHS.

In a separate series of experiments, tissues were relaxed with 1 mM NaHS in the absence or presence of tetraethylammonium (TEA; 1 or 10 mM, non-selective K^+ channel blocker), glibenclamide (3 μM, K_{ATP} channel blocker), paxilline (10 μM, BK_{Ca} channel blocker), 4-aminopyridine (4-AP, 1 mM, Kv channel blocker) or 10 μM HC-030031 (TRPA1 antagonist, Eid *et al.*, 2008). The roles of nitric oxide and cyclooxygenase were investigated using 300 μM N-Nitro-L-arginine methyl ester (L-NAME) and 10 μM indometacin respectively.

Effect of L-cysteine on porcine bronchioles

Tissues were setup as above. After pre-contraction with carbachol, tissues were exposed to different concentrations of L-cysteine (1 to 10 mM) in the absence or presence of 100 μM aminooxyacetic acid (AOAA).

In a separate set of experiments, tissues were exposed to 100 μM AOAA prior to construction of concentration-response curves to carbachol (1×10^{-8} M to 3×10^{-5} M).

Endogenous production of H_2S

Segments of small peripheral bronchioles, large main bronchi and trachea were homogenized in ice-cold Tris-EDTA

(50 mM, 1 mM, pH 7.4) and centrifuged at 4°C at 1000× *g* for 20 min. The supernatant layers were removed and recentrifuged at 30 000× *g* at 4°C for 40 min. Supernatant layers were then removed again and the pellet discarded.

Samples were assessed for H_2S production by the methylene blue method (Webb *et al.*, 2007), by incubating with 10 mM L-cysteine, in the absence and presence of pyridoxal phosphate (1 mM) in Tris buffer (0.1 M, pH 9.0) in a total volume of 300 µL. Samples were then incubated at 37°C in a shaking water bath for the indicated periods. Blank samples contained L-cysteine, but no tissue. Reactions were stopped by the addition of 500 µL 4% (w/v) zinc acetate in distilled water and kept on ice for 30 min. Two hundred microlitre of 0.1% (w/v) dimethylphenylene-diamine sulphate in 5 M HCl and 100 µL ferric chloride (50 mM) were then added. Samples were mixed and then centrifuged at 5000× *g* to precipitate the protein. Samples were then analysed at 670 nm on a Unicam spectrometer. Blank values were subtracted from test samples and data were expressed as nmoles per mg of protein.

The 3-MST activity was assessed by measuring the production of H_2S from 3-mercaptopyruvate using a slight modification of the method by Shibuya *et al.*, 2009. Briefly, samples, prepared as above, were incubated with 0.3 mM 3-mercaptopyruvate in Tris buffer (0.1 M, pH 9.0) containing 0.5 mM dithiothreitol in a total volume of 300 µL. Samples were then incubated at 37°C in a shaking water bath for 30 min. Reactions were stopped and estimation of methylene blue production assessed as above.

Western blotting

Samples prepared as for the enzyme assays were diluted 1:1 in 2× Laemmli sample buffer. A 10-µg sample from five different animals were then separated by polyacrylamide gel electrophoresis using a 4–20% gradient polyacrylamide gradient gel. Proteins were transferred to nitrocellulose membrane by Western blotting, followed by blocking in 5% fish skin gelatine in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). After 1 h incubation, blots were incubated with either rabbit anti-CBS (0.1 µg mL^{-1}, clone 3E1; Abnova, London, UK), mouse anti-CSE (2 µg mL^{-1} dilution; ab80643, Abcam, Cambridge, UK) or rabbit anti-MPST (0.2 µg mL^{-1}, HPA001240; Atlas antibodies, Stockholm, Sweden) antibodies in 5% fish skin gelatin overnight at 4°C. Blots were also probed for β-actin (1:50 000 dilution in 5% fish skin gelatin) using a mouse monoclonal antibody (Sigma-Aldrich, Poole, Dorset, UK). After washing in TBS-T, blots were then incubated with goat-anti-mouse and goat-anti-rabbit secondary antibodies conjugated to 680 nm and 800 nm infrared dyes (IRDye, Licor, Cambridge, UK) respectively. Immunoblots were then visualized using an Odyssey system from Licor.

Data analysis and statistics

Relaxation responses were expressed as a percentage of the carbachol-induced contraction and are presented as means ± SEM. Comparisons between more than two data sets were made using ANOVA followed by a Bonferroni *post hoc* test. For comparisons between two data sets, a two-tailed unpaired Student's *t*-test was used, or, for paired data, a two-tailed paired Student's *t*-test. A *P*-value of less than 0.05 indicated a significant difference between the data sets.

Materials

AOAA, D,L-propargylglycine (PGG); NaHS, TEA, L-cysteine, 3-mercaptopyruvate, paxilline, glibenclamide, 4-aminopyridine, L-NAME, indometacin were all obtained from Sigma-Aldrich. HC-03001 was obtained from Tocris Biosciences, Abingdon, UK.

Results

Effect of NaHS on isolated bronchioles

NaHS caused a concentration-dependent relaxation of porcine small peripheral bronchioles (Figure 1). The relaxation to each concentration was transient but did not appear to be due to desensitization as a second addition of NaHS produced an almost identical level of relaxation (Figure 1A & B). Owing to the transient nature of the response, non-cumulative additions of NaHS were used in subsequent experiments.

In order to determine whether the relaxation could also be obtained after pre-contraction with another agonist, tissues were pre-contracted with endothelin-1 prior to exposure to NaHS. A similar relaxation response to NaHS was obtained under these conditions (75 ± 5% relaxation after addition of 1 mM NaHS, n = 8). Although NaHS relaxed tissues with agonist-induced tone, 3 mM NaHS had no effect on basal tone. Denudation of the epithelium had no effect on the relaxation to NaHS (Figure 1C).

Role of K^+ channels in relaxations to NaHS

Previous studies of H_2S relaxations in isolated blood vessels have suggested that the mechanism of relaxation is, at least in part, through activation of K^+ channels (Kubo *et al.*, 2007b). In tissues contracted with KCl instead of carbachol, there was a much reduced relaxation response compared to that obtained after pre-contraction with carbachol (Figure 2). Exposure to the K^+ channel blocker TEA (1 mM) had no effect on the relaxation to 1 mM NaHS, although a higher concentration (10 mM) slightly inhibited the relaxation to 1 mM NaHS (Table 1). On the other hand, glibenclamide (3 µM), paxilline (10 µM) and 4-AP (1 mM) had no significant effect on the relaxation to 1 mM NaHS (Table 1). Similarly, the TRPA1 receptor antagonist HC-030031 (10 µM) had no effect on the relaxation to 1 mM NaHS (Table 1). Pre-incubation with a combination of 300 µM L-NAME to block nitric oxide production and 10 µM indometacin to inhibit cyclooxygenase also failed to inhibit the relaxation to 1 mM NaHS (49 ± 14% relaxation in control vs. 56 ± 20% relaxation in the presence of L-NAME and indometacin, n = 4).

Determination of H_2S production in homogenates of peripheral bronchioles

Biochemical assays showed an increase in the production of H_2S in porcine peripheral bronchiole tissue samples incubated with L-cysteine, which was enhanced in the presence of the cofactor pyridoxal phosphate (Figure 3). As a comparison, H_2S production was also measured in samples of large bronchioles and trachea. There was a similar increase in H_2S production at 60 min in the large bronchioles and the trachea,

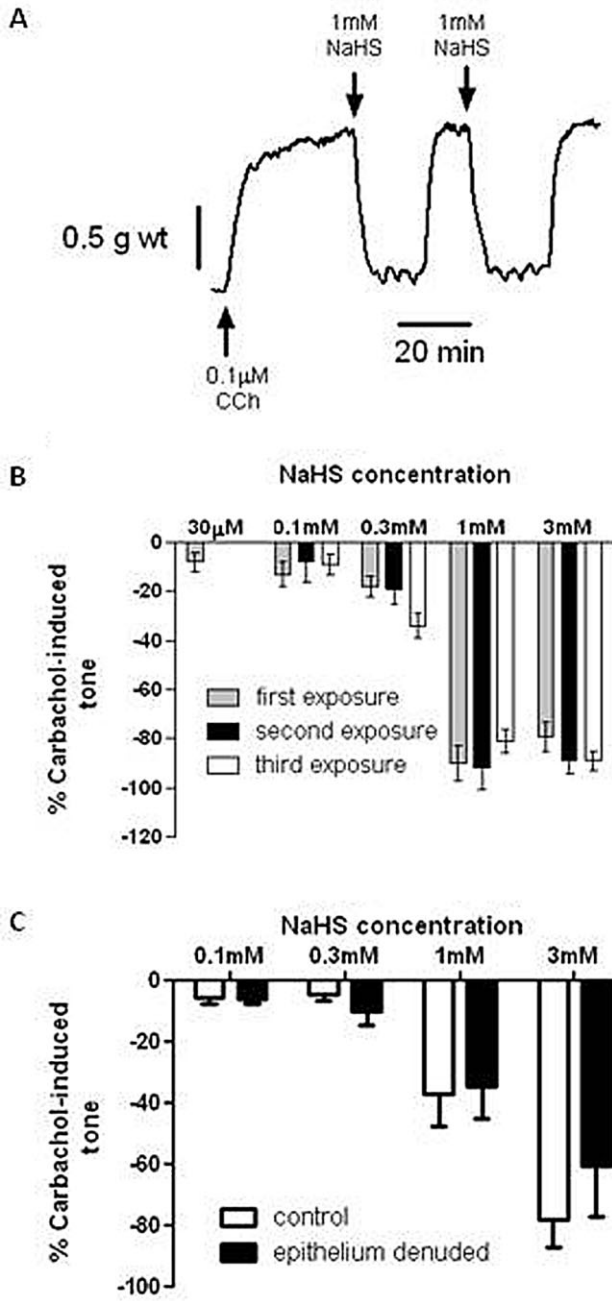

Figure 1

(A) A typical trace showing the effect of sodium hydrosulphide (NaHS) on bronchiole tone. Tissues were pre-contracted with the muscarinic agonist carbachol (CCh) to ~100% of the response to 60 mM KCl. After the contraction had reached a plateau, tissues were exposed to a single concentration of NaHS (1 mM). This produced a transient relaxation. After the relaxation had returned to the pre-contraction level, tissues were exposed to a second, single addition of NaHS (1 mM), without re-addition of carbachol. (B) Bar chart showing mean relaxations to repeated administration of NaHS in porcine bronchioles. Data are expressed as a % relaxation and are means ± SEM. of eight experiments. (C) Bar chart showing mean relaxations to NaHS in porcine bronchioles with and without denudation of the epithelium. Data are expressed as a % relaxation and are means ± SEM of six experiments.

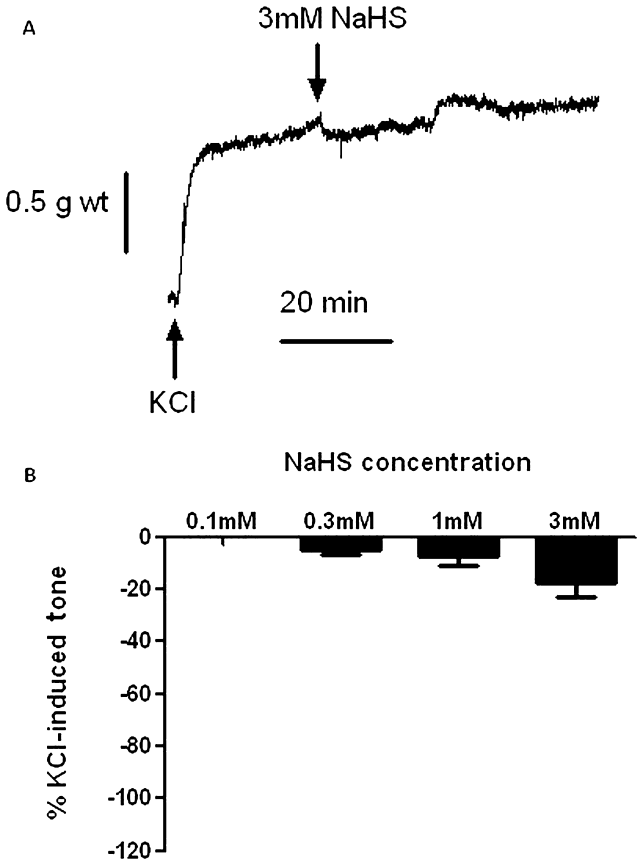

Figure 2

(A) A typical trace showing the effect of 3 mM sodium hydrosulphide (NaHS) on bronchiole tone after pre-contraction with 60 mM KCl. After the contraction had reached a plateau, tissues were exposed to a single concentration of NaHS (3 mM). (B) Maximum relaxation responses to NaHS after pre-contraction with 60 mM KCl in porcine peripheral bronchioles. Data are expressed as a percentage relaxation from the KCl-induced contraction and are means ± SEM of four to six experiments.

Table 1

Relaxation responses to 1 mM sodium hydrosulphide (NaHS) in porcine small bronchioles after pre-contraction with carbachol, in the presence of various K^+ channel blockers or the TRPA1 channel inhibitor HC-030031

	% control response
+10 mM TEA	55.6 ± 11.4% (n = 7)*
+3 μM glibenclamide	135.0 ± 17.0% (n = 4)
+10 μM paxilline	127.4 ± 21.8% (n = 4)
+1 mM 4-AP	103.3 ± 20.6% (n = 4)
+10 μM HC-030031	134.4 ± 19.4% (n = 4)

Data are expressed as a % of the control relaxation to 1 mM NaHS in the absence of inhibitor.
*indicates $P < 0.05$, Student's two-tailed, unpaired t-test
4-AP, 4-aminopyridine; TEA, tetraethylammonium.

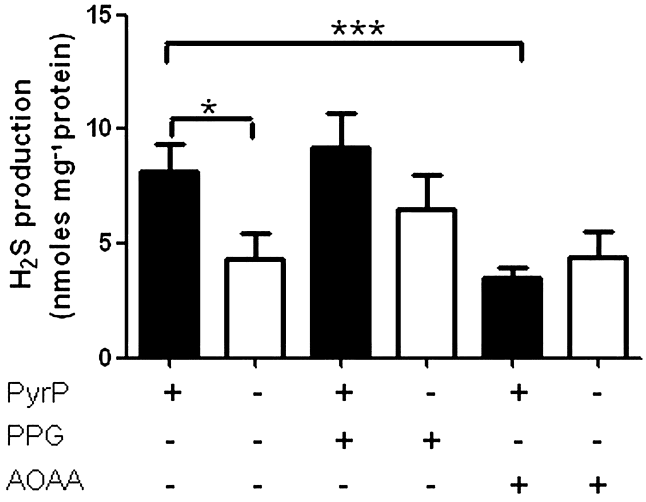

Figure 3

Production of H_2S after 60 min from L-cysteine (expressed as nmol mg^{-1} protein) in porcine small, peripheral bronchioles with and without the cofactor pyridoxal phosphate (PyrP; 1 mM) and in the presence or absence of propargylglycine (PPG; 10 μM) or amino-oxyacetic acid (AOAA) (100 μM). Data are mean ± SEM of nine experiments. * indicates $P < 0.05$, one-way ANOVA, followed by a Bonferroni *post hoc* test. *** indicates $P < 0.001$, one-way ANOVA, followed by a Bonferroni *post hoc* test.

Table 2

Production of H_2S from L-cysteine (expressed as nmole mg^{-1} protein) in porcine large bronchioles and trachea at 60 min, with and without the cofactor pyridoxal phosphate (PyrP; 1 mM)

	–PyrP	+ PyrP
Large bronchioles	6.6 ± 1.5 (n = 5)	11.0 ± 2.9 (n = 5)*
Trachea	7.9 ± 1.7 (n = 5)	11.4 ± 2.2 (n = 5)**

Data are mean ± SEM.
*indicates $P < 0.05$, two-tailed Student's paired t-test versus without pyridoxal phosphate.
**indicates $P < 0.01$, two-tailed Student's paired t-test versus without pyridoxal phosphate.

which was enhanced in the presence of pyridoxal phosphate (Table 2). There was no production of H_2S in solutions containing L-cysteine and pyridoxal phosphate indicating that the production of H_2S detected was dependent upon the presence of the tissue.

Production of H_2S in the porcine small bronchioles in the presence of pyridoxal phosphate was inhibited by 100 μM AOAA, but not by 10 μM PPG (Figure 3). Neither inhibitor had any effect on the production of H_2S in the absence of pyridoxal phosphate (Figure 3). Although 10 μM PPG had no effect on H_2S production in the bronchioles, the same concentration reduced the production of H_2S in Sprague Dawley rat brain from 3.3 ± 0.5 nmoles mg^{-1} protein to 0.06 ± 0.003 nmoles mg^{-1} protein.

These data suggest that H_2S could be produced through a pyridoxal phosphate-insensitive pathway in the porcine

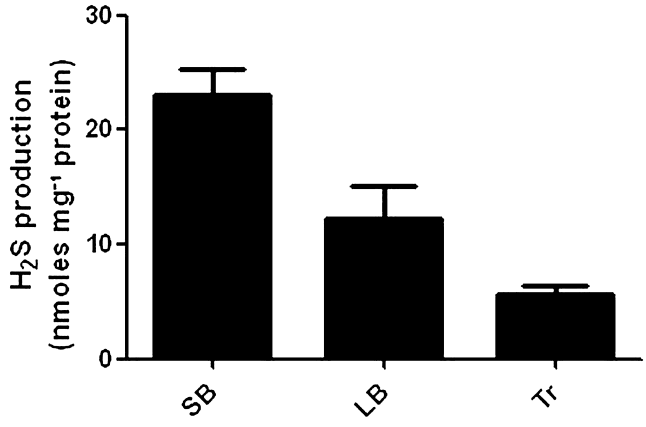

Figure 4

Production of H_2S from 3-mercaptopyruvate (expressed as nmol mg^{-1} protein) in porcine small, peripheral bronchioles (SB), porcine large bronchioles (LB) and porcine trachea (Tr). Data are mean ± SEM of four experiments.

airways, which might involve 3-MST. Therefore, we determined whether H_2S production could be detected using 3-mercaptopyruvate as a substrate. As seen in Figure 4, H_2S production was detected in small bronchioles using this alternative substrate. As a comparison, H_2S production could also be detected in large bronchioles and trachea using 3-mercaptopyruvate as substrate (Figure 4).

Detection of H_2S synthesizing enzymes using Western blotting

Using Western blotting, we were able to observe immunoreactivity for CSE, CBS and 3-MST in porcine peripheral bronchioles (Figure 5). Bands of similar molecular size were found in large bronchioles, trachea and pulmonary artery samples. The antibody against CSE produced a band at the predicted MW of 45 kDa (Jurkowska *et al.*, 2010) in porcine airways, pulmonary artery, porcine liver and the rat liver. In the porcine samples, a second band at 50 kDa was also detected. The antibody against CBS produced a strong band in all the porcine lung samples at the expected MW (~63 kDa; Skovby *et al.*, 1984) but no band in the rat liver samples. The antibody against 3-MST produced a strong band within the porcine lung samples at the predicted MW of ~30 kDa (Nagahara *et al.*, 1998), as did the rat liver cytosol. Both the porcine lung samples and the rat liver samples produced another band at a higher MW (at ~50 kDa in the porcine lung samples and ~43 kDa in the rat liver samples). Additional bands for CBS, CSE and 3-MST have also been detected by Western blot in mouse and rat tissue samples (Pong *et al.*, 2007; Shibuya *et al.*, 2009). Whether these additional bands represent alternative versions of the enzymes, or cross-reactivity with other, similar enzymes, is unknown.

Effect of L-cysteine on isolated bronchioles

In order to assess whether endogenously generated H_2S influenced airway tone *in vitro*, supplementation of tissue bath medium with different concentrations of L-cysteine was

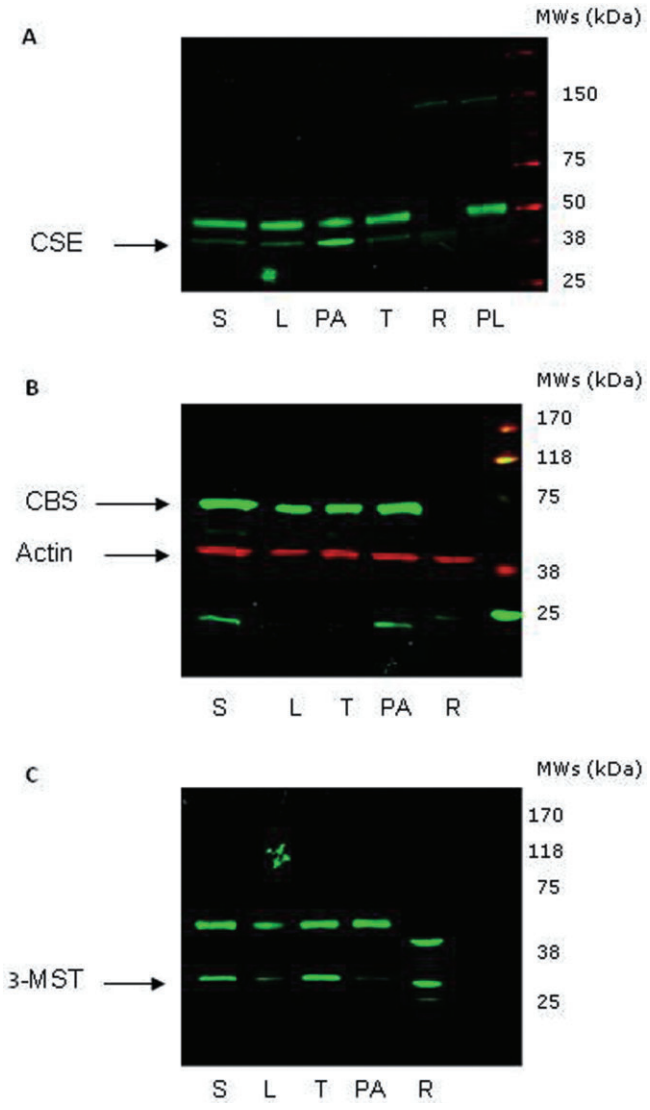

Figure 5

Typical Western blots showing immunoreactivity to antibodies against: (A) Cystathionine γ lyase (CSE), (B) cystathionine β-synthase (CBS) and (C) 3-mercaptopyruvate sulphanotransferase (3-MST) in cytosolic fractions from porcine small bronchioles (S), porcine large bronchioles (L) and porcine trachea (T). Also shown for comparison are porcine pulmonary artery (PA), rat liver cytosol (R) and pig liver cytosol (PL). In some experiments, smooth muscle actin was also detected as a positive control.

investigated. L-cysteine produced a concentration-dependent relaxation in small bronchioles pre-contracted with carbachol (Figure 6), although there was no effect on basal tone. Like the responses to NaHS, the relaxations to L-cysteine were transient (Figure 6A). Relaxations to 1 mM and 3 mM L-cysteine were significantly reduced in the presence of 100 μM AOAA (Figure 6B). There was also an apparent reduction in the relaxation to 10 mM L-cysteine in the presence of AOAA, although this was not significant. Pre-incubation with 10 mM TEA caused a complete inhibition of the relaxation to 10 mM L-cysteine (Figure 6C).

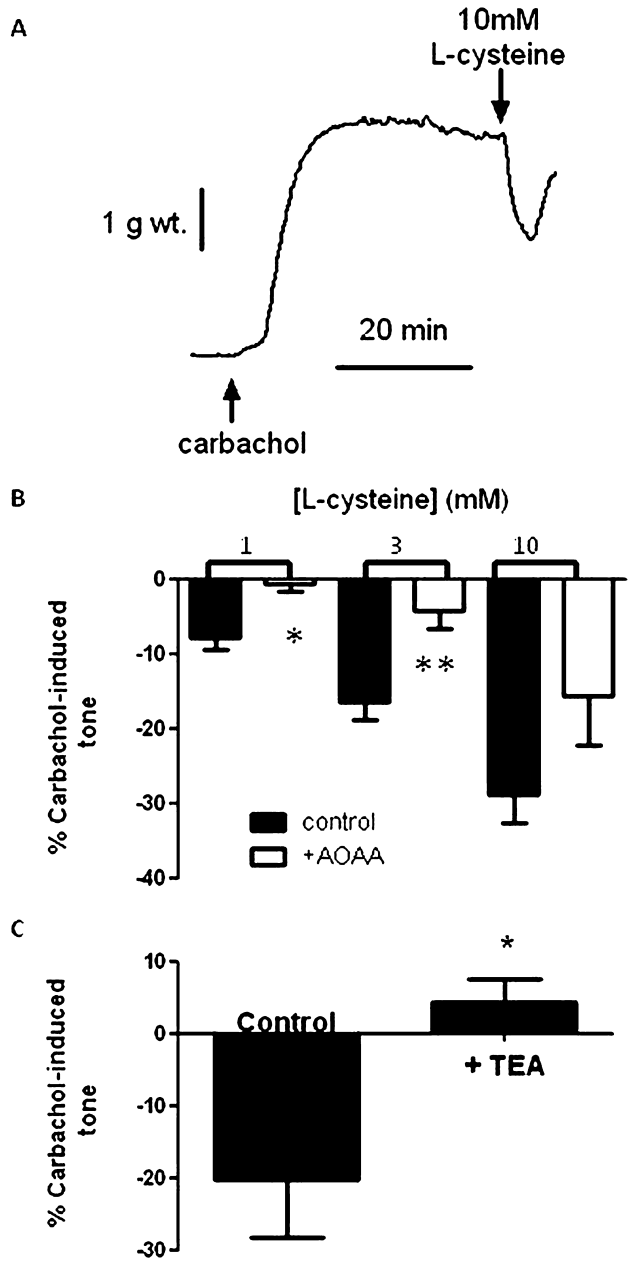

Figure 6

(A) A typical trace showing the effect of L-cysteine on bronchiole tone. Tissues were pre-contracted with the muscarinic agonist carbachol (CCh) to ~100% of the response to 60 mM KCl. After the contraction had reached a plateau, tissues were exposed to a single concentration of L-cysteine (10 mM). (B) Relaxation responses to different concentrations of L-cysteine in porcine small, peripheral bronchioles measured as a percentage change from the carbachol-induced contraction, in the absence (control) or presence of 100 μM aminooxyacetic acid (AOAA). Each point represents the mean ± SEM of 8–16 experiments. * indicates $P < 0.05$, Mann–Whitney *U*-test versus control, ** indicates $P < 0.01$, Student's two-tailed, unpaired *t*-test versus control. (C) Relaxation responses to 10 mM L-cysteine in porcine small, peripheral bronchioles measured as a percentage change from the carbachol-induced contraction, in the absence (control) or presence of 10 mM tetraethylammonium (+TEA). Each point represents the mean ± SEM of four experiments. * indicates $P < 0.05$, two-tailed Student's unpaired *t*-test versus control.

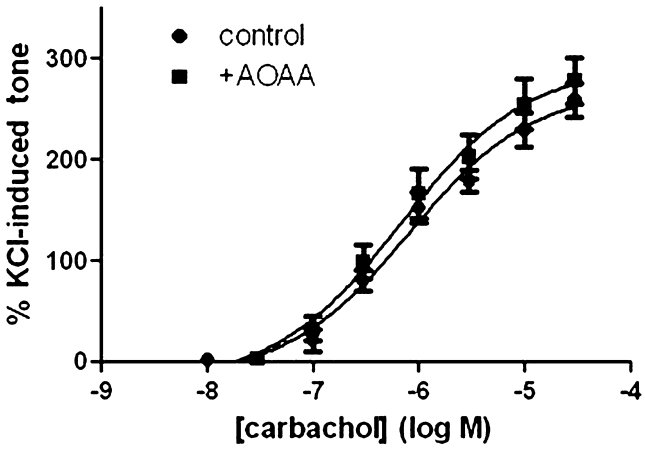

Figure 7

Concentration-response curves to carbachol in the absence (control) or presence of 100 μM AOAA. Data are expressed as a percentage of the contraction to 60 mM KCl and are means ± SEM mean of four experiments.

In order to determine whether basal levels of H_2S production could regulate airway tone, tissues were incubated with 100 μM AOAA prior to the construction of a concentration-response curve to carbachol. Pre-incubation with AOAA had no significant effect on the carbachol-induced contraction (Figure 7).

Discussion

Recent studies, particularly in the vasculature, have demonstrated that H_2S is an endogenous regulator of smooth muscle tone. The effect of H_2S on blood vessel tone is complex, with some studies reporting a contractile response at low concentrations and a relaxation response at higher concentrations (Kubo *et al.*, 2007b). This present study is the first to demonstrate the production of H_2S in airways and the presence of H_2S-synthesizing enzymes, in combination with H_2S-stimulated relaxation of the airways. Although other studies have demonstrated that exogenously applied H_2S produces responses in airways, none of these have demonstrated endogenous production. Trevisani *et al.* showed that the H_2S donor NaHS caused a contractile response within guinea pig bronchi (Trevisani *et al.*, 2005). However, Kubo *et al.* failed to observe a contractile response in the same tissue, but instead observed a small relaxation response (Kubo *et al.*, 2007a). Furthermore, in mouse bronchi, NaHS produced a large relaxation response (Kubo *et al.*, 2007a). Neither study linked the effects seen to endogenous production of H_2S in the airways. Indeed, in the mouse bronchi, the H_2S precursor L-cysteine had no effect on airway tone (Kubo *et al.*, 2007a). In this present study, we have demonstrated that the H_2S donor NaHS produces a large, concentration-dependent, transient relaxation in porcine peripheral bronchioles. There was no evidence of a contractile response to NaHS even at low concentrations, unlike the contractions seen at these lower concentrations in isolated blood vessels (Kubo *et al.*, 2007b). The large relaxation response supports the observations by Kubo *et al.* in the mouse bronchi. The transient nature of the relaxation did not appear to be due to desensitization of the relaxation mechanism as subsequent addition of NaHS after recovery of the tone caused a similar relaxation response across the whole range of concentrations investigated. This transient response, therefore, is likely to be due to the short-lived nature of H_2S in solution, as well as the reversibility of the relaxation mechanism.

The relaxation response to NaHS was reduced in the presence of a high concentration of K^+. Similarly, incubation with the non-selective K^+ channel blocker TEA at a concentration expected to block multiple K^+ channels (10 mM; Nelson and Quayle, 1995) caused a significant reduction in the relaxation response, although a lower concentration (1 mM) had no effect. These data suggest that the relaxation response is dependent, at least in part, on the opening of K^+ channels, similar to that observed in isolated blood vessels (Kubo *et al.*, 2007b). However, these data are at odds with those obtained in rat trachea in which it was found that high K^+ had no effect on NaHS relaxations (Chen *et al.*, 2011). In the mouse bronchi, the relaxation response was not blocked by the K_{ATP} channel inhibitor glibenclamide, although general blockade of all K^+ channels was not investigated in this study (Kubo *et al.*, 2007a). In airway epithelial cells, blockade of K_{Ca} or Kv channels, but not K_{ATP} channels reduces NaHS-induced changes in Na^+ channel current (Althaus *et al.*, 2012). In this current study, we found that blockade of individual K^+ channels with glibenclamide, paxilline (BKCa channel blocker) or 4-AP (Kv channel blocker) at concentrations shown to inhibit K^+ channel function in other smooth muscle preparations (Cheang *et al.*, 2010; Jackson-Weaver *et al.*, 2011; Montano *et al.*, 2011) had no effect on the relaxation responses to NaHS in the porcine bronchioles. Each of these channels has been implicated in vasodilator responses to H_2S (Kubo *et al.*, 2007b; Cheang *et al.*, 2010; Liang *et al.*, 2012). These data suggest that either high K^+ and TEA are blocking the relaxation to NaHS through a mechanism independent of an effect on K^+ channels, or that blockade of multiple K^+ channel types is required to cause significant inhibition of the relaxation.

More recently, it has been shown that H_2S can cause the release of sensory neuropetides in rat trachea through stimulation of TRPA1 and this may mediate the vasodilatation effect of H_2S in mouse ears (Pozsgai *et al.*, 2012). However, in the current study, we found that the TRPA1 receptor antagonist HC-030031 had no effect on the relaxation to NaHS in the porcine bronchioles, suggesting that this is not the mechanism of relaxation in the airways. Similarly, inhibition of nitric oxide synthase and cyclooxygenase had no effect on the relaxation, indicating that neither NO nor cyclooxygenase products are involved. The relaxation responses to NaHS were also unaltered by removal of the epithelium, suggesting that the main site of action is likely to be the smooth muscle cells.

The relaxation responses to NaHS reported in the mouse bronchi were not replicated by L-cysteine, suggesting that either there is no endogenous production of H_2S, or that it is not produced in large enough amounts to affect airway tone in the mouse bronchi (Kubo *et al.*, 2007a). However, in this present study, we have demonstrated that L-cysteine produces a concentration-dependent relaxation in porcine

peripheral bronchioles. Also, using an assay for H_2S, we demonstrated the production of H_2S from L-cysteine in homogenates of the bronchioles. These data suggest that H_2S is produced within porcine peripheral bronchioles and that this production is able to relax the airways. Using Western blotting, we also demonstrated the expression of three distinct enzymatic pathways for H_2S production in peripheral bronchioles: CBS, CSE and 3-MST. These data provide additional support that H_2S can be produced endogenously within peripheral bronchioles. Only AOAA inhibited the production of H_2S from L-cysteine in the bronchioles, indicating CBS, rather than CSE (Whiteman *et al.*, 2011), is involved in H_2S production in these tissues. In bronchiolar rings, AOAA also inhibited the relaxation to L-cysteine suggesting that CBS-generated H_2S is responsible for the relaxation seen with L-cysteine. However, it must be noted that AOAA inhibits other enzymes and, therefore, a non-selective effect cannot be ruled out. On the other hand, AOAA only inhibited the production of H_2S in the presence of pyridoxal phosphate in tissue homogenates, indicating that it is inhibiting H_2S production through an effect on a pyridoxal phosphate-sensitive enzyme. Although CBS immunoreactivity could be detected in bronchiolar tissue homogenates, whether CBS is the enzyme involved in production of H_2S in the porcine peripheral bronchioles cannot be stated for certain. A major problem with current research in this area is a lack of selective inhibitors of the H_2S-synthesizing enzymes. Until selective inhibitors become available, then the role of CBS in the production of H_2S in the bronchioles cannot be confirmed.

These data do not rule out the possibility that 3-MST is also a source of H_2S in the airways to regulate tone. Indeed using 3-mercaptopyruvate as a substrate, we were able to detect H_2S production in the airways. Furthermore, only part of the H_2S production from L-cysteine was dependent upon pyridoxal phosphate, which is required for CBS and CSE, but not 3-MST, activity (Li *et al.*, 2011). There is the possibility that pyridoxal phosphate is present in the cytosolic fractions in high enough levels to stimulate basal production of H_2S. However, AOAA only inhibited the H_2S production stimulated by exogenously applied pyridoxal phosphate. Taken together, these data suggest that part of the H_2S production from L-cysteine could be through the 3-MST pathway. To date there is no inhibitor of 3-MST and, therefore, it is not possible to test whether this is the case.

In order to determine whether basal production of H_2S could influence airway tone, we investigated the effect of AOAA on carbachol-induced contractions. Although this compound inhibited H_2S production in the airways and inhibited L-cysteine-induced relaxations, there was no effect of AOAA on either basal tone, or on carbachol-induced contractions. This suggests that H_2S may not be produced in high enough concentrations at basal levels to regulate airway tone. On the other hand, H_2S may play a greater role in the regulation of airway tone in conditions in which H_2S synthesis is enhanced, for example, in inflammation or after exposure to airborne irritants, such as cigarette smoke (Chen *et al.*, 2011).

The data presented here demonstrate that exogenously applied H_2S causes a relaxation of the porcine peripheral airways, which is mediated, at least in part, through a K^+-channel-dependent mechanism. This is similar to the effect seen in mouse bronchi and rat trachea (Kubo *et al.*, 2007a; Chen *et al.*, 2011), although neither of these studies were able to identify the mechanism of relaxation. The K^+ channel-dependent nature of the relaxation is similar to that observed in isolated blood vessels (Kubo *et al.*, 2007b). Although exogenous H_2S produces a relaxation in the mouse bronchi, L-cysteine did not replicate this relaxation, suggesting that H_2S is not produced endogenously within mouse trachea, or that endogenous L-cysteine is already saturating for H_2S production in the mouse (Kubo *et al.*, 2007a). This difference could be due to differences in the diameter of the airway tissues used, in that it is possible that H_2S is only produced within the lower airways. However, we have also demonstrated that H_2S production can be detected all through the respiratory tract in the pig (Figure 4). Furthermore, CBS, CSE and 3-MST immunoreactivity could also be detected in large and small airways from the pig. Kubo *et al.* did not measure H_2S production in the mouse bronchi, and so it is not known whether H_2S is produced in the mouse bronchi, but not at a sufficient concentration to initiate a relaxation (Kubo *et al.*, 2007a).

In conclusion, our data demonstrate that H_2S can be produced endogenously within airways from the pig. This production is likely to be through the enzymes CBS and 3-MST. The data presented here demonstrate that H_2S production in the airways is involved in the regulation of airway tone. Its role as both a pro- and anti-inflammatory mediator suggests that it may play a role in the pathogenesis of chronic obstructive pulmonary disease or asthma. Indeed, a recent study suggests that endogenous H_2S may play a protective role to reduce lung damage caused by cigarette smoke (Chen *et al.*, 2011) and other airborne irritants.

Acknowledgements

We would like to thank G. Woods & Son, Clipstone, Nottinghamshire for supply of the abattoir tissue.

Conflicts of interest

None.

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