The anti-proliferative and anti-inflammatory response of COPD airway smooth muscle cells to hydrogen sulfide

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Abstract

Background: COPD is a common, highly debilitating disease of the airways, primarily caused by smoking. Chronic inflammation and structural remodelling are key pathological features of this disease caused, in part, by the aberrant function of airway smooth muscle (ASM). We have previously demonstrated that hydrogen sulfide (H2S) can inhibit ASM cell proliferation and CXCL8 release, from cells isolated from non-smokers.

Methods: We examined the effect of H2S upon ASM cells from COPD patients. ASM cells were isolated from non-smokers, smokers and patients with COPD (n = 9). Proliferation and cytokine release (IL-6 and CXCL8) of ASM was induced by FCS, and measured by bromodeoxyuridine incorporation and ELISA, respectively.

Results: Exposure of ASM to H2S donors inhibited FCS-induced proliferation and cytokine release, but was less effective upon COPD ASM cells compared to the non-smokers and smokers. The mRNA and protein expression of the enzymes responsible for endogenous H2S production (cystathionine-β-synthase [CBS] and 3-mercaptopyruvate sulphur transferase [MPST]) were inhibited by H2S donors. Finally, we report that exogenous H2S inhibited FCS-stimulated phosphorylation of ERK–1/2 and p38 mitogen activated protein kinases (MAPKs), in the non-smoker and smoker ASM cells, with little effect in COPD cells.

Conclusions: H2S production provides a novel mechanism for the repression of ASM proliferation and cytokine release. The ability of COPD ASM cells to respond to H2S is attenuated in COPD ASM cells despite the presence of the enzymes responsible for H2S production.

Keywords: COPD, Hydrogen sulfide, Proliferation, IL-6, CXCL8, Airway smooth muscle

Background

Hydrogen sulfide (H2S) was discovered in human tissues over 15 years ago, as emerged as an important gaseous mediator of several biological processes [1]. H2S is now considered the third member of a family of gasotransmitters, together with nitric oxide (NO) and carbon monoxide [2]. The bulk of endogenous H2S synthesis in mammalian tissues appears to be from the pyridoxal-5′-phosphate-dependent enzymes, cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS), and also by 3-mercaptopyruvate sulphur transferase (MPST) [3].

Chronic obstructive pulmonary disease (COPD) is a common, highly debilitating disease of the airways, primarily caused by smoking [4]. Serum H2S levels are significantly increased in patients with stable COPD as compared to age matched control subjects or those with acute exacerbation of COPD [5]. Serum H2S levels were negatively correlated with the severity of airway obstruction in patients with stable COPD whereas they were positively correlated with the lung function in all patients with COPD and healthy controls. Patients with acute exacerbations and increased pulmonary artery pressure (PASP) had lower levels of H2S than those with normal PASP, suggesting a negative relation between H2S and PASP in COPD exacerbations. Serum H2S levels are also lower in smokers than non-smokers.
regardless of their health status (COPD or healthy controls). Furthermore, patients with acute exacerbations, whose serum H2S levels were decreased, demonstrated greater neutrophil numbers but lower lymphocyte numbers in sputum than patients with stable COPD, suggesting a potential role of H2S in regulating inflammatory response at different types or stages of COPD.

We have previously demonstrated that mitogen stimulation increases inflammatory mediator release from both ASM IL-6 and CXCL8 release in COPD patients to a greater degree than those from non-smoker subjects [4]. Furthermore, we have shown that H2S donors inhibit mitogen-induced inflammatory mediator release and proliferation of cells from healthy non-smoking subjects [6]. We therefore set out to determine the effect of H2S in ASM cells isolated from healthy smokers and patients with COPD. We hypothesized that H2S may also mediate ASM proliferation, and cytokine release to varying degrees in these diseased cells. We examined the effect of both exogenous and intracellular sources of H2S in human ASM from 9 donors in each group upon proliferation induced by fetal calf serum (FCS). We used two extracellular H2S donors; the rapidly releasing H2S donor, sodium hydrogen sulfide (NaSH), and modelled endogenous H2S synthesis with the slow H2S-releasing molecule, GYY4137 [6, 7]. To examine the role of endogenously synthesized H2S, we used an inhibitor of H2S synthesis (O-(carboxymethyl)-hydroxylamine hemihydrochloride (CHH)) to inhibit CBS [6]. Finally, we also investigated the role of mitogen-activated protein kinase (MAPK) activation in this process.

**Methods**

**Primary human ASM cell culture**

Primary human ASM cells were previously dissected from the lungs of healthy non-smokers, healthy smokers and patients with COPD disease and smoking status were defined according to guidelines produced by the American Thoracic Society [8]. Healthy smokers had a smoking history of at least 10 pack years. There were significant differences between FEV1 in litres, FEV1 percent predicted, and FEV1/FVC ratio between smokers and patients with COPD compared with non-smokers but matched for age and smoking history (Table 1).

ASM cells were cultured and plated as previously described [4, 6, 9–11]. ASM cells were plated onto 96-well plates for the measurement of cytokine release, and six well plates for RNA and protein extraction. Confluent cells were growth-arrested by FCS deprivation for 24 h in Dulbecco’s Modified Eagle’s Medium supplemented with sodium pyruvate (1 mM), L-glutamine (2 mM), nonessential amino acids (1:100), penicillin (100 U/ml)/streptomycin (100 mg/ml), amphotericin B (1.5 mg/ml), and BSA (0.1%) [6]. Cells at passages 3–4 from nine different donors per group were used.

Cells were stimulated in triplicate ±2.5% FCS for 1 h before treatment with a H2S donor (NaSH or GYY4137 [100 μM]), 24 h prior CBS, CSE and MPST mRNA and protein expression was measured. At 8 days, cellular proliferation was measured by BrdU assay [14], and IL-6 and CXCL8 levels were determined by DuoSet ELISA (R&D Systems, Abingdon, UK), cellular viability by MTT assay [14], and IL-6 and CXCL8 levels were determined by DuoSet ELISA (R&D Systems, Abingdon, UK) as previously described [6]. For the inhibitor studies, cells were treated with 1 mM O-(carboxymethyl)-hydroxylamine hemihydrochloride (CHH), 5 μM PD098059 (a MEK-1/2 inhibitor) or 5 μM SB 203580 (a p38 MAP kinase inhibitor) for 30 min before treatment with NaSH (100 μM) for a further 8 days.

**Table 1** Characteristics of subjects providing airway smooth muscle cells for culture

|               | Non-smokers | Smokers | COPD  |
|---------------|-------------|---------|-------|
| n             | 9           | 9       | 9     |
| Age (y)       | 66.4 ± 12.72| 59.2 ± 7.6 | 65.4 ± 6.6 |
| Sex (? - ♀)   | 7–2         | 4–5     | 5–5   |
| Pack years smoking | N/A         | 29.25 ± 3.3 | 9.2 ± 3.92 |
| FEV1 (L)      | 4.02 ± 0.48 | 3.12 ± 0.78 | 1.76 ± 0.45 |
| FEV1 (% predicted) | 104.23 ± 7.28 | 101.5 ± 8.7 | 77.1 ± 20.97 |
| FEV1/FVC (%)  | 78.89 ± 5.98 | 77.1 ± 3.31 | 38.08 ± 15.75 |

Definition of abbreviations: FEV1; forced expiratory volume, FVC; forced vital capacity

Data shown as mean ± SEM

**RNA isolation and detection of mRNA expression**

mRNA was isolated and CBS, CSE and 3-MST mRNA expression levels were measured as previously described [4, 6, 9–11].

**Western blotting**

Proteins were measured as previously described [4, 6, 9] using mouse anti-CBS (A-2) antibody, a mouse anti-CSE (30.7) antibody, mouse anti-MPST (H-11), rabbit anti-p38 MAPK antibody and rabbit anti-phospho–p38 MAPK (Thr180/Tyr182) antibody (all from Santa Cruz Biotechnology, Middlesex, UK) and, rabbit anti–extracellular signal–regulated kinase (ERK)–1/2 (137F5) and rabbit anti–phospho–ERK-1/2 (Thr202/Tyr204; purchased from Cell Signalling Technology, Ely, Cambridgeshire, UK).

**Immunohistochemistry analysis of CSE, CBS and MPST in bronchial biopsies**

Cryostat sections from historical biopsies were stained and scored as previously described [15]. Briefly, after blocking non-specific binding sites with horse serum, 1:
200 primary antibody was applied in TRIS-buffered saline (0.15 M saline containing 0.05 M TRIS-hydrochloric acid at pH 7.6) and incubated for 1 h at room temperature in a humid chamber. Antibody binding was demonstrated with a secondary horse anti-mouse (Vector, BA 2000) antibody followed by ABC kit HRP Elite, PK6100, Vectastain and diaminobenzidine (DAB) substrate (brown colour). Human tonsil or nasal polyp were used as positive controls. For the negative control, normal mouse, rabbit or goat non-specific immunoglobulins (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at the same protein concentration as the primary antibody.

Data analysis
Data were analysed using GraphPad Prism, version 5.03 (GraphPad Software, San Diego, CA). Data were not normally distributed (as assessed by the Kolmogorov-Smirnov test), and therefore groups were compared using the Dunn nonparametric test. All data are expressed as means ± SEMs. Significance was defined as a $P$ value of less than 0.05.

Results
The presence of H$_2$S producing enzymes in ASM cells in-situ was determined by immunohistochemical staining of bronchial biopsies from healthy non-smokers, healthy smokers and subjects with COPD. The most intense staining was seen for CSE but no difference in the expression of CSE, CBS or MPST was observed between patient groups (Additional file 1: Figure S1).

Effect of H$_2$S on FCS-induced ASM proliferation and inflammatory mediator release
After 8 days of culture in the absence of FCS, neither NaSH (100 μM) nor GYY4137 (100 μM) had any significant effect upon cell viability in cells from any subject group (Fig. 1a). ASM proliferation increased in the presence of 2.5% FCS ($p < 0.001$), an effect that was inhibited by both NaSH and GYY4137 ($p < 0.05$) in all ASM groups. However, there was a significant increase in the level of proliferation between the patient groups, with the highest level seen in the COPD patients compared to non-smokers ($p < 0.01$). Furthermore, although the H$_2$S donors returned proliferation levels to baseline in the non-smoker ASM cells, this was not the case in the smokers or COPD patients (Fig. 1b). Similar results were observed with regards to FCS-induced IL-6 (Fig. 1c), and CXCL8 release (Fig. 1d) with both being greatest in ASM from COPD subjects and having a lesser response to NaSH and GYY.

Effect of H$_2$S on CSE, CBS and MPST mRNA expression in non-smokers, smokers and COPD patients
We next examined the effect of exogenous H$_2$S upon basal and FCS-exposed CSE, CBS and MPST mRNA expression. CSE mRNA expression did not alter under any of the experimental parameters studied in any of the patient cohorts (Fig. 2a). NaSH (100 μM) alone had no effect on CBS mRNA levels at 24 h in any of the patient groups. FCS (2.5%) enhanced CBS mRNA expression only in the smokers ($p < 0.05$) and this was reversed back
to baseline by treatment with NaSH (100 μM). In addition, NaSH also suppressed FCS-treated CBS mRNA levels in ASMs from non-smokers (p < 0.05) (Fig. 2b).

FCS (2.5%) treatment for 24 h significantly up-regulated the expression of MPST mRNA in ASMs from non-smokers and smokers with significantly higher levels in the smokers (p < 0.05) compared with non-smokers (Fig. 2c). There was no effect of FCS on MPST mRNA expression in cells from COPD patients. NaSH (100 μM) alone had no effect on MPST mRNA expression but reduced FCS-stimulated expression back to baseline in cells from non-smokers and smokers (p < 0.05). No effect of NaSH was observed on FCS-treated COPD ASM cells (Fig. 2c).

Effect of H2S on CSE, CBS and MPST protein expression in non-smokers, smokers, and COPD patients
Neither FCS nor NaSH had any effect on CSE protein expression in ASM cells from any group studied (data not shown). NaSH (100 μM) treatment alone had no effect on CSE or MPST expression at 24 h (Fig. 3). FCS (2.5%) increased CBS protein in the non-smokers (p < 0.05) and smoker ASM cells (p < 0.01) but no effect on CBS expression in COPD ASM cells. The effect of FCS on CBS expression was significantly greater in cells from smokers than non-smokers (p < 0.05). NaSH (100 μM) suppressed FCS-induced CBS protein production at 24 h in cells from smokers and non-smokers (p < 0.01, p < 0.05 respectively) without affecting expression in COPD cells (Fig. 3a & b).

Similarly, FCS (2.5%) enhanced MPST protein expression in ASMs from non-smokers (p < 0.05) and smokers (p < 0.001). Treatment with NaSH (100 μM) completely attenuated the ability of FCS to induce MPST protein at 24 h in cells from smokers and non-smokers (p < 0.01, p < 0.05 respectively) (Fig. 3a & c). Again, no effect on MPST protein expression was observed in COPD ASM cells.

Effect of inhibiting CBS on ASM proliferation induced by FCS in samples isolated from non-smokers, smokers, and COPD patients
ASM cells were pre-treated with an inhibitor of CBS (CHH) for 30 min before treatment with 2.5% FCS with or without NaSH (100μM) for 8 days. In ASMs from non-smokers, CHH (1μM) significantly enhanced FCS-stimulated proliferation (p < 0.01) to levels seen with FCS alone in ASM cells from the COPD patients (Fig. 4a). A similar ability of CHH to stimulate FCS-induced proliferation to levels seen in COPD cells was observed with ASM cells from the smokers (p < 0.05). In contrast, CHH had no effect on FCS-induced proliferation in ASMs from patients with COPD (Fig. 4a). The ability of NaSH to suppress FCS-induced proliferation of ASMs from non-smoker and healthy smoker cells was attenuated by the presence of CHH (p < 0.01, p < 0.05 respectively) (Fig. 4a). In contrast, the reduced ability of NaSH to suppress FCS-stimulated proliferation in COPD ASM cells was not affected by CHH.

A similar profile to that observed for proliferation was seen in relation to FCS-stimulated IL-6 and CXCL8
release (Fig. 4b & c). CHH enhanced FCS-induced IL-6 (Fig. 4b) and CXCL8 (Fig. 4c) release from cells from non-smokers (p < 0.01) and smokers (p < 0.05) to levels with COPD cells. In contrast, CHH had no effect on FCS-induced proliferation in ASMs from patients with COPD (Fig. 4b & c). The ability of NaSH to suppress FCS-induced IL-6 (Fig. 4b) and CXCL8 (Fig. 4c) release from ASMs from non-smoker and healthy smoker cells was attenuated by the presence of CHH (p < 0.01). In contrast, the reduced ability of NaSH to suppress FCS-stimulated IL-6 (Fig. 4b) and CXCL8 (Fig. 4c) from COPD ASM cells was not affected by CHH.

Fig. 4 Effect of inhibiting cystathionine-β-synthase (CBS) on ASM from non-smokers, smokers and COPD patients. ASM cells were incubated with FCS (2.5%) for 1 h and NaSH (100 μM) was added for another 24 h. CBS, MPST and β-actin were detected by Western blotting (a). Further examples are shown in Additional file 2: Figure S2. Changes in protein expression were quantified by densitometry, normalized against β-actin expression, and then expressed as the percent change versus untreated controls (b & c). Bars represent means (± SEM) of nine ASM donors. */+/# P < 0.05; **/++/## P < 0.01; ***/+++/### P < 0.001

Fig. 3 Effect of the H₂S donor, NaSH on CBS and MPST protein expression in human ASM cells from non-smokers, smokers and COPD patients. ASM cells were incubated with FCS (2.5%) for 1 h and NaSH (100 μM) was added for another 24 h. CBS, MPST and β-actin were detected by Western blotting (a). Further examples are shown in Additional file 2: Figure S2. Changes in protein expression were quantified by densitometry, normalized against β-actin expression, and then expressed as the percent change versus untreated controls (b & c). Bars represent means (± SEM) of nine ASM donors. */+/# P < 0.05; **/++/## P < 0.01; ***/+++/### P < 0.001
Effect of NaSH on activation of ERK-1/2 and p38 MAPK

We have previously reported that 2.5% FCS significantly phosphorylates both ERK-1/2 and p38 MAPK in ASM cells from non-smokers and that this is prevented by NaSH [6]. We confirmed that FCS stimulates ERK-1/2 activation in non-smokers and demonstrate a similar increase in cells from smokers but a greater activation from COPD cells (Fig. 5a & b). Phosphorylation of ERK-1/2 was significantly reduced by NaSH only in cells from non-smokers ($p < 0.05$) (Fig. 5a & b). FCS induced a greater level of p38 MAPK phosphorylation in cells from smokers and COPD patients compared to that seen in non-smokers (Fig. 5a & c). This phosphorylation was attenuated by NaSH in cells from non-smokers and smokers but not in cells from COPD patients (Fig. 5a & c).

Finally, we examined the role of the MAPKs, ERK-1/2 and p38, on FCS-induced proliferation (Fig. 6a), IL-6 (Fig. 6b) and CXCL8 (Fig. 6c) release in human ASM cells. The ERK-1/2 inhibitor, PD98059 (5 mM), significantly inhibited FCS-induced proliferation, IL-6 and CXCL8 release ($p < 0.05$) in all patient groups (Fig. 6a, b & c). However, the magnitude of the effect in COPD cells was less than that observed in smoker and non-smoker cells. The p38 MAPK inhibitor, SB202190 (5 mM), had a reduced effect compared to that seen with PD98059. The combination of PD98059 and SB202190 had a greater suppressive effect on all parameters than the individual inhibitors across all subject groups but again the magnitude of the effect in COPD cells was less.

Furthermore, when the ASM cells were further treated with NaSH (100μM) in addition to the MAPK inhibitors, a further decrease in IL-6 and CXCL8 release was observed in the COPD patients ($p < 0.05$) although this still failed to reach baseline levels as seen in cells from smokers and non-smokers (Fig. 6b & c).

Discussion

For the first time, we demonstrate that both endogenous and exogenous H$_2$S inhibits human ASM cell proliferation and cytokine release induced by FCS, and that this effect was dependent on the patient. Specifically; proliferation and cytokine release from non-smoker ASM cells returned to basal levels (as previously reported [6]) whereas in smokers, both IL-6 and CXCL8 release were reduced but did not return to basal levels. In contrast, the effect of H$_2$S on proliferation and cytokine release from ASM cells isolated from COPD patients was impaired compared to smokers and non-smoker
cells. Furthermore, we have shown that endogenous H$_2$S is produced by the enzymes CBS and MPST, and not by CSE. We found that H$_2$S differentially inhibited phosphorylation of the MAPKs, ERK-1/2 and p38, according to the patient group and propose that this could be a mechanism by which H$_2$S inhibits cellular proliferation and cytokine release [4, 16–19].

ASM proliferation is increased in response to FCS [9, 10, 20] and studies have examined the role of H$_2$S upon cell proliferation. These have concluded that this gas can induce proliferation [21] or, conversely, inhibit it [6, 22, 23] depending upon the cell type examined. Both the fast-release H$_2$S donor, NaSH, and the slow-release donor, GYY4137, have been used previously to affect inflammation in both in-vivo and in-vitro models of inflammation, including a mouse model of vascular inflammation and oxidative stress [24], asthma [25], COPD [26], and a rat model of colitis [27]. Our data extends our previous report demonstrating the inhibitory action of H$_2$S in non-smoker ASM cells [6] and examined its role in smoker and COPD ASM cells. Both NaSH and GYY4137 caused similar inhibitory effects on FCS-

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**Fig. 6** Effect of mitogen-activated protein kinase 1/2 (MEK-1/2) and p38 MAPK inhibition upon FCS-induced proliferation, IL-6 and CXCL8 release in human ASM cells from non-smokers, smokers and COPD patients. ASM cells were incubated with an MEK-1/2 (5 mM) inhibitor, a p38 (5 mM) inhibitor, or both for 30 min. The ASM cells were then further incubated with NaSH (100 μM) for another 8 days. DNA synthesis (a), IL-6 release (b), and CXCL8 release (c) were subsequently measured by bromodeoxyuridine (BrdU) ELISA, and DuoSet ELISA respectively. Bars represent means (± SEM) of nine ASM donors. *P < 0.05 versus cells plus 2.5% FCS; **P < 0.01 versus cells plus 2.5% FCS; ***P < 0.001 versus cells plus 2.5% FCS; #P < 0.05 versus cells plus 2.5% FCS plus NaSH; ##P < 0.01 versus cells plus 2.5% FCS plus NaSH.
induced ASM cell proliferation, IL-6 and CXCL8 release from smokers as well as non-smokers indicating that the rate of release does not modulate the inhibitory effect of H\textsubscript{2}S in ASM. We also show for the first time, an effect upon primary ASM cells isolated from patients with COPD. However, the effect of H\textsubscript{2}S donors is reduced compared to that seen in cells from smokers and non-smokers which may explain, in part, the increased inflammatory and proliferative status of COPD cells. Indeed, the H\textsubscript{2}S enzyme inhibitor CHH had no significant effect on FCS-induced inflammatory protein release from COPD cells in contrast to the effect seen in cells from other subject groups.

We found that, all three H\textsubscript{2}S producing enzymes are expressed in ASM cells to a similar extent across the subject groups studied. However, our pharmacological studies suggest that endogenous H\textsubscript{2}S production is these cells is most likely to be through the enzymes, CBS, and MPST. In cultured ASM cells, FCS was able to induce CBS and MPST mRNA and protein in cells from non-smokers and smokers but not in COPD cells suggesting that mitogens may induce cells to produce more H\textsubscript{2}S. NaSH inhibited both CBS and MPST, likely as a negative-feedback inhibitory mechanism. Currently, CBS appears to be involved in the generation of endogenous H\textsubscript{2}S in neural pathways, the brain, vascular tissue, and non-smoker ASM cells [6, 28–32]. In contrast, "CSE is predominantly involved in endogenous H\textsubscript{2}S production in rodent smooth muscle and the lung [33–37], and MPST maintains mitochondrial function [extensively reviewed in [38, 39]]. Clearly cell, species and pathology differences should be taken into consideration when investigating the production of endogenous H\textsubscript{2}S.

A role for the ERK-1/2 and p38 MAPKs in regulating ASM cell proliferation and cytokine release is well documented [4, 16–19] and it has been shown to affect the phosphorylation of these kinases [6, 26, 40–43]. Hence, we examined the degree of phosphorylation of these kinases in our COPD ASM cells. We noted that FCS induced both ERK-1/2 and p38 MAPK phosphorylation, which was reduced by NaSH in both the non-smoker and smoker ASM cells, but no effect was seen in the COPD cells. Inhibiting these kinases significantly reduced the ASM proliferation and cytokine release and, when they were used before treatment with NaSH, a further decrease in an asthma phenotype was observed, further supporting the possibility that the mechanism of H\textsubscript{2}S, at least in part, is via the inhibition of these kinases.

Finally, our data shows that ASM cells of COPD patients indicate an attenuated response to H\textsubscript{2}S, as compared to the non-smoker and smoker-groups. But the question remains, why? There are numerous reviews discussing both the importance of H\textsubscript{2}S in chronic respiratory diseases [3, 44] and smooth muscle itself [45], however recent studies demonstrate further actions of this gasotransmitter. For example, Fitzgerald et al. demonstrate that H\textsubscript{2}S causes the relaxation of human ASM and implicate the role for sarcolemmal KATP channels [46]. In mouse models, Huand et al. indicate that H\textsubscript{2}S can induce mouse ASM relaxation by activating BK\textsubscript{Ca} [47], and Castro-Piedras et al. indicate that H\textsubscript{2}S causes ASM relaxation by inhibiting Ca\textsuperscript{2+} release through InsP3Rs and consequent reduction of const-

\section*{Conclusion}

In conclusion, we have shown for the first time that H\textsubscript{2}S inhibits both human ASM proliferation and cytokine release induced by FCS, differentially between ASM cells isolated from non-smokers, smokers and patients with COPD. It is likely that exogenous H\textsubscript{2}S targets the production of endogenous H\textsubscript{2}S by inhibiting the transcription and subsequent translation of the CBS and MPST enzymes, and proliferation is controlled by H\textsubscript{2}S through a negative-feedback pathway. H\textsubscript{2}S inhibits the activity of the ERK-1/2 and p38 MAPKs, in the non-smokers and smokers, but with little effect in the COPD ASM cells. We propose that H\textsubscript{2}S may provide a novel therapeutic avenue in the stabilization of ASM proliferation but that its effectiveness in COPD may be more limited.

\section*{Additional files}

\begin{itemize}
\item \textbf{Additional file 1: Figure S1.} Immunohistochemistry staining of CSE, CBS and MPST in bronchial biopsies from non-smokers, smokers and COPD patients. Photomicrographs showing representative photomicro-grahs of cystathionine-β-lyase (CSE), cystathionine-γ-synthase (CBS) and 3-mercaptopyruvate sulphur transferase (MPST) staining in the bronchial mucosa from control non-smokers, control smokers with normal lung function and mild/moderate COPD patients. Immune-stained airway smooth muscle cells are indicated by brown staining. Results are representative of those from 13 non-smokers, 14 smokers with normal lung function, 15 mild/moderate COPD patients. Calibration bar represents 20 μm. Graphical representation of the results are shown in the right hand panels. (JPG 332 kb)
\end{itemize}
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Availability of data and materials
All data is available upon request.

Take Home Message
Hydrogen sulfide production provides a novel mechanism for regulating airway smooth muscle phenotype in COPD.

Authors’ contributions
MMF designed the study, performed the experiments, analysed the data and wrote the manuscript. AP, PC & GC performed the immunohistochemistry. KD & AH provided healthy smoker and COPD patients. ASM cells were incubated with FCS (2.5%) for 1 h and NaSH (100 μM) was added for another 24 h. CBS, MPST (A), total and phospho–ERK–1/2, total and phospho-p38 and β-actin (B) were detected by Western blotting. (JPG 212 kb)

Ethics approval and consent to participate
This study was approved by the Royal Brompton & Harefield NHS Trust Ethics committee and all subjects gave written informed consent.

Competing interests
The authors declare that they have no competing interests.

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References
1. Li L, Moore PK. Putative biological roles of hydrogen sulfide in lung and disease: a breath of not so fresh air? Trends Pharmacol Sci. 2008;29(6):265–70. https://doi.org/10.1016/j.tips.2007.11.003.
2. Gadalla MM, Snyder SH. Hydrogen sulfide as a gasotransmitter. J Neurochem. 2010;113(1):14–26. https://doi.org/10.1111/j.1471-4159.2010.06253.x.
3. Chung KF. Hydrogen sulfide as a potential biomarker of asthma. Expert Review of Respiratory Medicine. 2014;8(1):137–48.
4. O’Leary L, Sevink J, Papazoglou IM, Thomas B, Cheng Y, Karkkainen K, et al. Airway smooth muscle inflammation is regulated by microRNA-145 in COPD. FEBS Lett. 2016;590(9):1324–34. https://doi.org/10.1016/j.febslet.2016.02.033.
5. Chen Y-H, Yao W-Z, Geno D-Y, Li-K, Lu N, Lu-Chao M-W, et al. Clinical investigations: COPD: endogenous hydrogen sulfide in patients with COPD. Chest. 2005;128:3105–12. https://doi.org/10.1378/chest.128.5.3305.
6. Perry MM, Hui CW, Williams M, Wood ME, Adcock I, Kirkham P, et al. Hydrogen sulfide enhances generation and release of IL-8 from human airway smooth muscle cells. Am J Respir Cell Mol Biol. 2011;45(4):746.
7. Li L, Whitehouse M, Gonda S, New K, Cheng Y, Lee SW, et al. Characterization of a novel, transforming growth factor-β-inducible sulfide-releasing molecule (G14Y137): new insights into the biology of hydrogen sulfide. Circulation. 2008;117(18):2351–60. https://doi.org/10.1161/CIRCULATIONAHA.107.1573467.
8. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. American Thoracic Society. Am J Respir Crit Care Med. 2015;192(1 Pt 2):S7–S122.
9. Perry MM, Baker JE, Gibeon DS, Adcock IM, Chung KF. Airway smooth muscle hyperplasia is regulated by microRNA-211 in severe asthma. Am J Respir Cell Mol Biol. 2014;51(1):7–17. https://doi.org/10.1165/rcmb.2013-0067OC.
10. Perry MM, Tsiatsiou E, Austin PJ, Lindsay MA, Gibeon DS, Adcock IM, et al. Role of non-coding RNAs in maintaining primary airway smooth muscle cells. Respir Res. 2014;15:58. https://doi.org/10.1186/1465-9921-15-58.
11. Perry MM, Durham AL, Austin PJ, Adcock IM, Khan FC. BET Bromodomain regulate transforming growth factor-β-induced proliferation and cytokine release in asthmatic airway smooth muscle. J Biol Chem. 2015;290(14):9111–21. https://doi.org/10.1074/jbc.M114.612671.
12. Perry MM, Lavender P, Scott Kuo CH, Gallea F, Michaeloudes C, Flanagan JM, et al. DNA methylation modules in airway smooth muscle are associated with asthma severity. Eur Respir J. 2018; https://doi.org/10.1183/13993003.01068-2017.
13. Austin PJ, Tsiatsiou E, Boardman C, James SW, Lindsay MA, Adcock IM, et al. Transcriptional profiling identifies the long non-coding RNA plasmaacytoma variant translocation (PVT1) as a novel regulator of the asthmatic phenotype in human airway smooth muscle. J Allergy Clin Immunol. 2017;139(3):780–9. https://doi.org/10.1016/j.jaci.2016.06.014.
14. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1):55–63. https://doi.org/10.1016/0022-1759(83)90303-4.
15. Contoli M, Papu A, Cappello F, Adcock I, Durham A, Di Stefano A, et al. Innate immunity but not NLRP3 inflammasome activation correlates with severity of stable COPD. Thorax. 2014;69(6):516–24.
16. Xie S, Sukkar MB, Isa R, Khorsani NM, Chung KF. Mechanisms of induction of airway smooth muscle hyperplasia by transforming growth factor-beta. Am J Physiol Lung Cell Mol Physiol. 2007;293(1):L245–53.
17. Liu W, Liang Q, Gorska M, Alam R, Balzar S, Wenzel S, et al. Cell-specific activation profile of extracellular signal-regulated kinase 1/2, Jun N-terminal kinase, p38 mitogen-activated protein kinases in asthmatic airways. J Allergy Clin Immunol. 2008;121(4):893–902.
18. Nath P, Leung S-Y, Williams A, Noble A, Chakravarty S, Luedtke GR, et al. Importance of p38 mitogen-activated protein kinase pathway in allergic airway remodeling and bronchial hypersensitivity. Eur J Pharmacol. 2006;541(1–3):161–7.
19. Lamer-Svensson HM, Williams AE, Tsiatsiou E, Perry MM, Jiang X, Chung KF, et al. Pharmacological studies of the mechanism and function of interleukin-1beta-induced miRNA-146a expression in primary human airway smooth muscle. Respir Res. 2010;11:68. https://doi.org/10.1186/1465-9921-11-68.
20. Shiels IA, Taylor SM, Bowler SD. Airway smooth muscle proliferation in asthma: the potential of vascular leakage to contribute to pathogenesis. Med Hypotheses. 1995;45(1):37–40. https://doi.org/10.1016/0306-9877(95)90198-1.

21. Christl SU, Esner HD, Dusel G, Kasper H, Scheppach W. Antagonistic effects of sulfide and butyrate on proliferation of colonic mucosa: a potential role for these agents in the pathogenesis of ulcerative colitis. Dig Dis Sci. 1996;41(2):2477–81.

22. Du J, Hui Y, Cheung Y, Bin G, Jiang H, Chen X, et al. The possible role of hydrogen sulfide as a smooth muscle cell proliferation inhibitor in rat cultured cells. Heart Vessel. 2004;19(2):75–80.

23. Yang G. Cystathionine gamma-lyase overexpression inhibits cell proliferation of cultured cells. Heart Vessel. 2004;19(2):75–80.

24. https://doi.org/10.1111/j.1076-0296.1997.tb02901.x.

25. Zhang G, Wang P, Yang G, Cao Q, Wang R. The inhibitory role of hydrogen sulfide in airway hyperresponsiveness and remodeling in a mouse model of asthma. Am J Pathol. 2013;182(4):1188–95. https://doi.org/10.1016/j.ajpath.2012.12.008.

26. Li F, Zhang P, Zhang M, Sun X, Bao A, Zhou X, et al. Hydrogen sulfide prevents and partially reverses ozone-induced features of lung inflammation and emphysema in mice. Am J Respir Cell Mol Biol. 2016;55(1):72–81. https://doi.org/10.1165/rcmb.2015-0034OC.

27. Wallace JL, Vong L, McKnight W, Dicay M, Martin GR. Endogenous and Exogenous Hydrogen Sulfide Promotes Resolution of Collits in Rats. Gastroenterology. 2009;137(2):569–78.e1. https://doi.org/10.1053/j.gastro.2009.04.012.

28. Abe K, Kimura H. The possible role of hydrogen sulfide as an endogenous neuromodulator. J Neurosci. 1996;16(3):1066–71.

29. Chen X, Jhee K-H, Kruger WD. Production of the neuromodulator H2S by endothelium expresses 3-mercaptopyruvate sulfurtransferase and produces hydrogen sulfide in smooth muscle cells. Am J Physiol Heart Circ Physiol. 2002;283(2):H662–9. https://doi.org/10.1152/ajpheart.00198.2001.

30. Kimura H. Hydrogen sulfide as a neuromodulator. Mol Neurobiol. 2010;46(1):393–8. https://doi.org/10.1007/s12035-009-8133-z.

31. https://doi.org/10.1006/bbrc.1997.6878.

32. Shibuya N, Mikami Y, Kimura Y, Kimura H, Nagahara H. Endothelial cell expresses 3-mercaptoppyruvate sulfurtransferase and produces hydrogen sulfide. J Biochem. 2009;146(5):623–31. https://doi.org/10.1093/jb/mmpv111.

33. Yang G, Wu L, Blyant S, Min S, Yang R, Kifer A, Nystrom L. Cystathionine gamma-lyase deficiency and overexpression in smooth muscle cells. Cardiovasc Res. 2010;86(3):498–506. https://doi.org/10.1093/cvr/cvp420.

34. Yang G, Wang J, Liu L. Pro-apoptotic effect of endogenous H2S on human aorta smooth muscle cells. FASEB J. 2006;20(3):553–5. https://doi.org/10.1096/fasebj.2005.06.2522.

35. Chen Y-T, Wu R, Geng B, Ji Y-F, Wang P-P, Yao W-Z, et al. Endogenous hydrogen sulfide reduces airway inflammation and remodeling in a rat model of asthma. PloS One. 2009;4(5):e5717. https://doi.org/10.1371/journal.pone.0005717.

36. Yoshida JF, Fouillet C, Paris D, Paul V, Sinet PM, Kamoun P, et al. Genomic organization of the human cystathionine beta-synthase gene: evidence for intronic crosstalk in cardiovascular diseases. Am J Physiol Heart Circ Physiol. 2012;303(7):H3514–23. https://doi.org/10.1152/ajpheart.00924.2011.

37. Yang G, Módis K, Coletta C, Olah G, Yanagi K, Ransy C, et al. Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part II. Biochemical and physiological mechanisms. Br J Pharmacol. 2014;171(8):2099–122. https://doi.org/10.1111/bph.12369.

38. Módis K, Bos EM, Calza E, van Goor H, Coletta C, Papapetropoulos A, et al. Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part II. Pathophysiological and therapeutic aspects. Br J Pharmacol. 2014;171(8):2123–46. https://doi.org/10.1111/bph.12368.

39. Papapetropoulos A, Pyliochou A, Marziozi A, Altaayn Z, Yang G, Wang R, et al. Hydrogen sulfide is an endogenous stimulator of angiogenesis. Proc Natl Acad Sci U S A. 2009;106(15):6197–202. https://doi.org/10.1073/pnas.0908047106.