Hydrogen sulfide acts as a mediator of inflammation in acute pancreatitis: \textit{in vitro} studies using isolated mouse pancreatic acinar cells

Ramasamy Tamizhselvi, Philip K. Moore, Madhav Bhatia *
Department of Pharmacology, National University of Singapore, Singapore

Received: November 10, 2006; Accepted: January 18, 2007

Abstract

Hydrogen sulphide (H\textsubscript{2}S) is synthesized from L-cysteine via the action of cystathionine-\(\gamma\)-lyase (CSE) and cystathionine-\(\beta\)-synthase (CBS). We have earlier shown that H\textsubscript{2}S acts as a mediator of inflammation. However the mechanism remains unclear. In this study, we investigated the presence of H\textsubscript{2}S and the expression of H\textsubscript{2}S synthesizing enzymes, CSE and CBS, in isolated mouse pancreatic acini. Pancreatic acinar cells from mice were incubated with or without caerulein (10\textsuperscript{-7} M for 30 and 60 min). Caerulein increased the levels of H\textsubscript{2}S and CSE mRNA expression while CBS mRNA expression was decreased. In addition, cells pretreated with DL-propargylglycine (PAG, 3 mM), a CSE inhibitor, reduced the formation of H\textsubscript{2}S in caerulein treated cells, suggesting that CSE may be the main enzyme involved in H\textsubscript{2}S formation in mouse acinar cells. Furthermore, substance P (SP) concentration in the acini and expression of SP gene (preprotachykinin-A, PPT-A) and neurokinin-1 receptor (NK-1R), the primary receptor for SP, are increased in secretagogue caerulein-treated acinar cells. Inhibition of endogenous production of H\textsubscript{2}S by PAG significantly suppressed SP concentration, PPT-A expression and NK1-R expression in the acini. To determine whether H\textsubscript{2}S itself provoked inflammation in acinar cells, the cells were treated with H\textsubscript{2}S donor drug, sodium hydrosulphide (NaHS), (10, 50 and 100 \(\mu\)M), that resulted in a significant increase in SP concentration and expression of PPT-A and NK-1-R in acinar cells. These results suggest that the pro-inflammatory effect of H\textsubscript{2}S may be mediated by SP-NK-1R related pathway in mouse pancreatic acinar cells.

Keywords: cystathionine-\(\gamma\)-lyase • cystathionine-\(\beta\)-synthase • H\textsubscript{2}S • Substance P • pancreatic acinar cells

Introduction

Hydrogen sulphide (H\textsubscript{2}S) has long been known as a toxic gas [14]. However, it has emerged that H\textsubscript{2}S is also generated in mammalian tissues from the amino acids L-cysteine and homocysteine by pyridoxal-5'-phosphate-dependent enzymes such as cystathionine-\(\beta\)-synthase (CBS, EC4.2.1.22) and cystathionine-\(\gamma\)-lyase (CSE, EC4.4.1.1) [26, 28]. CBS converts homocysteine to cystathionine and hydrolyses L-cysteine to equimolar amounts of serine and H\textsubscript{2}S whereas CSE converts cystathionine to L-cysteine yielding pyruvate, NH\textsubscript{3} and H\textsubscript{2}S [28]. Nitric oxide (NO) and carbon monoxide (CO) now including H\textsubscript{2}S are growing family of regulatory gaseous molecules involved in various physiological and pathological functions in mammalian tissues [32]. In contrast to other gaseous mediators (NO and CO [31, 33]) very little information exists on the mechanism by which H\textsubscript{2}S influences cell function.
Physiological levels of $\text{H}_2\text{S}$ in rat serum vary from 30 to 45µM [24, 27, 35] and in mouse serum, ~23µM [10] and within this concentration range $\text{H}_2\text{S}$ induces relaxation of aorta tissues and induces transient blood pressure reduction [37]. Vascular effect of $\text{H}_2\text{S}$ may also be mediated by a direct stimulation of $\text{K}_{\text{ATP}}$ channels with subsequent hyperpolarization of rat aortic vascular smooth muscle in mesenteric artery, aorta, and portal vein [17, 37]. Consistent with this, a reduced CSE expression/activity and decreased $\text{H}_2\text{S}$ concentration contributes to the pathophysiology of pulmonary hypertension in rodents [34] whereas CBS deficiency leads to hyperhomocysteinemia, increased blood pressure and endothelial dysfunction [13]. In rodents, CBS deficiency induced by genetic deletion [13] or chronic treatment with DL-propargylglycine (PAG), an irreversible inhibitor of CSE [34], results in a severe hypertension and severe loss of endothelial function.

We have previously reported that caerulein-induced acute pancreatitis up-regulates pancreas CSE mRNA expression and $\text{H}_2\text{S}$ biosynthesis. Therapeutic administration of PAG reduces the severity of caerulein-induced pancreatitis and affords protection against associated lung injury in mice [10]. A similar pro-inflammatory effect of $\text{H}_2\text{S}$ has also been reported in other animal models of hindpaw edema [9], lipopolysaccharide (LPS) induced endotoxemia [24] as well as cecal ligation and puncture (CLP) induced sepsis [36]. In these studies, tissue CSE expression is up-regulated leading to increased $\text{H}_2\text{S}$ biosynthesis whereas inhibition of $\text{H}_2\text{S}$ formation has been shown to display distinct anti-inflammatory activity [9, 24, 36]. Therefore, it is emerging that $\text{H}_2\text{S}$ is an additional and important physiological mediator in inflammation.

Acute pancreatitis is a common clinical condition, the incidence of which has been increasing over recent years [2–4, 6, 7]. It is generally believed that the earliest events in acute pancreatitis occur within acinar cells. In vitro and in vivo data [8] indicate that the earliest event and most important factor responsible for acute pancreatitis is the increase and the activation of intra-acinar digestive enzymes. After this initial insult, other components (ischemia, pancreatic glutathione deficiency, oxygen free radicals, cell death and inflammatory mediators) intervene to worsen pancreatitis. Increasing evidence indicates that neurogenic pro-inflammatory factors, such as substance P (SP), can play important roles in determining the severity of acute pancreatitis [19] both in mice and rats. SP belongs to the tachykinin family of neuropeptides, derived from the preprotachykinin (PPT-A) gene. It is a neurotransmitter and pain mediator released from both central and peripheral endings of primary afferent neurons as well as from various inflammatory cells [16, 25]. The biological actions of SP are mediated primarily by neurokinin-1 receptors (NK-1R). Previous data [1] showed that SP levels and NK-1R in pancreatic acinar cells are up-regulated during experimental pancreatitis in mice. In addition, NK-1R deletion or antagonism protects mice against pancreatitis [5, 15]. Further study showed that the antagonist treatment suppressed the increase in pancreatic and lung PPT-A mRNA expression and SP protein levels in acute pancreatitis [21].

Although recent studies have demonstrated that $\text{H}_2\text{S}$ plays a key role in inflammation, the cellular source of $\text{H}_2\text{S}$ and the mechanism by which $\text{H}_2\text{S}$ acts as an inflammatory mediator in pancreas is yet to be found. To this end we have identified $\text{H}_2\text{S}$ synthesizing enzyme activity, CSE and CBS mRNA expression in pancreatic acinar cells. The effect of $\text{H}_2\text{S}$ concentration, CBS and CSE mRNA in cholecystokinin (CCK) secretagogue caerulein-treated acinar cells has been monitored. Moreover we have investigated whether $\text{H}_2\text{S}$ as pro-inflammatory molecule is involved in SP-NK-1R related pathway in mouse pancreatic acinar cells. We induced acinar cells with caerulein ($10^{-7}$ M) and studied the effect of PAG (3 mM) an irreversible inhibitor of $\text{H}_2\text{S}$-synthesizing enzyme CSE, on SP levels, PPT-A and NK-1R mRNA expression. We have also assessed the pro-inflammatory effect of the $\text{H}_2\text{S}$ donor, sodium hydro sulphide (NaHS) in acinar cells.

**Materials and methods**

**Preparation of pancreatic acini**

All experiments were approved by the animal ethics committee of National University of Singapore, Singapore and carried out in accordance with the established Guiding Principles for Animal Research. Pancreatic acini were obtained from mouse pancreas by collagenase treatment as described earlier [1]. Briefly, pancreata from male Swiss albino mice (25–30 g) were removed under aseptic conditions, infused with collagenase buffer A (in mM: 140 NaCl, 4.7 KCl, 1.13 MgCl$_2$, 1 CaCl$_2$, 10 glucose, and 10 HEPES,
H₂S biosynthesis in pancreatic acini was measured essentially as described previously [30]. Frozen cells were thawed on ice and homogenized in ice-cold 100 mM potassium phosphate buffer (pH 7.4). The reaction mixture (total volume, 500 µl) contained homogenate (430 µl), L-cysteine (10 mM; 20 µl), pyridoxal 5'-phosphate (2 mM; 20 µl), and saline (30 µl). The reaction was performed in parafilm ed eppendorf tubes and initiated by transferring the tubes from ice to a water bath at 37°C. In some experiments, the enzymatic reaction was stopped immediately by addition of trichloroacetic acid (10% w/v, 250 µl), and saline (30 µl). The reaction was performed in parafilm ed eppendorf tubes and initiated by transferring the tubes from ice to a water bath at 37°C. In some experiments, the enzymatic reaction was stopped immediately by addition of trichloroacetic acid (10% w/v, 250 µl) to denature protein prior to addition of L-cysteine. After incubation for 30 min, zinc acetate (1% w/v, 250 µl) was added to trap evolved H₂S, followed by trichloroacetic acid (10% w/v, 250 µl). Subsequently, N,N-dimethyl-p-phenylenediamine sulphate (20 µM; 133 µl) in 7.2 M HCl and FeCl₃ (30 µM; 133 µl) in 1.2 M HCl were added and the absorbance of the resulting solution (670 nm) measured 15 min thereafter, using a 96-well microplate reader (Tecan Systems Inc.). The H₂S concentration of each sample was calculated against a calibration curve of sodium hydrosulphide (NaHS; 3.12–250 µM) and results are expressed as nmol H₂S formed mg⁻¹ DNA [24].

Measurement of Substance P levels

Pancreatic acinar cells were homogenized in 2 ml ice-cold assay buffer for 20 sec. The homogenates were centrifuged (13,000 x g, 20 min, 4°C) and the supernatants were collected. They were adsorbed on C₁₈ cartridge columns (Bachem) as described [21]. The adsorbed peptides were eluted with 1.5 ml 75% v/v acetonitrile. The samples were freeze-dried and reconstituted in assay buffer. Substance P content was then determined using an ELISA kit (Bachem) according to the manufacturer’s instructions and expressed as ng/µg DNA [20].

RT-PCR

Total RNA from isolated pancreatic acinar cell was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol [10]. The concentration of isolated nucleic acids were determined spectrophotometrically by measuring the absorbance at 260 nm, and the integrity was verified by ethidium bromide staining of 18S and 28S rRNA bands on a denaturing agarose gel. All samples were thereafter stored at −80°C until required. RNA (1 µg) was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) at 25°C for 5 min and 42°C for 30 min, followed by 85°C for 5 min. The cDNA was used as a template for PCR amplification by iQ Supermix (Bio-Rad). The PCR primers (Table 1) for detection of CSE, CBS, PPT-A and NK-1R were synthesized by Proligo (Singapore). The reaction mixture was first subjected to 95°C for 3 min for the activation of polymerase. This was followed by an optimal cycle of amplifications (Table 1), consisting of 95°C for 30 sec optimal annealing temperature (Table 1) for 72°C for 30 sec. PCR amplification was carried out in MyCycler (Bio-Rad, Hercules, CA, USA). PCR products were analyzed on 1.5% w/v agarose gels containing 0.5 µg/ml ethidium bromide.
Statistical analysis

Data are expressed as the mean ± standard error of the mean (S.E.M.). In all figures, vertical error bars denote the S.E.M. The absence of such error bars indicates that the S.E.M. falls within the dimensions of the data bar. The significance of differences between groups was evaluated by analysis of variance (ANOVA), with post hoc Tukey’s test when comparing three or more groups. A P-value of less than 0.05 was considered statistically significant.

Results

Production of H₂S by pancreatic acini in response to caerulein treatment

Pancreatic acinar cells were stimulated with caerulein (10⁻⁷ M) for 30 and 60 min at 37°C. After which the pellet was used to assess the levels of H₂S. Figure 1 shows a significant increase in H₂S levels following 60 min incubation with caerulein. In freshly prepared acinar cells, levels of H₂S were 10.5 ± 0.8 μM. Whereas H₂S levels increased from 11.2 ± 1.1 and 12.1 ± 0.63 μM in control cells to 12.1 ± 0.65 and 25.1 ± 1.3 μM in cells incubated with caerulein for 30 and 60 min, respectively (P < 0.001 when caerulein treated cells (60 min) were compared with control cells (60 min); Fig. 1).

CSE and CBS mRNA expression and H₂S synthesis in the pancreatic acini

Pancreatic acinar cells were found to express the gene for the H₂S forming enzyme CSE and CBS, as evidenced by RT-PCR extracted from these cells (Fig. 2A and B). In addition, incubation of acinar cell homogenates with L-cysteine resulted in the formation of H₂S, showing the presence of H₂S synthesizing enzyme activity in the cells (Fig. 3A).

To determine whether CSE and CBS mRNA expression is regulated by caerulein, we extracted total RNA from isolated acinar cells incubated for 30 and 60 min with or without caerulein. Figure 2A (P < 0.001 when caerulein treated cells (60 min) were compared with control cells (60 min)) shows a significant up-regulation of CSE mRNA expression by caerulein, whereas CBS mRNA expression was decreased following 60 min incubation with caerulein (P < 0.001 when caerulein treated cells (60 min) were compared with control cells (0 hr); Fig. 2B).

Table 1 PCR primer sequences, optimal amplification cycles, optimal annealing temperatures and product sizes

| Gene   | Primer sequence                      | Optimal conditions | Size (bp) |
|--------|--------------------------------------|--------------------|-----------|
| r18S   | Sense: 5’_GTAACCCGTTGAACCCATT-3_     | 22 cycles          | 150       |
|        | Antisense: 5’_CCATCCAATCGGTAGTCGGC-3’|                    |           |
|        |                                      | Annealing: 59°C    |           |
| CSE    | Sense: 5’_GAC CTC AAT AGT CGG TTT GTC TGC GTA ATG -3_ | 22 cycles          | 618       |
|        | Antisense: 5’_CAG TGC GTA TGC TCC GTA ATG -3’ |                    |           |
|        |                                      | Annealing: 60°C    |           |
| CBS    | Sense: 5’_GAT TTG ATT CCC CCG AGT CCC AG -3_ | 22 cycles          | 618       |
|        | Antisense: 5’_CAC TTA TCC ACC ACC GCC CTG TC -3’ |                    |           |
|        |                                      | Annealing: 60°C    |           |
| NK1R   | Sense: 5’_CTTGCCCTTTTGGAACCGTGTG-3’  | 42 cycles          | 501       |
|        | Antisense: 5’_CAGCTTCCTTATCTGTGGG-3’ |                    |           |
|        |                                      | Annealing: 59.7°C  |           |
| PPT-A  | Sense: 5’_ACCTGCTCCACTCTGGCAACCGCGGAAAG-3’ | 43 cycles          | 239       |
|        | Antisense: 5’_GAACGTGAGGCTTTGGTTCGCTGCGCAGT-3’ |                    |           |
|        |                                      | Annealing: 68°C    |           |
| B-actin| Sense: 5’_GGCG CGT TAT TCC CCT CCA TC-3’ | 22 cycles          | 552       |
|        | Antisense: 5’_GTC ACG CAC GAT TCC CCT CTC-3’ |                    |           |
|        |                                      | Annealing: 61°C    |           |
Effect of DL-propargylglycine (PAG) on H$_2$S synthesizing activity

As CSE mRNA is up-regulated after caerulein treatment in acinar cells, we decided to investigate whether an inhibitor of CSE enzyme might exhibit anti-inflammatory activity. PAG was chosen for these experiments since it produces an irreversible inhibition of CSE [29], crosses biological membranes readily, and has been shown to cause long-lasting CSE inhibition after administration in animals [19]. Isolated pancreatic acinar cells were treated for 30 and 60 min at 37°C with different concentrations of PAG (2–5 mM). Only 60 min treatment with PAG at a dose of 3 mM showed maximum decrease in H$_2$S production when compared with control cells (Fig. 3A). The dose of 3 mM of PAG was used to carry out subsequent experiments.

As shown in Figure 3B treatment of pancreatic acini with caerulein caused a significant increase in H$_2$S production. Pretreatment of acini with 3 mM of PAG followed by stimulation with caerulein significantly attenuated the production of H$_2$S when compared with caerulein treated cells. ($P < 0.001$). Since PAG does not block CBS activity either in vitro [30] or after administration in experimental animals in vivo [29], it is therefore likely that the observed rise in H$_2$S in caerulein treated acinar cells may be due to enhanced CSE enzyme activity.

Fig. 1 Hydrogen sulphide (H$_2$S) concentration in caerulein-induced mouse pancreatic acinar cells. Freshly prepared pancreatic acinar cells were incubated with or without caerulein (10$^{-7}$ M for 30 and 60 min). H$_2$S concentration was measured as described in Section ‘Materials and methods’. Results shown are the mean ± S.E.M. of at least six separate determinations. ***$P < 0.001$ when caerulein treated cells (60 min) were compared with control cells (60 min).

Effect of PAG pretreatment on substance P level and PPT-A mRNA expression in pancreatic acinar cells

Figure 4 shows the concentration of SP in the pancreatic acinar cells. As expected, hyper stimulation by caerulein increased the levels of SP in acinar cells ($P < 0.001$). Pretreatment of acini with 3 mM PAG followed by stimulation with caerulein significantly suppressed the SP concentration when compared with caerulein treated cells ($P < 0.05$). Densitometry analysis of the PCR products on agarose gel shows that the PPT-A mRNA expression increased in caerulein induced acinar cells ($P < 0.001$) and pretreatment with 3 mM PAG resulted in a significant decrease in PPT-A gene expression ($P < 0.001$; Fig. 5A).

Effect of PAG treatment on NK-1R mRNA expression in pancreatic acinar cells

A significant increase in NK-1R mRNA expression was observed in caerulein-treated pancreatic acinar cells ($P < 0.001$; Fig. 5B). However, pretreatment with 3 mM PAG followed by stimulation with caerulein suppressed NK-1R expression when compared with caerulein treated acinar cells ($P < 0.05$; Fig. 5B).
Fig. 2 Cystathionine-\(\gamma\)-lyase (CSE) and cystathionine-\(\beta\)-synthase (CBS) mRNA expression in control and caerulein-treated mouse pancreatic acinar cells. (A) RT-PCR of CSE in control and caerulein (10\(^{-7}\) M for 30 and 60 min) treated acinar cells. The graphs represent the optical density of the bands of CSE generated from six independent experiments that were normalized with the expression of 18S. ***P < 0.001 when caerulein treated cells (60 min) were compared with control cells (60 min). (B) RT-PCR of CBS in control and caerulein treated acinar cells. The graphs represent the optical density of the bands of CBS generated from six independent experiments that were normalized with the expression of 18S. ***P < 0.001 when caerulein treated cells (60 min) were compared with control cells (0 hr).
Fig. 3 Effect of DL-propargylglycine (PAG) on H_{2}S synthesizing activity in caerulein-treated mouse pancreatic acinar cells. (A) Freshly prepared pancreatic acinar cells were incubated for 30 and 60 min at 37°C with different concentration of PAG (2–5 mM). After which the pellet was used to detect the H_{2}S synthesizing activity. Results shown are the mean ± S.E.M. of at least six separate determinations. ***P < 0.001 when 3 mM PAG treated cells (60 min) were compared with control cells (60 min). (B) Freshly prepared pancreatic acinar cells were pretreated with PAG (3 mM) 60 min before caerulein (10^{-7} M for 30 and 60 min) treatment. Pancreatic acinar cell homogenates were assessed for ability to synthesize H_{2}S from exogenous L-cysteine. Results shown are the mean ± S.E.M. of at least six separate determinations. ***P < 0.001 when caerulein treated cells (60 min) were compared with control cells (60 min). ***P < 0.001 when caerulein treated cells (60 min) were compared with PAG pretreated cells (60 min).
These experiments suggest that the inhibition of endogenous H2S biosynthesis results in an anti-inflammatory effect in caerulein-induced pancreatic acinar cells and thereby provides indirect evidence that H2S exerts pro-inflammatory activity in acinar cells. To investigate a possible direct role of H2S in acinar cells, we treated the cells with the H2S donor drug, NaHS (10 and 100 μM) and evaluated its effect.
on SP level. As shown in Fig. 6 (P < 0.01) 100 µM of NaHS significantly increased SP level in pancreatic acinar cells after 30 min treatment. In addition, PPT-A (P < 0.05; Fig 7A) and NK-1R expression were markedly increased 30 min after treatment with NaHS (P < 0.01; Fig 7B).

**Discussion**

H₂S is increasingly recognized as a functionally relevant mediator of a number of physiological functions in mammalian tissues. It is generated endogenously during L-cysteine metabolism in many types of mammalian cells in the reaction catalyzed by cystathionine β-synthase and cystathionine γ-lyase [26,28].

We have previously reported that caerulein induced acute pancreatitis up regulates pancreas CSE mRNA expression and H₂S biosynthesis. Therefore it causes a significant increase in plasma H₂S concentration [10]. Blockage of H₂S formation by administration of PAG, an inhibitor of CSE enzyme activity, reduced the severity of acute pancreatitis.

**Fig. 6** Effect of H₂S donor, sodium hydro-sulphide (NaHS), on SP level in pancreatic acinar cells. Freshly prepared pancreatic acinar cells were treated with NaHS (10, 50 and 100 µM) for 30 min. Substance P level was measured as described in Materials and Methods. Results shown are the mean ± S.E.M. of at least six separate determinations. **P < 0.01 between control and NaHS-induced cells.

**Fig. 7** H₂S donor, sodium hydrosulphide (NaHS), induces PPT-A and NK-1R mRNA expression in mouse pancreatic acinar cells. (A) PPT-A mRNA expression in control and NaHS-treated (10, 50 and 100 µM) cells. The graphs represent the optical density of the bands of PPT-A generated from six independent experiments that were normalized with the expression of β-actin. *P < 0.05 between control and NaHS-treated cells. (B) NK-1R mRNA expression in control and NaHS-treated (10, 50 and 100 µM) cells. The graphs represent the optical density of the bands of NK-1R generated from six independent experiments that were normalized with the expression of β-actin. ***P < 0.001 between control and NaHS-induced cells.
whereas exogenous H2S results in augmented MPO activity as well as histological changes in lung and pancreas [10]. A similar pro-inflammatory effect of H2S has also been reported in other animal models of inflammatory diseases, such as hindpaw edema [9], cecal ligation-induced sepsis [26] and lipopolysaccharide induced endotoxemia [24]. Based on these findings, we have proposed that H2S acts as a pro-inflammatory mediator. However, the precise mechanism by which H2S acts as an inflammatory mediator during acute pancreatitis remains unknown. Therefore, we went on to investigate the role of endogenous H2S in caerulein-treated pancreatic acinar cells.

We show here that mRNA for CSE and CBS are expressed in acinar cells (Fig. 2A and B) and the pancreatic acinar cell homogenates convert L-cysteine to H2S ex vivo (Fig. 1). With the use of caerulein stimulated pancreatic acini, a well-established in vitro model for pancreatitis [23], our findings indicated an important role of H2S in pancreatic acinar cells. Stimulation of pancreatic acini with caerulein has significantly increased CSE mRNA expression (Fig. 2A). CBS mRNA expression, however reduced on caerulein treatment (Fig. 2B). The H2S synthesizing enzyme activity was also found to be elevated in the cells (Fig. 3B). It is reasonable to conclude that caerulein treatment up-regulates CSE expression and the CSE activity. The present study shows that, although both CBS and CSE are expressed by the pancreatic acinar cells, CSE may be the main enzyme involved in the H2S generation in the caerulein-induced pancreatic acinar cells. Consistent with this concept, exposure of the acinar cells to PAG, inhibits H2S formation (Fig. 3B). Inhibition of H2S synthesis using pharmacological agents acting on CSE has been shown to reduce inflammation [24] and swelling [9]. In our previous study, we have reported that therapeutic administration of PAG reduces the severity of caerulein-induced pancreatitis and affords protection against associated lung injury in mice [10]. As PAG elicits a complete and irreversible inactivation of CSE activity in vitro [18, 27], our finding suggests that CSE may be the enzyme mainly responsible for H2S formation in acinar cells.

In the present study, we observed a novel interaction between two groups of pro-inflammatory mediators – H2S and the neuropeptide substance P. Inhibition of endogenous H2S, with specific CSE inhibitor PAG resulted in attenuation in caerulein-induced SP release (Fig. 4), PPT-A and NK-1R expression (Fig. 5A and B). This suggests the pro-inflammatory effect of H2S are mediated by the release of SP. SP in turn is mediated by NK-1R in pancreatic acini.

Previously, it has been demonstrated that SP has an important role in mediating H2S-induced lung inflammation [11]. Genetic deletion of PPT-A and NK-1R reduced the severity of acute pancreatitis and associated lung injury [1, 5, 15]. We have earlier showed that NK-1R antagonist protected mice against caerulein-induced acute pancreatitis and associated lung injury (21). Further study showed that the antagonist treatment suppressed the increase in pancreatic and lung PPT-A expression and SP release in acute pancreatitis [22]. Our recent study demonstrated that H2S-induced SP release is inhibited by CP 96,345, a specific antagonist for NK-1R [11]. The above result suggests that part of the anti-inflammatory action of NK-1R antagonist may be mediated by a lowering of intracellular H2S production at sites of inflammation. Our result suggests the possibility that part of the known anti-inflammatory actions of CSE inhibitor may be mediated through an inhibition of SP-induced inflammation, although obviously other inflammatory mediators such as adhesion molecules, cytokines and nitric oxide in the interplay between H2S and SP may also be involved.

Since inhibition of endogenous H2S biosynthesis by PAG significantly reduced the SP release, we thought it of value to determine whether H2S itself is involved in SP-NK-1R-related pathway in acinar cells. For this purpose, we used the H2S donor, NaHS, to produce H2S. When mouse pancreatic acini were treated with NaHS, there was a significant increase in SP (Fig. 6), and up regulation of PPT-A and NK-1R expression in acinar cells (Fig. 7A and B), indicating that H2S by itself induces SP release and SP is mediated through the NK-1R. Our results demonstrate a novel role of H2S in SP-NK-1R-related pathway in pancreatic acinar cells.

In conclusion, we have shown that caerulein up-regulates CSE gene expression and lead to enhanced H2S biosynthesis in acinar cells. Caerulein induced inflammation is attenuated by inhibition of CSE activity by PAG. These results suggest that H2S plays a role in caerulein-induced acinar cell inflammation and inhibition of H2S synthesis may be useful in the therapy of inflammation. An extended knowledge of the biological and molecular alterations linked to
H2S and acute pancreatitis will open the way for new therapies for this life-threatening condition.

Acknowledgements

This work was supported by Biomedical Research Council Grant No. R-184-000-094-305 and Office of Life Sciences Cardiovascular Biology Research Program (Grant no. R-184-000-074-712).

References

1. Bhatia M, Saluja AK, Hofbauer B, Frossard JL, Lee HS, Castagliuolo I, Wang CC, Gerard N, Pothoulakis C, Steer ML. Role of substance P and the neurokinin 1 receptor in acute pancreatitis and pancreatitis-associated lung injury. *Proc Natl Acad Sci USA*. 1998; 95: 4760–65.

2. Bhatia M, Brady M, Shokuhi S, Christmas S, Neoptolemos JP, Slavin J. Inflammatory mediators in acute pancreatitis. *J Pathol*. 2000; 190: 117–25.

3. Bhatia M, Neoptolemos JP, Slavin J. Inflammatory mediators as therapeutic targets in acute pancreatitis. *Curr Opin Investig Drugs*. 2001; 2: 496–501.

4. Bhatia M. Novel therapeutic targets for acute pancreatitis and associated multiple organ dysfunction syndrome. *Curr Drug Targets – Inf Allergy*. 2002; 1: 343–51.

5. Bhatia M, Slavin J, Cao Y, Basbaum AI, Neoptolemos JP. Preprotachykinin-A gene deletion protects mice against acute pancreatitis and associated lung injury. *Am J Physiol Gastrointest Liver Physiol*. 2003; 284: G830–6.

6. Bhatia M. Novel therapeutic targets for acute pancreatitis and associated multiple organ dysfunction syndrome – an update. *Med Chem Rev Online*. 2004; 1: 25–6.

7. Bhatia M, Moochhala S. Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. *J Path*. 2004; 202: 145–56.

8. Bhatia M, Wong FL, Cao Y, Lau HY, Huang J, Puneet P, Chevali L. Pathophysiology of acute pancreatitis. *Pancreatology*. 2005; 5: 132–44.

9. Bhatia M, Sidhapuriwala J, Moochhala SM, Moore PK. Hydrogen sulfide is a mediator of carrageenan-induced hindpaw edema in the rat. *Br J Pharmacol*. 2005; 145: 141–4.

10. Bhatia M, Wong FL, Fu D, Law HY, Moochhala SM, Moore PK. Role of hydrogen sulfide in acute pancreatitis and associated lung injury. *FASEB J*. 2005; 19: 623–5.

11. Bhatia M, Zhi L, Zhang H, Moore PK. Role of substance P in hydrogen sulfide-induced pulmonary inflammation in mice. *Am J Physiol Lung Mol Physiol*. 2006; 291: L896–904.

12. Chunyu Z, Junbao D, Dingfang B, Hui Y, Xiuying T, Chaoshu T. The regulatory effect of hydrogen sulfide on hypoxic pulmonary hypertension in rats. *Biochem Biophys Res Commun*. 2003; 302: 810–6.

13. Eberhardt RT, Forgione MA, Cap A, Leopold JA, Rudd MA, Trollet M. Endothelial dysfunction in a murine model of mild hyperhomocysteinemia. *J Clin Invest*. 2000; 106: 483–91.

14. Gosselin RE, Smith RP, Hodge HC. Clinical toxicology of commercial products. 5th ed. Baltimore, USA: Williams and Wilkins Ltd; 1984.

15. Grady EF, Yoshimi SK, Maa J, Valeroso D, Vartanian RA, Rahim S, Kim EH, Gerard C, Gerard N, Bunnett NW, Kirkwood KS. Substance P mediates inflammatory oedema in acute pancreatitis via activation of the neurokinin-1 receptor in rats and mice. *Br J Pharmacol*. 2000; 130: 505–12.

16. Haines KA, Kolasinski SL, Cronstein BN, Reibman J, Gold LI, Weissmann G. Chemoattraction of neutrophils by substance P and transforming growth factor-beta 1 is inadequately explained by current models of lipid remodeling. *J Immunol*. 1993; 151: 1491–9.

17. Hosoki R, Matsuki N, Kimura H. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem Biophys Res Commun*. 1991; 237: 527–31.

18. Johnston M, Jankowski D, Mercotte P, Tanaka H, Esaki N, Soda K, Walsh C. Suicide inactivation of bacterial cystathionine gamma synthase and methionine gamma-lyase during processing of L-propargylglycine. *Biochem*. 1979; 18: 4690–701.

19. Kodama H, Mikasa H, Sasaki K, Awata S, Nakayama K. Unusual metabolism of sulfur-containing amino acids in rats treated with DL-propargylglycine. *Arch Biochem Biophys*. 1983; 225: 25–32.

20. Labarca C, Paigen K. A simple, rapid, and sensitive DNA assay procedure, *Anal Biochem*. 1980; 102: 344–52.

21. Lau HY, Wong FL, Bhatia M. A key role of neurokinin 1 receptors in acute pancreatitis and associated lung injury. *Biochem Biophys Res Commun*. 2005; 327: 509–15.

22. Lau HY, Bhatia M. Effect of CP96,345 on the expression of tachykinins and neurokinin receptors in acute pancreatitis. *J Pathol*. 2006; 208: 364–71.

23. Leach SD, Modlin IM, Scheele GA, Gorelick FS. Intracellular activation of digestive zymogens in rat pancreatic acini. Stimulation by high doses of cholecystokinin. *J Clin Invest*. 1991; 87: 362–6.

24. Li L, Bhatia M, Zhu YZ, Ramnath RD, Wang ZZ, Anuar FM, Whiteman M, Salto-Tellez M, Moore PK.
Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. FASEB J. 2005; 19: 1196–8.

25. Maggi CA. The effects of tachykinins on inflammatory and immune cells. *Regul Pept.* 1997; 70: 75–90.

26. Mester A, Fraser PE, Tice SV. Enzymatic desulphuration of β-mercaptopyruvate to pyruvate. *J Biol Chem.* 1954; 206: 516–75.

27. Mok YYP, Shirhan M, Cheong YP, Wang ZZ, Bhatia M, Moochhala SM, Moore PK. Role of hydrogen sulfide in haemorrhagic shock in the rat: protective effect of inhibitors of hydrogen sulfide biosynthesis. *Br J Pharmacol.* 2004; 143: 882–9.

28. Navarra P, Dello Russo C, Mancuso C, Preziosi P, Grossman A. Gaseous neuromodulators in the control of neuroendocrine stress axis. *Ann NY Acad Sci USA.* 2000; 917: 638–46.

29. Patacchini R, Santicioli P, Giuliani S, Maggi CA. Hydrogen sulfide (H₂S) stimulates capsaicin-sensitive primary afferent neurons in the rat urinary bladder. *Br J Pharmacol.* 2004; 142: 31–34.

30. Stipanuk MH, Beck PW. Characterization of the enzymatic capacity for cysteine desulphhydration in liver and kidney of the rat. *Biochem J.* 1982; 206: 267–77.

31. Thippeswamy T, McKay JS, Quinn JP, Morris R. Nitric oxide, a biological double-faced janus-is this good or bad? *Histol Histopathol.* 2006; 21: 445–58.

32. Wang R. Two’s company, three’s a crowd: can H₂S be the third endogenous gaseous transmitter? *FASEB J.* 2002; 16: 1792–8.

33. Wu L, Wang R. Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. *Pharmacol Rev.* 2005; 57: 585–630.

34. Yan H, Du J, Tang C. The possible role of hydrogen sulfide on the pathogenesis of spontaneous hypertension in rats. *Biochem Biophys Res Commun.* 2004; 313: 22–7.

35. Yusuf M, Tan BKH, Hsu A, Bhatia M, Moore PK. Streptozotocin-induced diabetes in the rat is associated with enhanced tissue hydrogen sulphide biosynthesis. *Biochem Biophys Res Commun.* 2005; 333: 1146–52.

36. Zhang H, Zhi L, Moore PK, Bhatia M. The role of hydrogen sulfide in cecal ligation and puncture induced sepsis in the mouse. *Am J Physiol Lung Cell Mol Physiol.* 2006; 290: L1193–201.

37. Zhao W, Zhang J, Lu Y, Wang R. The vasorelaxant effects of endogenous gaseous KATP channel opener. *EMBO J.* 2001; 20: 6008–16.