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Pro-inflammatory effects of hydrogen sulphide on substance P in caerulein-induced acute pancreatitis

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Abstract

Hydrogen sulphide (H_2S), a novel gasotransmitter, has been recognized to play an important role in inflammation. Cystathionine-γ-lyase (CSE) is a major H_2S synthesizing enzyme in the cardiovascular system and DL-propargylglycine (PAG) is an irreversible inhibitor of CSE. Substance P (SP), a product of preprotachykinin-A (*PPT-A*) gene, is a well-known pro-inflammatory mediator which acts principally through the neurokinin-1 receptor (*NK-1R*). We have shown an association between H_2S and SP in pulmonary inflammation as well as a pro-inflammatory role of H_2S and SP in acute pancreatitis. The present study was aimed to investigate the interplay between pro-inflammatory effects of H_2S and SP in a murine model of caerulein-induced acute pancreatitis. Acute pancreatitis was induced in mice by 10 hourly intraperitoneal injections of caerulein (50 (g/kg). PAG (100 mg/kg, i.p.) was administered either 1 hr before (prophylactic) or 1 hr after (therapeutic) the first caerulein injection. PAG, given prophylactically as well as therapeutically, significantly reduced plasma H_2S levels and pancreatic H_2S synthesizing activities as well as SP concentrations in plasma, pancreas and lung compared with caerulein-induced acute pancreatitis. Furthermore, prophylactic as well as therapeutic administration of PAG significantly reduced *PPT-A* mRNA expression and *NK-1R* mRNA expression in both pancreas and lung when compared with caerulein-induced acute pancreatitis. These results suggest that the pro-inflammatory effects of H_2S may be mediated by SP-*NK-1R* pathway in acute pancreatitis.

Keywords: hydrogen sulphide • substance P • caerulein • DL-propargylglycine (PAG)

Introduction

Acute pancreatitis is a common clinical condition, the incidence of which has been increasing over recent years [1]. Most cases develop as a result of biliary disease or excess alcohol consumption. About 25% of patients suffer a severe attack and between 30% and 50% of these will die [1]. The exact mechanisms by which diverse etiological factors induce an attack of acute pancreatitis are still unclear but once the disease process is initiated, common inflammatory and repair pathways are invoked. If this inflammatory reaction is very pronounced, it leads to a systemic inflammatory response syndrome (SIRS), and the

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systemic response is ultimately accountable for the majority of the morbidity and mortality [1].

Hydrogen sulphide (H_2S) has recently been identified as a biological mediator [20, 22, 31]. Cystathionine-β-synthase (CBS, EC4.2.1.22) and Cystathionine-γ-lyase (CSE, EC4.4.1.1) are the key enzymes involved in H_2S synthesis. Both CBS and CSE are widely distributed in tissues. However, CBS is a predominant source of H_2S in the central nervous system whereas CSE is a major H_2S-producing enzyme in the cardiovascular system. DL-propargylglycine (PAG) is an irreversible inhibitor of CSE. H_2S dilates blood vessels and relaxes gastrointestinal smooth muscles by opening muscle K^+ATP channels and promotes hippocampal long-term potentiation by enhancing the sensitivity of N-methyl-D-aspartate receptors to glutamate [36]. H_2S has been shown to act as an important endogenous regulator of leukocyte activation and trafficking during an inflammatory response [33]. Furthermore, H_2S has been shown to stimulate the activation of human monocytes with the generation of pro-inflammatory cytokines, and this response is, at least partially, through the ERK-NF-κB signaling pathway [37].

Substance P (SP), a neuropeptide product of the preprotachykinin-A gene (*PPT-A*) plays an important role in inflammatory disorders including acute pancreatitis [4, 13]. SP binds preferentially to neurokinin-1 receptor (*NK-1R*), causing vasodilatation, plasma extravasation, leukocyte adhesion and subsequent accumulation at the site of tissue injury [10–13]. SP can specifically stimulate the chemotaxis of neutrophils. SP also enhances cytokine secretion from lymphocytes, monocytes, macrophages and mast cells. Inflammatory mediators, such as cytokines and histamine potentiate tissue injury, and stimulate further leukocyte recruitment, thereby amplifying the inflammatory response. Previously, we have shown increased levels of H_2S and CSE mRNA expression in pancreas in caerulein-induced pancreatitis and associated lung injury [2, 30] and treatment with PAG, a CSE inhibitor, significantly reduced the severity of caerulein-induced pancreatitis and associated lung injury [2]. The effects of CSE blockade suggest an important pro-inflammatory role of H_2S in acute pancreatitis and associated lung injury. Earlier studies have shown that knockout mice deficient in *NK-1R* and knockout mice deficient in *PPT-A* gene are protected against acute pancreatitis and associated lung injury [4, 5, 12]. These results suggest an important pro-inflammatory role of SP in neurogenic inflammation as well as in acute pancreatitis and associated lung injury. Increased concentrations of plasma, pancreatic and pulmonary SP have been found in caerulein-induced pancreatitis in mice [4, 17], in sodium hydrosulphide (NaHS, H_2S donor)-stimulated mouse pancreatic acinar cells [30] and NaHS-induced lung inflammation [6]. Therefore, the present study was aimed to investigate pro-inflammatory effect of H_2S on SP in caerulein-induced acute pancreatitis and associated lung injury.

Materials and methods

Induction of acute pancreatitis

All animal experiments were approved by the Animal Ethic Committee of National University of Singapore and carried out in accordance with established International Guiding Principles for Animal Research. Caerulein was obtained from Bachem (Bubendorf, Switzerland) and DL-PAG was obtained from Sigma. Swiss mice (male, 20–25 g) were randomly assigned to control or experimental groups using 12 animals for each group. Animals were given hourly intraperitoneal (i.p.) injections of normal saline or saline containing caerulein (50 μg/kg) for 10 hrs [2, 4, 5]. PAG (100 mg/kg, i.p.) dissolved in saline was administered either 1 hr (prophylactic) before or 1 hr after (therapeutic) the first caerulein injection. One hour after the last caerulein injection animals were sacrificed by an i.p. injection of a lethal dose of 50 mg/kg pentobarbital (Nembutal, CEVA Sante Animale, Naaldwijk, Netherlands). Blood, pancreas and lung tissues were collected. Harvested heparinized blood was centrifuged (8000 rpm, 10 min, 4°C), the plasma was aspirated and stored at (80°C for subsequent detection of plasma H_2S and SP concentrations. Samples of pancreas and lung were removed, weighed and then stored at (80°C for subsequent measurement of tissue H_2S synthesizing activities, SP concentrations and RT-PCR assay as described below.

Measurement of plasma H_2S

Aliquots (300 μl) of plasma were mixed with distilled water (250 μl; depending on volume of plasma used), trichloroacetic acid (10% w/v, 300 μl), zinc acetate (1% w/v, 150 μl), N,N-dimethyl-p-phenylenediamine sulphate (20 μM; 100 μl) in 7.2 M HCl and $FeCl_3$ (30 μM; 133 μl) in

1.2 M HCl and then the solution (300 μl) were added into 96-well plates. The absorbance of the resulting solution (670 nm) was measured 10 min thereafter by a microplate reader (SPECTRAFluor Plus, Tecan Austria GmbH, Grödig, Austria) [34]. All samples were assayed in duplicate and H_2S was calculated using a calibration curve of sodium hydrosulphide (NaHS; 3.12–200 μM). The plasma H_2S concentrations were expressed as μM.

Assay of tissue H_2S synthesizing activity

H_2S synthesizing activity in pancreatic and lung homogenates was measured essentially as described elsewhere [3]. Briefly, pancreatic and lung tissue were homogenized in 1 ml of 100 mM ice-cold potassium phosphate buffer (pH 7.4). The reaction mixture (total volume, 500 μl) contained L-cysteine (20 μl, 10 mM), pyridoxyal 5′-phosphate (20 μl, 2 mM), saline (30 μl) and tissue homogenate (430 μl). The reaction was performed in tightly sealed microcentrifuge tubes and initiated by transferring the tubes from ice to a shaking water bath at 37°C. After incubation for 30 min, 1% w/v zinc acetate (250 μl) was added to trap evolved H_2S followed by 10% v/v trichloroacetic acid (250 μl) to denature the protein and stop the reaction. Subsequently, N,N-dimethyl-p-phenylenediamine sulphate (20 μM; 133 μl) in 7.2 M HCl was added, immediately followed by FeCl3 (30 μM; 133 μl) in 1.2 M HCl. The absorbance of the resulting solution at 670 nm was measured by spectrophotometry in a 96-well microplate reader. The H_2S concentration was calculated as described earlier. Results were then corrected for the DNA content of the tissue sample [15] and were expressed as nmoles H_2S formed/μg DNA.

Measurement of SP concentrations

Pancreas and lung samples were homogenized in 2 ml ice-cold assay buffer for 20 sec using Heidolph Diax 900 (Schwabach, Germany). The homogenates were centrifuged (13,000g, 20 min, 4°C) and the supernatants were collected. The supernatants were adsorbed on Sep-Pak C_{18} cartridge columns (Waters Associates, Milford, MA) as described [27]. The adsorbed peptides were eluted with 1.5 ml of 75% v/v acetonitrile. The samples were freeze-dried and reconstituted in assay buffer. SP content was then determined with an ELISA kit (Peninsula Laboratories, San Carlos, CA) according to the manufacturer's instructions and expressed as ng/μg of DNA for pancreas and lungs or ng/ml for plasma. SP can be measured in the range of 0–10 ng/ml in this assay.

RT-PCR

RT-PCR experiments were carried out as described previously (17). Total RNA from the pancreas and lungs was extracted with TRizol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol with modifications. Briefly, pancreatic or pulmonary tissues were isolated and rapidly homogenized in TRizol® reagent. Aqueous phase separation was carried out after adding chloroform and centrifugation at 12,000 × *g* for 15 min at 4°C. The aqueous layer was separated and the RNA was precipitated using isopropyl alcohol. After RNA was pelleted by centrifugation (12,000 × *g* for 10 min at 4°C), the pellet was washed in 70% v/v ethanol, air-dried and dissolved in RNase-free water. RNA was quantitated spectrophotometrically by absorbance at 260 nm (1 OD = 40 μg/ml). The purity of RNA was assessed by a 260/280 ratio between 1.6 and 2.0 and the integrity of RNA was verified by ethidium bromide staining of 18S and 28S rRNA bands on a denaturing agarose gel. Total RNA (1 μg) was reverse-transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) at 25°C for 5 min, 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 r18S, *NK-1R* and *PPT-A* were synthesized by Proligo (Singapore). The primers were intronspanning, such that genomic DNA contamination was excluded. cDNA synthesized from 1 μg total RNA was included in a typical PCR. 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 for 30 sec and 72°C for 30 sec. PCR amplification was performed in MyCycler™ (Bio-Rad). PCR products were analysed on 1% w/v agarose gels containing 0.05 mg/100 ml ethidium bromide and photographed using Gel Doc-It Imaging System (UVP). Product sizes were identified by comparison with DNA size standards included in the gels. Densitometry results from PCR products were normalized to 18S internal controls.

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 significance of differences between groups was evaluated by analysis of variance (ANOVA) when comparing three or more groups and the data were analysed by Tukey's method as a post hoc test for the difference between groups. A P value of <0.05 was considered to indicate a statistically significant difference.

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

Gene	Primer sequence	Optimal conditions	Size (bp)
r18S	Sense: 5'-GTAACCCGTTGAACCCCATT-3' Antisense: 5' -CCATCCAATCGGTAGTAGCG-3'	Lung: 22 cycles Pancreas: 22 cycles	150
NK-1R	Sense: 5'-CTTGCCTTTTGGAACCGTGTG-3' Antisense: 5'-CACTGTCCTCATTCTCTTGTGGG-3'	Lung: 35 cycles Pancreas: 42 cycles	501
PPT-A	Sense: 5'-ACCTGCTCCACTCCTGCACCGCGGCCAAG-3' Antisense: 5'-GAACTGCTGAGGCTTGGGTCTTCGGGCGAT-3'	Lung: 43 cycles Pancreas: 42 cycles	239

Results

Plasma H_2S concentration in acute pancreatitis and effect of PAG on H_2S concentrations

Figure 1A shows plasma H_2S concentrations measured after 10 hrs of saline/caerulein injections. The H_2S concentrations were significantly ($P < 0.05$) increased in caerulein-induced acute pancreatitis compared with saline-injected mice. Inhibition of endogenous H_2S synthesis by either prophylactic or therapeutic treatment of PAG resulted in significant ($P < 0.05$) reduction in plasma H_2S compared with caerulein group.

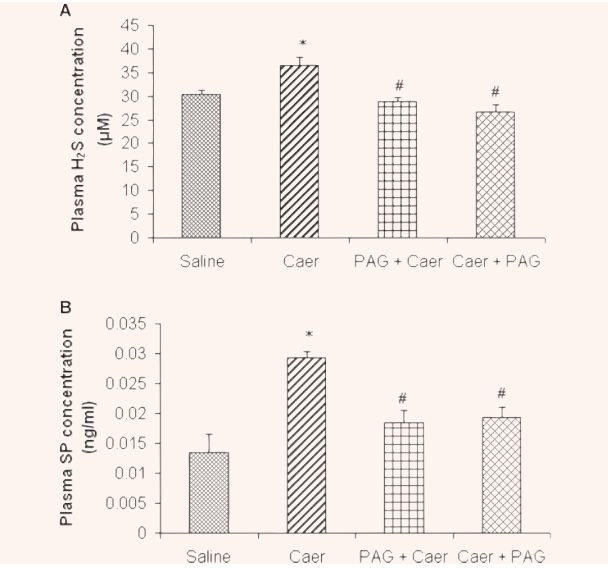

Fig. 1 Effect of propargylglycine (PAG) on plasma H_2S (**A**) and SP concentrations (**B**) in acute pancreatitis. Mice were given 10 hourly injections of caerulein (50 μg/kg, i.p.) to induce acute pancreatitis. PAG (100 mg/kg, i.p.) was administered either 1 hr before or 1 hr after the first caerulein injection. One hour after the last caerulein injection, plasma H_2S and SP concentrations were measured as described in Materials and methods. Results shown are the mean ± S.E.M. ($n = 8$–10 animals in each group). Asterisk (*): $P < 0.05$ when caerulein induced pancreatitis mice were compared with control mice. Hash (#): $P < 0.05$ when PAG-treated acute pancreatitis animals were compared with saline-treated acute pancreatitis animals. Abbreviations used: Caer: Caerulein

Plasma SP concentration in acute pancreatitis and effect of PAG on SP concentrations

As shown in Fig. 1B, caerulein-induced acute pancreatitis resulted in a significant increase (twofold increase, $P < 0.05$) in plasma concentrations of SP compared with saline-injected mice. Both prophylactic and therapeutic treatment of PAG resulted in significant ($P < 0.05$) reduction in plasma SP concentrations compared with caerulein group.

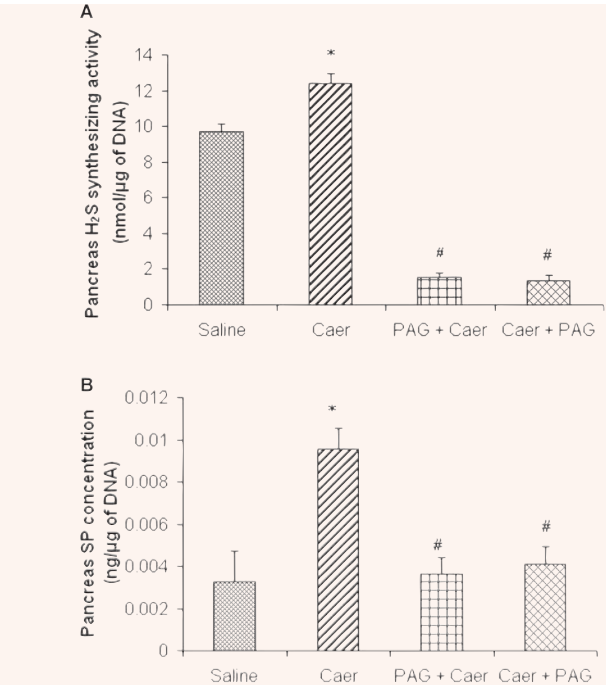

Fig. 2 Effect of PAG on pancreatic H_2S synthesizing activities (**A**) and SP concentrations (**B**) in acute pancreatitis. Mice were given 10 hourly injections of caerulein (50 μg/kg, i.p.) to induce acute pancreatitis. PAG (100 mg/kg, i.p.) was administered either 1 hr before or 1 hr after the first caerulein injection. One hour after the last caerulein injection, pancreatic H_2S synthesizing activities and SP concentrations were measured as described in Materials and Methods. Results shown are the mean ± S.E.M. (n = 8–10 animals in each group). Asterisk (*): $P < 0.05$ when caerulein-induced pancreatitis mice were compared with control mice. Hash (#): $P < 0.05$ when PAG-treated acute pancreatitis animals were compared with saline-treated acute pancreatitis animals. Abbreviations used: Caer: Caerulein

Effect of PAG treatment on pancreatic H_2S synthesizing activity

To determine whether the elevated level of plasma H_2S was due to high H_2S production in pancreas, we measured H_2S synthesizing activity in pancreas after 10 hrs of saline/caerulein injections. As shown in Fig. 2A, pancreatic H_2S synthesizing activity was significantly increased ($P < 0.05$) in caerulein-induced acute pancreatitis compared with saline-injected mice. PAG treatment, both prophylactic and therapeutic, resulted in a significant ($P < 0.05$) reduction in pancreatic H_2S synthesizing activity compared with caerulein group.

Effect of PAG treatment on pancreatic SP concentrations

To assess the effect of H_2S on pancreatic SP concentrations in acute pancreatitis, we measured SP concentrations in pancreas (Fig. 2B). Similar to high pancreatic H_2S synthesizing activity in acute pancreatitis, pancreatic SP concentration was also significantly increased (three fold increase, $P < 0.05$) in caerulein-induced acute pancreatitis compared with saline-treated mice. PAG treatment, both prophylactic and therapeutic, resulted in a significant ($P < 0.05$) reduction in pancreatic SP concentrations.

Effect of PAG treatment on pancreatic *PPT-A* mRNA expression

Pancreatic mRNA expression of *PPT-A* was determined using RT-PCR. Caerulein-induced acute pancreatitis resulted in a significant increase ($P < 0.05$) in expression of *PPT-A* mRNA in pancreas compared with saline-treated mice (Fig. 3A). Densitometry analysis of the PCR products on agarose gel shows twofold increase of *PPT-A* compared with control. PAG treatment, both prophylactic and therapeutic, resulted in significant ($P < 0.05$) reduction in *PPT-A* mRNA in pancreas.

Effect of PAG treatment on pancreatic *NK-1R* mRNA expression

Pancreatic mRNA expression of *NK-1R* was significantly increased ($P < 0.05$) in caerulein-induced acute pancreatitis (Fig. 3B). Densitometry analysis of the PCR products on agarose gel shows six-fold increase of *NK-1R*. PAG treatment, both prophylactic and therapeutic, resulted in significant (30% reduction, $P < 0.05$) reduction in *NK-1R* mRNA in pancreas.

Effect of PAG treatment on pulmonary H_2S synthesizing activity

In contrast to high H_2S synthesizing activities in pancreas, lung H_2S synthesizing activity was not affected by caerulein treatment compared with saline-injected mice (Fig. 4A). PAG treatment, either prophylactic or therapeutic, did not alter lung H_2S synthesizing activities. No significant differences in

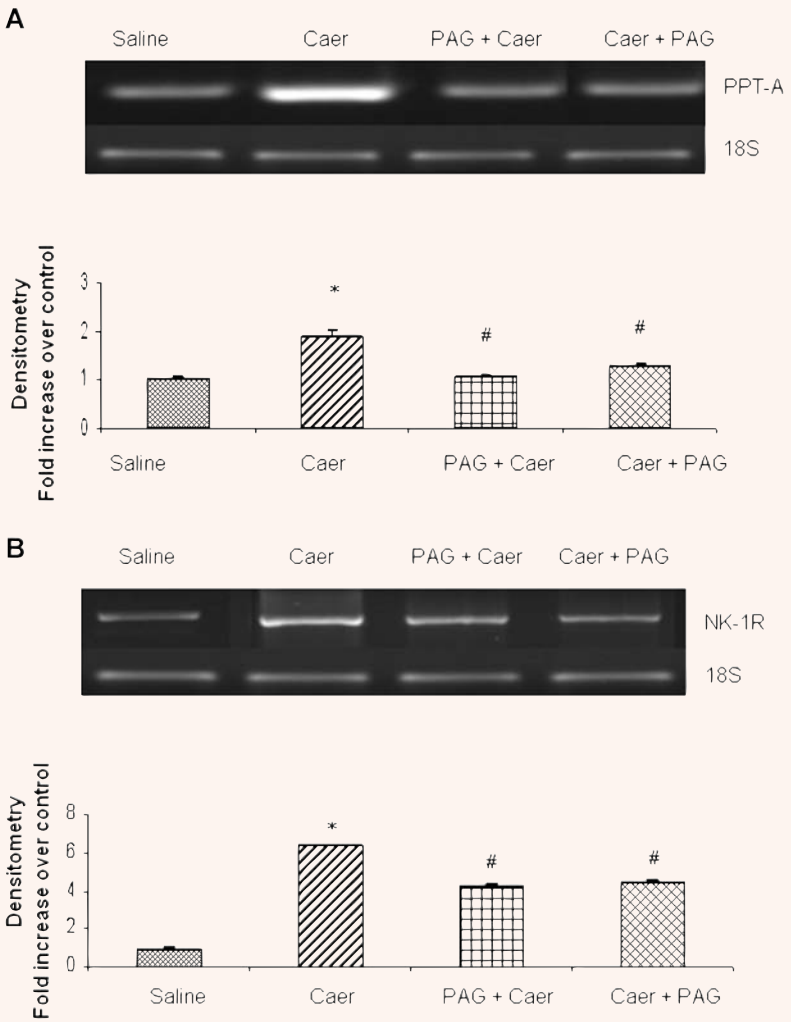

Fig. 3 Effect of PAG on pancreatic PPT-A (**A**) and NK-1R mRNA (**B**) expression in acute pancreatitis. Mice were given 10 hourly injections of caerulein (50 μg/kg, i.p.) to induce acute pancreatitis. PAG (100 mg/kg, i.p.) was administered either 1 hr before or 1 hr after the first caerulein injection. One hour after the last caerulein injection, pancreatic mRNA expression of PPT-A and NK-1R were carried out as described in Materials and methods. Histograms of results mRNA expression of PPT-A are the mean ± S.E.M. (n = 6 animals in each group). Asterisk (*): $P < 0.05$ when caerulein-induced pancreatitis mice were compared with control mice. Hash (#): $P < 0.05$ when PAG-treated acute pancreatitis animals were compared with saline-treated acute pancreatitis animals. Abbreviations used: Caer: Caerulein

pulmonary H_2S synthesizing activity were found between all four groups.

Effect of PAG treatment on pulmonary SP concentrations

Although there were no significant differences in pulmonary H_2S synthesizing activity between caerulein-induced acute pancreatitis and saline-injected mice, pulmonary SP concentrations were significantly increased (fivefold increase, $P < 0.05$) in caerulein-induced acute pancreatitis (Fig. 4B). PAG treatment, both prophylactic as well as therapeutic, unexpectedly resulted in significant ($P < 0.05$) reduction in pulmonary SP concentrations.

Effect of PAG treatment on pulmonary *PPT-A* mRNA expression

Caerulein-induced acute pancreatitis resulted in a significant increase ($P < 0.05$) in the expression of pulmonary *PPT-A* mRNA (Fig. 5A). Densitometry analysis of the

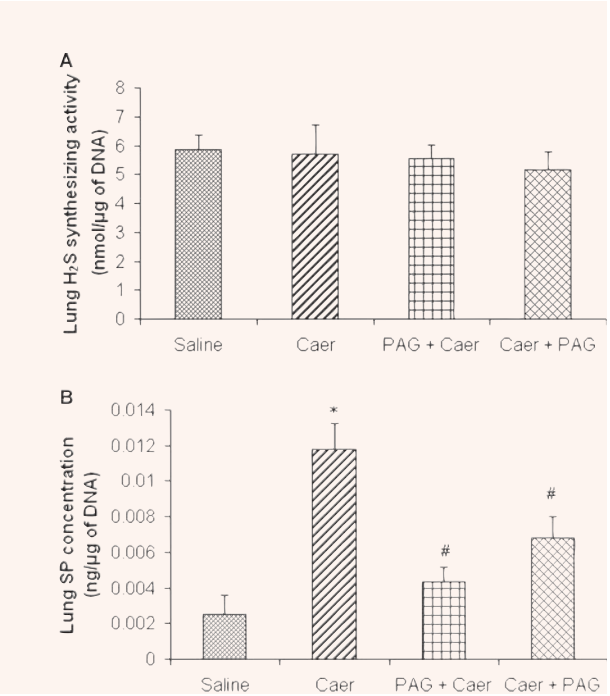

Fig. 4 Effect of PAG on pulmonary H_2S synthesizing activities (**A**) and SP concentrations (**B**) in acute pancreatitis. Mice were given 10 hourly injections of caerulein (50 µg/kg, i.p.) to induce acute pancreatitis. PAG (100 mg/kg, i.p.) was administered either 1 hr before or 1 hr after the first caerulein injection. One hour after the last caerulein injection, pulmonary H_2S synthesizing activities and SP concentrations were measured as described in Materials and methods. Results shown are the mean ± S.E.M. (n = 8–10 animals in each group). Asterisk (*): $P < 0.05$ when caerulein-induced pancreatitis mice were compared with control mice. Hash (#): $P < 0.05$ when PAG-treated acute pancreatitis animals were compared with saline-treated acute pancreatitis animals. Abbreviations used: Caer: Caerulein

PCR products on agarose gel shows twofold increase of *PPT-A* when compared with control. PAG treatment, both prophylactic and therapeutic, resulted in significant ($P < 0.05$) reduction in *PPT-A* mRNA in lungs.

Effect of PAG treatment on pulmonary *NK-1R* mRNA expression

Pulmonary mRNA expression of *NK-1R* was significantly increased ($P < 0.05$) in caerulein-induced acute pancreatitis (Fig. 5B). Densitometry analysis of the PCR products on agarose gel shows almost twelvefold increase of *NK-1R* in lungs of caerulein-treated animals when compared with saline-treated animals. PAG treatment, both prophylactic and therapeutic, resulted in significant ($P < 0.05$) reduction in *NK-1R* mRNA in lungs.

Discussion

H_2S has been recognized as a biologically active gaseous mediator in mammals. CBS and CSE are the key enzymes involved in H_2S synthesis. Both enzymes are pyridoxal phosphate dependent and are expressed in a range of mammalian cells and tissues. Although other enzymes can catalyse the production of H_2S, CBS seems to be the main H_2S-forming enzyme in the CNS whereas CSE is the main H_2S-forming enzyme in the cardiovascular system. Several research studies have demonstrated increased biosynthesis of H_2S in various animal models of inflammation, for example, acute pancreatitis, septic shock, endotoxic shock and carrageenan-induced hind paw oedema and suggested a pro-inflammatory role of H_2S in inflammation. Furthermore, pre-treatment with PAG, an irreversible inhibitor of CSE enzyme activity, reduced tissue H_2S formation in all these inflammatory models and exhibited marked anti-inflammatory effects [2–3, 19, 33–34]. Our recent *in vitro* study using pancreatic acinar cells has also shown that caerulein increased the levels of H_2S and CSE mRNA expression, indicating that CSE may be the main enzyme involved in H_2S formation in mouse pancreatic acinar cells [30].

SP is a major mediator of neurogenic inflammation in several tissues including skin [13], cardiovascular tissue [21], cephalic structures [9, 23], respiratory tract [7, 24], genitourinary tract [25] and gastrointestinal tract [8, 10, 21]. Our earlier results have demonstrated that plasma, lung and pancreatic concentrations of SP were increased in caerulein-induced acute pancreatitis [17]. We have earlier shown that *PPT-A* gene knockout mice and *NK-1R* knockout mice were protected against caerulein-induced acute pancreatitis and associated lung injury [4, 5]. These studies showed the pro-inflammatory role of SP in the pathogenesis of acute pancreatitis and associated lung injury [4–5, 17–18]. Other investigators also reported the role of *PPT-A* gene in polymicrobial sepsis [27], airway inflammation [14],

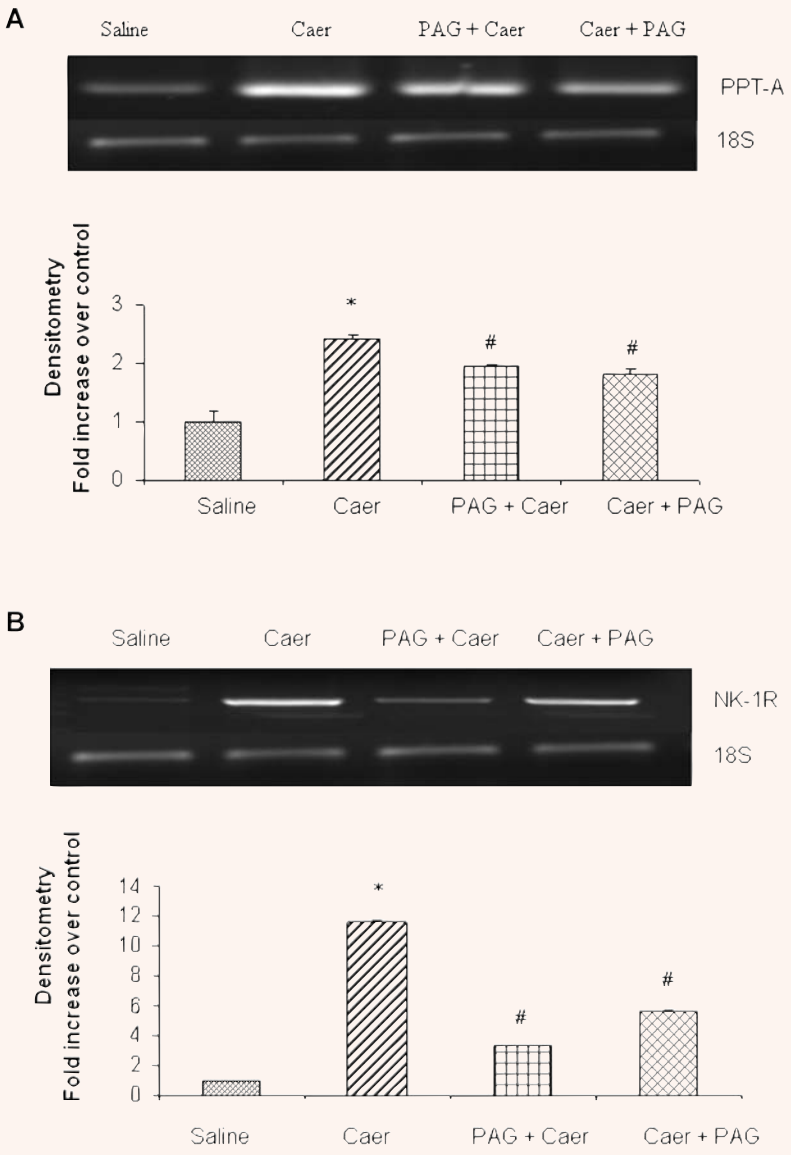

Fig. 5 Effect of PAG on pulmonary PPT-A (**A**) and NK-1R mRNA (**B**) expression, in acute pancreatitis. Mice were given 10 hourly injections of caerulein (50 μg/kg, i.p.) to induce acute pancreatitis. PAG (100 mg/kg, i.p.) was administered either 1 hr before or 1 hr after the first caerulein injection. One hour after the last caerulein injection, pulmonary mRNA expression of PPT-A and NK-1R were carried out as described in Materials and methods. Histograms of results mRNA expression of PPT-A shown are the mean ± S.E.M. (n = 6 animals in each group). Asterisk (*): $P < 0.05$ when caerulein-induced pancreatitis mice were compared with control mice. Hash (#): $P < 0.05$ when PAG-treated acute pancreatitis animals were compared with saline-treated acute pancreatitis animals. Abbreviations used: Caer: Caerulein

arthritis [16], cystitis [29] and inflammatory bowel disease [14]. Studies using either *NK-1R* antagonists or mice genetically deficient in the *NK-1R* have proven a role for this receptor in asthma and chronic bronchitis, intestinal inflammation and resistance to infection.

Similarly, both H_2S and SP play a key role in the pathogenesis of various forms of inflammation. Several reports suggest that H_2S stimulates sensory nerve endings, thereby releasing endogenous tachykinins, such as SP, calcitonin gene-related peptide (CGRP) and neurokinin A [26]. A previous study [6] from our group demonstrated that administration of NaHS (H_2S donor) to mice resulted in an increase in plasma SP concentration and lung inflammation. In SP deficient mice, the *PPT-A* knockout mice, H_2S did not cause any lung inflammation [6]. These results pointed to H_2S acting *via* SP in lung.

The present study was aimed to investigate pro-inflammatory effect of H_2S on SP in caerulein-induced

acute pancreatitis. Significantly higher concentrations of plasma H_2S (Fig. 1A) were found in caerulein-injected mice compared to saline-injected mice. This rise in H_2S was abolished in animal treated with PAG either prophylactically or therapeutically. Increased plasma H_2S concentration in caerulein-treated animals could be mainly due to increased H_2S synthesizing activity in pancreas as there was no change in H_2S synthesizing activity in lung (Figs 2A, 4A). However, PAG treatment significantly reduced the H_2S synthesizing activity in pancreas. These results are consistent with our previous results [2]. A recent *in vitro* study has shown that treatment of isolated pancreatic acinar cells with NaHS resulted in a significant increase in the production of SP and expression of *PPT-A* and *NK-1R* in acinar cells [30]. In the present study, there was a significant increase in plasma SP as well as plasma H_2S in caerulein-injected mice compared to saline-injected mice. Also, high concentrations of SP were found in pancreas and lung which was associated with the up-regulation of *PPT-A* and *NK-1R* mRNA expression. Furthermore, prophylactic or therapeutic treatment of PAG caused significant reduction in levels of plasma, pancreatic and lung SP and attenuated mRNA expression of *PPT-A* and *NK-1R*.

Our findings indicate that the elevated concentration of H_2S in acute pancreatitis seems to up-regulate the pancreatic and pulmonary expression of *PPT-A* gene and thereby leads to a substantial rise in the production of SP in pancreas and lung. Moreover, the increased H_2S concentration causes an up-regulation of pancreatic and pulmonary *NK-1R* gene expression. As it has been shown earlier, this increase in SP production and *NK-1R* gene expression in the pancreas and lungs leads to increased inflammation and tissue injury in the pancreas and lung as evidenced by hyperamylasemia, myeloperoxidase (MPO) activities and histological examination of the tissue injury [17–18]. In present study, although pulmonary H_2S synthesizing activity was not increased in caerulein group, it seems that increased plasma H_2S due to increased pancreatic H_2S synthesizing activity may up-regulate *PPT-A* and *NK-1R* mRNA expression in lung and thereby caused increased production of SP in lung. Although inhibition of CSE enzyme activity had no impact on pulmonary H_2S synthesizing activity, it caused significant reduction in pulmonary SP in acute pancreatitis (Fig. 4A and B). Likewise, inhibition of H_2S formation by PAG treatment decreased the levels of *PPT-A* gene expression and SP in lung whereas exogenous NaHS administration increased the pulmonary concentration of SP in caecal ligation and puncture (CLP)-operated sepsis [32].

Although the present study offers the possibility that H_2S may modulate the production of SP (and its receptor) at the gene level, the precise mechanism by which H_2S induces the transcription of *PPT-A* and *NK-1R* remains to be investigated. An earlier study using the polymicrobial sepsis model has shown H_2S up-regulates the production of pro-inflammatory mediators and exacerbates the systemic inflammation in sepsis through a mechanism involving NF-κB activation [35]. We have also shown that SP-induced chemokine synthesis in mouse pancreatic acinar cells is NF-κB dependent [28]. These results suggest that H_2S may modulate the production of SP through NF-κB activation.

Nevertheless, these results, for the first time, show a critical role of SP on the pro-inflammatory action of H_2S in acute pancreatitis.

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