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## ORIGINAL PAPER

# Gastric acid induces mucosal H₂S release in rats by upregulating mRNA and protein expression of cystathionine gamma lyase

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**Abstract** It is well known that hydrogen sulfide (H₂S) protects the gastric mucosa against gastric acid and other noxious stimulants by several mechanisms but until now the effect of gastric acid on H₂S production has not been evaluated. This study was performed to determine the effect of basal and stimulated gastric acid secretion on mRNA and protein expression of cystathionine gamma lyase (CSE) and cystathionine beta synthase (CBS), and on mucosal release of H₂S in rats. Seventy-two male rats were randomly assigned into 9 groups (8 in each)—control, distention, and pentagastrin-induced gastric acid secretion groups. The effects of 15 % alcohol solution, propargyl-glycine (PAG), ʟ-NAME, and pantoprazole were also investigated. Under anesthesia, animals underwent tracheostomy and midline laparotomy. A catheter was inserted into the stomach through the duodenum for gastric washout. At the end of the experiments, the animals were killed and the gastric mucosa was collected to measure H₂S concentration and to quantify mRNA expression of CSE and CBS by quantitative real-time PCR, and expression of their proteins by western blot. Basal and stimulated gastric acid secretion increased mucosal levels of H₂S, and mRNA and protein expression of CSE. Pantoprazole and ʟ-NAME reversed H₂S release and restored protein expression of CSE to the control level. Pantoprazole, but not propargylglycine, pretreatment inhibited the elevated level of protein expression of eNOS in response to distention-induced gastric acid secretion. Our findings indicated that NO mediated the stimulatory effect of gastric acid on H₂S release and protein expression of CSE.

**Keywords** Distention-induced gastric acid secretion · Cystathionine gamma lyase · H₂S · Rat

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## Introduction

There is increasing evidence that endogenous gaseous signaling molecules, including NO, CO, and H₂S, regulate physiological functions of mammalian tissues. Under physiological conditions, mucosal defensive barriers in the stomach protect the gastric mucosa against such aggressive compounds as gastric acid and pepsin. Recently, hydrogen sulfide has been shown to protect and maintain gastric mucosal integrity [1, 2] against physiological corrosive factors, including hydrochloric acid and pepsin, by stimulating mucus and bicarbonate secretion and by inhibiting gastric acid secretion [3–5]. It is also well documented that H₂S promotes the effect of the gastric mucosal defensive barrier against such irritants as ischemia and reperfusion [6, 7], ethanol [8], NSAID [9], and water immersion restraint stress-induced mucosal gastric ulcers [10] by increasing the gastric mucosal blood flow, stimulating bicarbonate secretion [3], reducing plasma levels of proinflammatory cytokines [7], increasing prostaglandin synthesis [4], and reducing the production of reactive oxygen species [6].

Moreover, endogenous and exogenous H₂S have been shown to stimulate duodenal secretion of bicarbonate in rats [3]. Mucosal acidification has been shown to stimulate HCO₃⁻ secretion concomitant with increasing PGE₂ and

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NO production, and these responses were mitigated by propargylglycine, a selective CSE inhibitor, implying that endogenous $H_2S$ is involved in acid-induced bicarbonate secretion in the rat duodenum [3]. In addition, pharmacological inhibition of the endogenous production of $H_2S$ by PAG has been reported to worsen acid-induced duodenal damage, showing that $H_2S$ stimulates $HCO_3^-$ secretion in the duodenum [11]. Last, exogenous, but not endogenous, $H_2S$ has been reported to induce bicarbonate secretion in the rat stomach, with involvement of NO, prostaglandins, and CGRP (calcitonin gene-related peptie) neurons [12].

As far as we are aware, there is no report of the effect of basal and stimulated gastric acid secretion on hydrogen sulfide release in the gastric mucosa of the rat. The purpose of this study was, therefore, to evaluate the effect of basal and stimulated (by distention and pentagastrin) gastric acid secretion on mucosal $H_2S$ release and on gene and protein expression of two key enzymes, CBS and CSE, which are involved in endogenous generation of $H_2S$, and to determine the involvement of NO in this effect.

## Materials and methods

### Animals

Male Wistar rats (200–250 g) were purchased from the animal house of Ahvaz Jundishapur University of Medical Sciences. The animals were fed on a conventional diet and had free access to tap water. They were maintained under standard conditions of humidity, temperature ($22 \pm 2$ °C), and 12-h light–dark cycle. The animals were deprived of food, but not water, overnight before experiments. All experiments were performed in accordance with regulations set by Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (PRC154).

### Animal grouping and experimental procedures

Seventy-two adult male Wistar rats were randomly assigned to 9 groups (8 in each), the control, and distention-induced and pentagastrin-induced gastric acid secretion groups. Effects of L-NAME, pantoprazole, and PAG treatment on 4 groups of animals were also investigated.

Under anesthesia induced by a mixture of ketamine and xylazine (60 + 15 mg/kg, respectively, i.p.), animals underwent tracheostomy and midline laparotomy. For gastric distention and gastric washout a catheter was inserted into the stomach through the duodenum and fixed in place by a ligature on the pylorus. Depth of anesthesia was checked throughout the experiment by use of the pedal withdrawal (toe pinch) reflex every 30 min. If the pedal withdrawal reflex was observed, a supplemental dose of

ketamine + xylazine (1/3 of the initial dose) was administered to maintain adequate anesthesia. Animal body temperature was measured with a rectal thermometer and maintained at 37 °C by use of a homeothermic blanket control system (Harvard, UK). At the beginning of each experiment, the lumen of the stomach was gently rinsed with isotonic saline (37 °C, pH 7) until gastric washout was clear. Thirty minutes after surgery, 1 ml normal saline was instilled into the stomach and 15 min later the gastric content was washed out and a new volume of normal saline was instilled through duodenal catheter into the stomach; this was repeated until the end of experiment. The acid content of each sample was measured with an autotitrator pH meter (Radiometer, Copenhagen, Denmark) by automatic potentiometric titration to pH 7 with NaOH (0.01 M); results were expressed as $\mu EqH^+/15$ min. Total acid output was expressed as $\mu EqH^+/90$ min (6 consecutive washouts).

In the first set of experiments, to evaluate the effect of stimulated gastric acid secretion on mucosal $H_2S$ release and mRNA and protein expression of CBS and CSE, 3 groups of animals were used. In the control group, 1 ml isotonic saline (pH 7 and 37 °C) was instilled into the stomach. In the distention-induced acid secretion group, acid secretion was stimulated by gastric distention (isotonic saline, 1.5 ml/100 g body weight, pH 7 and 37 °C) [13]. In the pentagastrin-induced acid secretion group, 1 ml of isotonic saline (pH 7 and 37 °C) was instilled into the stomach and gastric acid secretion was stimulated by a single dose of pentagastrin (20 µg/kg, i.p.).

In the second set of experiments, to investigate the effect of distention-induced gastric acid secretion on mucosal $H_2S$ release and mRNA and protein expression of CBS and CSE when the mucus gel layer is permeable to gastric acid [2], the stomach was distended by 15 % alcohol solution (1.5 ml/100 g body weight, pH 7 and 37 °C) to stimulate gastric acid secretion.

In the third set of experiments, to verify the stimulatory effect of gastric acid on mucosal $H_2S$ production and on mRNA and protein expression of CSE, one group of animals received 3 doses of the proton pump inhibitor, pantoprazole (50 mg/kg, i.p.), for 2 days before the experiment; the last dose of pantoprazole was given 60 min before gastric distention (isotonic saline, 1.5 ml/100 g body weight, pH 7 and 37 °C). This treatment was performed to ensure maximum inhibition of gastric acid secretion [14].

In the fourth set of experiments, to investigate the effect of nitric oxide in mediating the effect of distention-induced gastric acid secretion on mucosal $H_2S$ release and on CSE expression, two groups of rats received a single dose of $N^G$-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg, i.p.) [15] simultaneous with stimulation of gastric acid secretion

by distention (isotonic saline or 15 % alcohol solution, 1.5 ml/100 g body weight, pH 7 and 37 °C).

In the fifth set of experiments, to investigate the interaction between NO and $H_2S$, one group of rats was pretreated with a single dose of propargylglycine (50 mg/kg, i.p.) 30 min before stimulating gastric acid secretion by distention (isotonic saline, 1.5 ml/100 g body weight, pH 7 and 37 °C) and the level of protein expression of eNOS in response to distention-induced gastric acid secretion was compared for PAG-treated and untreated rats.

In the sixth set of experiments, to determine the effect of basal acid output on $H_2S$ production and gene and protein expression of CSE, one group of animals, control (pantoprazole-pretreated control; PC), received 3 doses of proton pump inhibitor, pantoprazole (50 mg/kg, i.p.), for 2 days before the experiment; the last dose of pantoprazole was given 60 min before the experiment. This treatment was performed to ensure complete inhibition of gastric acid secretion [14].

At the end of experiment, animals were killed by administration of an overdose of anesthetic. Their stomachs were removed, opened along the greater curvature, rinsed with physiological saline, and pinned out on paraffin covered with ice-cold saline. Two samples of gastric mucosal tissue were quickly excised, snap-frozen and stored in liquid nitrogen. One sample was used for measurement of mRNA expression of CSE and CBS by quantitative real-time PCR and expression of their proteins by western blot. Another sample was used for determination of mucosal $H_2S$ release by use of an ELISA kit.

## Quantitative real-time PCR

The mRNA levels of CBS, CSE, eNOS, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by quantitative real-time PCR (qRT-PCR) by use of step-one systems (Applied Biosystems, USA). Specific primers (Bioneer, South Korea) were used for measurement of CBS, CSE, eNOS, and GAPDH; the lengths of the amplified products were: GAPDH, 5′-TGCTGGTGCTGAGTATGTCGTG-3 and 5′-CGGAGAT GATGACCCTTTTGG-3′, 101 bp; CSE, 5′-TGTTGT CATGGGCTTAGTG-3′ and 5′-CCATCCCATTCCTGAA GTG-3, 167 bp; CBS, 5′-CTGTGAAGGGCTATCGCTG C-3′ and 5′-CTGGCATTGCGGTACTGGTC-3′, 205 bp; and eNOS, 5′-TCCGATTCAACAGTGTCTCCT-3′ and 5′-ACAGAAGTGCGGGTATGCTC-3′, 251 bp. All PCR amplifications were performed in duplicate reactions and in final volume of 20 μl containing 2 μl cDNA, 50 nm of the specific primers, and 10 μl master mix SYBR green (2 × SYBR® Green PCR Master Mix; Applied Biosystems, USA) by use of the procedure: incubation at 95 °C for 10 min to activate DNA Taq polymerase and 40 two-step

cycles with denaturation at 95 °C for 20 s, and annealing and extension at 60 °C for 1 min. In addition, the no-template negative control ($H_2O$) was routinely run in every PCR. The melting curve was examined at the end of the amplification process to ensure the specificity of the PCR products. The purity of each amplicon for each reaction was further confirmed by agarose gel electrophoresis. Expression levels of the CBS, CSE, and eNOS, genes were normalized against GAPDH expression (internal calibrator for equal RNA template loading and normalization). For relative quantification of gene expression, the comparative cycle of threshold (Ct) method with arithmetic formulae ($2^{-\Delta\Delta Ct}$) was used.

## Protein extraction

Frozen mucosal tissue was extracted with RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS) containing protease inhibitor cocktail (Complete mini; Roche, Indianapolis, IN, USA).To analyze the protein fraction, protein pellets obtained from gastric mucosa by use of RIPA buffer were resuspended in 1 % SDS. The total recovery and integrity of these fractions were determined by Bradford assay and SDS-polyacrylamide gel electrophoresis.

## Western blot analysis

Mucosal proteins were separated by SDS-PAGE on 10 % acrylamide gels and were transferred on to a nitrocellulose membrane. The membranes were blocked with 5 % non-fat dried milk dissolved in tris-buffered saline with 0.1 % Tween 20 (TBST, pH 7.6) for 6 h and then incubated overnight at 4 °C with anti-CBS antibody (mouse monoclonal, dilution 1:200; Santa Cruz Biotechnology; SC-133154), anti-CSE antibody (rabbit polyclonal, dilution 1:150; Santa Cruz Biotechnology; SC-135203), anti-eNOS antibody (rabbit polyclonal, dilution 1:250; ab66127), or anti-beta actin antibody (mouse monoclonal, dilution 1:5000; Abcam (ab20272), USA) was added to the membrane. After five washes with TBST, membranes were incubated with a rabbit polyclonal secondary antibody to mouse IgG HRP; dilution 1:7000) for 90 min at room temperature. Labeled proteins were detected by use of a chemiluminescence western blotting system. Expression of the proteins studied was semi-quantified by use of Image J image-analysis software and the values were normalized to β-actin.

## Determination of mucosal $H_2S$ levels

To investigate the effect of basal and stimulated gastric acid on the mucosal $H_2S$ production and release, we

performed an enzyme-linked immunosorbent assay (ELISA) by use of a commercial ELISA kit (ABIN771902, antibodies-online, USA) in accordance with the manufacturer's instructions.

### Statistical analysis

Data are shown as mean ± SEM. Statistical analysis was performed by one-way ANOVA and followed by the post-hoc Tukey test. Significance was set at $P < 0.05$.

## Results

### Effect of gastric distention by saline, 15 % alcohol solution, pentagastrin, and pantoprazole on total acid output

As illustrated in Table 1, the total acid content of gastric effluents in the distention (saline and 15 % alcohol solutions) and pentagastrin-induced gastric acid secretion groups was significantly increased compared with the control group ($P < 0.001$). The total acid content of gastric effluents in the distention-induced gastric acid secretion group pretreated with pantoprazole was significantly lower than in the distention (saline or 15 % alcohol solutions) and pentagastrin-induced gastric acid secretions groups ($P < 0.001$). The acid output in response to pentagastrin was significantly higher than in response to gastric distention (saline or 15 % alcohol solution) ($P < 0.05$). Pretreatment with 3 doses of pantoprazole completely inhibited basal acid secretion.

### Effect of distention (by saline and 15 % alcohol solutions), and pentagastrin-induced gastric acid secretions on mucosal $H_2S$ release and involvement of NO

As shown in Fig. 1, analysis of ELISA results showed that mucosal release of $H_2S$ in response to distention and pentagastrin-induced gastric acid secretion was significantly increased compared with basal gastric acid secretion ($P < 0.001$). These levels in response to distention-induced gastric acid secretion by 15 % alcohol solution and pentagastrin-induced gastric acid secretion were significantly higher than in distention-induced acid secretion by saline ($P < 0.05$). Acid inhibition by pantoprazole pretreatment inhibited the stimulatory effect of basal and distention-induced gastric acid secretion on mucosal $H_2S$ release. The stimulatory effect of distention (by saline and alcoholic solutions)-induced gastric acid secretion on mucosal $H_2S$ release was also prevented by L-NAME. The lowest level of $H_2S$ concentration was detected in PAG-treated rats.

### Effect of distention (by saline and 15 % alcohol solutions) and pentagastrin-induced gastric acid secretions on mucosal mRNA and protein expression of CBS

As shown in Fig. 2a, b, both mRNA and protein of CBS were expressed in gastric mucosa in the control and distention (by saline and 15 % alcohol solutions) and pentagastrin-induced gastric acid secretion groups. Analysis of qRT-PCR and western blot results revealed no significant differences between control and experimental groups.

**Table 1** Effect of distention (saline and 15 % alcohol solutions) and pentagastrin-induced gastric acid secretion, and of pantoprazole pretreatment, on total acid output in male Wistar rats

| Group | Total acid content (mEqH$^+$/90 min) of gastric effluents | Instilled volume (6 consecutive instilled volumes combined) | Collected volume (6 consecutive washout volumes combined ) |
|---|---|---|---|
| Control | 20.12 ± 1.8 | 6 ± 0.00 | 6.7 ± 0.2 |
| Distention (saline) | 92 ± 7.2*** | 20.5 ± 0.5 | 23.5 ± 0.5 |
| Distention (15 % alcohol solution) | 93.5 ± 5.8*** | 20.7 ± 0.6 | 24.1 ± 0.4 |
| Pentagastrin (20 μg/kg) | 131 ± 6***$^\beta$ | 6 ± 0.00 | 9.1 ± 0.3 |
| PPI + Dis (pantoprazole-pretreated distention-induced gastric acid secretion) | 13 ± 1.2$^\alpha$ | 20.1 ± 0.2 | 20.5 ± 0.35 |
| PPI + C (pantoprazole-pretreated control group) | 0 | 6 ± 0.00 | 6 ± 0.1 |

Data are expressed as mean ± SEM

$^\beta$ $P < 0.05$, significant increase compared with distention-induced gastric acid secretion group

*** $P < 0.001$ versus control rats

$^\alpha$ $P < 0.001$ versus distention (saline and 15 % alcohol solutions) and pentagastrin-induced gastric acid secretion groups
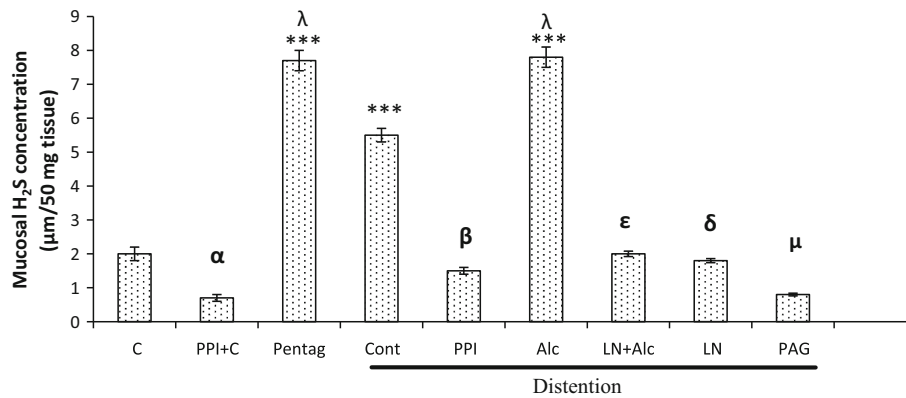
**Fig. 1** Effect of distention (by saline and 15 % alcohol solutions) and pentagastrin-induced gastric acid secretion on the gastric mucosal release of H₂S. *C* control, *PPI + C* pantoprazole-pretreated control rats, *Cont* distention-induced gastric acid secretion group, *Pentag* pentagastrin-induced acid secretion group, *Alc* gastric acid secretion was stimulated by distention by use of 15 % alcohol solution, *PPI* proton pump inhibitor, *pantoprazole* pantoprazole-pretreated distention-induced gastric acid secretion animals, *LN + Alc* and *LN + Dis* rats administered a single dose of L-NAME (10 mg/kg, i.p.) concomitant with gastric distention with 15 % alcohol solutions or with physiological saline, *PAG* propargylglycine-treated rats.

Analysis of ELISA results showed that basal and stimulated gastric acid secretion increase mucosal release of H₂S. PAG, pantoprazole, and that L-NAME inhibited this increase. $^{\alpha}P < 0.05$ versus control group; $^{\beta}P < 0.001$ significant decrease compared with distention and pentagastrin-induced gastric acid secretion groups; $***P < 0.001$ significant increase compared with control rats; $^{\lambda}P < 0.05$ significant increase versus distention-induced gastric acid secretion group; $^{\varepsilon}P < 0.001$ versus 15 % alcohol solution group; $^{\delta}$ and $^{\mu}P < 0.001$ versus distention-induced gastric acid secretion. Results are expressed as mean ± SEM

## Effect of distention (by saline and 15 % alcohol solutions) and pentagastrin-induced gastric acid secretions on mucosal mRNA expression of CSE

As illustrated in Fig. 3, analysis of qRT-PCR results showed the level of mRNA expression of CSE in distention (by saline and 15 % alcohol solutions) and pentagastrin-induced gastric acid secretion groups was significantly higher than in control rats ($P < 0.01$ and $P < 0.001$, respectively). The level of mRNA expression of CSE in response to distention-induced gastric acid secretion by 15 % alcohol solution and in response to pentagastrin-induced gastric acid secretion was significantly higher than in distention-induced acid secretion by saline ($P < 0.05$). Pantoprazole pretreatment reversed the level of mRNA expression of CSE in the distention-induced group to control level. The up-regulatory effect of distention-induced gastric acid secretion on mucosal mRNA expression of CSE was not affected by L-NAME treatment (Fig. 3). The basal level of mRNA expression of CSE was totally inhibited by pantoprazole pretreatment (Fig. 3).

## Effect of distention (by saline and 15 % alcohol solutions) and pentagastrin-induced gastric acid secretion on mucosal protein expression of CSE

As shown in Fig. 4, analysis of western blot results showed protein expression of CSE increased significantly in response to pentagastrin and distention-induced gastric

acid secretion compared with control rats ($P < 0.01$ and $P < 0.001$, respectively). The level of protein expression of CSE in response to distention-induced gastric acid secretion by 15 % alcohol solution and in response to pentagastrin-induced gastric acid secretion was significantly higher than in distention-induced acid secretion by saline ($P < 0.05$). Pantoprazole and L-NAME treatment prevented the up-regulatory effects of distention-induced gastric acid secretion on protein expression of CSE. Basal level of protein expression of CSE was completely inhibited by pantoprazole pretreatment (Fig. 4).

## Effect of distention (by saline and 15 % alcohol solutions)-induced gastric acid secretion and propargylglycine on mucosal mRNA and protein expression of eNOS

As shown in Figs. 5 and 6, analysis of qRT-PCR and western blot results showed mRNA and protein expression of eNOS were significantly increased in response to distention-induced gastric acid secretion by isotonic saline and by 15 % alcohol solution compared with the control group ($P < 0.01$). Gene and protein expression of eNOS in response to gastric distention by 15 % alcohol solution was significantly higher than in response to gastric distention by isotonic solution ($P < 0.05$). Pretreatment with pantoprazole inhibited the up-regulatory effect of distention-induced gastric acid secretion on protein expression of eNOS. The increased level of protein expression of eNOS
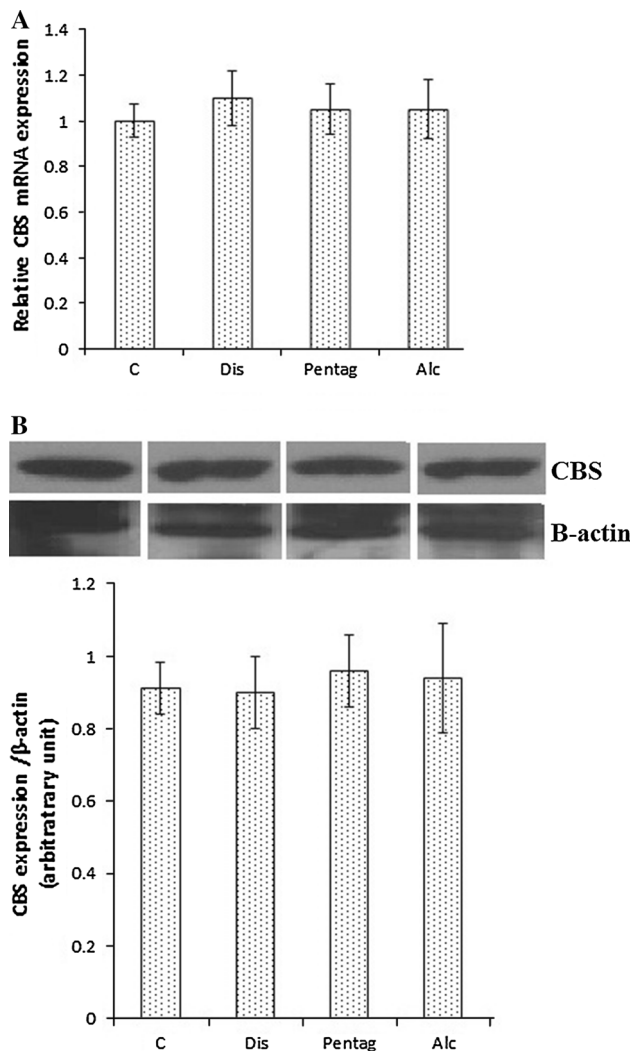
Fig. 2 Effect of distention (saline and 15 % alcohol solutions) and pentagastrin-induced gastric acid secretion on mRNA and protein expressions of cystathionine beta synthase (CBS) in gastric mucosa. Analysis of qRT-PCR (a) and western blot (b) results showed that the level of mRNA and protein expression of CBS in control and experimental groups were equal. Results are expressed as mean ± SEM

in response to distention-induced gastric acid secretion was not affected by PAG pretreatment.

## Discussion

The findings of this study indicated that:

1. distention and pentagastrin-induced gastric acid secretion increased mucosal $H_2S$ release and up-regulated mRNA and protein expression of CSE in the gastric mucosa of rat;
2. the increased level of CSE mRNA and protein expression and $H_2S$ release in response to distention-

induced gastric acid secretion was returned to the control level by pantoprazole pretreatment;
3. distention-induced gastric acid secretion (by isotonic saline and 15 % alcohol solutions) increased mRNA and protein expression of eNOS;
4. the increased level of protein expression of eNOS in response to distention-induced gastric acid secretion was returned to the control level by pantoprazole pretreatment;
5. up-regulation of protein expression of CSE and increased mucosal release of $H_2S$ by distention-induced gastric acid secretion was inhibited by L-NAME treatment;
6. pretreatment with propargylglycine did not affect the increased level of protein expression of eNOS in response to distention-induced gastric acid secretion; and
7. basal levels of gene and protein expression of CSE were completely inhibited by pantoprazole pretreatment.

This study showed, for the first time, that the mucosal $H_2S$ production significantly increased in response to stimulation of gastric acid secretion by pentagastrin and distention (saline or 15 % alcohol solutions). The inhibition of acid secretion by pantoprazole pretreatment prevented this increase. These findings showed that this response is dependent on gastric acidity, and thus that stimulated gastric acid secretion increases mucosal $H_2S$ production. These results also showed that L-NAME treatment prevented $H_2S$ response to distention-induced gastric acid secretion. Therefore, NO is involved in this response. These results showed that any decrease in intragastric pH stimulated mucosal $H_2S$ synthesis. These results also showed that the basal $H_2S$ release was significantly reduced but not completely inhibited by pantoprazole pretreatment, which suggests that this amount of hydrogen sulfide is produced by activation of other enzymatic pathways of $H_2S$ production, for example CBS [16].

Analysis of quantitative real-time PCR results in this study indicated that mRNA expression of CSE increased in response to distention (both by isotonic saline and by 15 % alcohol solutions) and pentagastrin-induced gastric acid secretion. Pantoprazole pretreatment but not L-NAME inhibited the CSE elevation in response to distention-induced gastric acid secretion. Therefore, these findings show that mRNA expression of CSE is upregulated in response to gastric acid but not to gastric distention. The results of this study also indicated that pantoprazole pretreatment completely inhibited the basal level of mRNA expression of CSE in pantoprazole-pretreated control rats. This is strongly implies that both stimulated and basal gastric acid secretion upregulated CSE transcription. Taken together
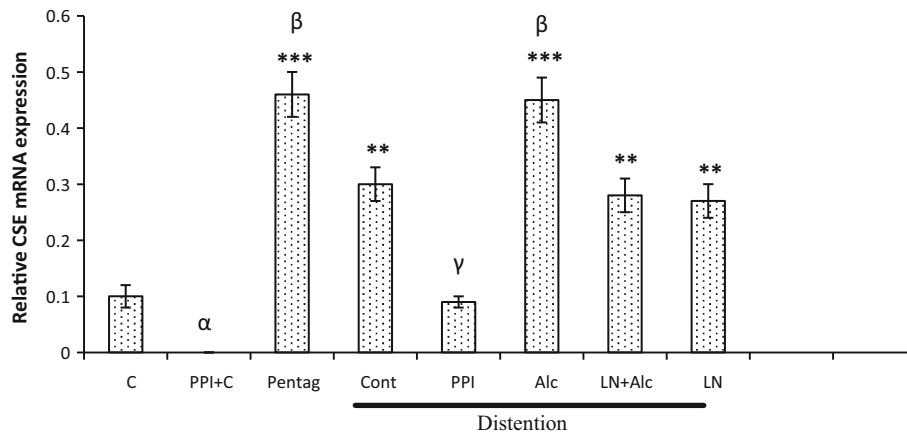
**Fig. 3** Effect of distention (saline and 15 % alcohol solutions) and pentagastrin-induced gastric acid secretion on mRNA expression of cystathionine gamma lyase (CSE) in gastric mucosa. Analysis of qRT-PCR results showed that distention and pentagastrin-induced gastric acid secretion upregulated gene expression of CSE. Pantoprazole but not L-NAME inhibited this increase. $^{\alpha}P < 0.05$ significant decrease compared with control group; $^{**}P < 0.001$ and $^{***}P < 0.01$ versus control rats; $^{\beta}P < 0.05$ significant increase compared with distention-induced gastric acid secretion group; $^{\gamma}P < 0.01$ versus distention and pentagastrin-induced gastric acid secretion groups. Results are expressed as mean ± SEM
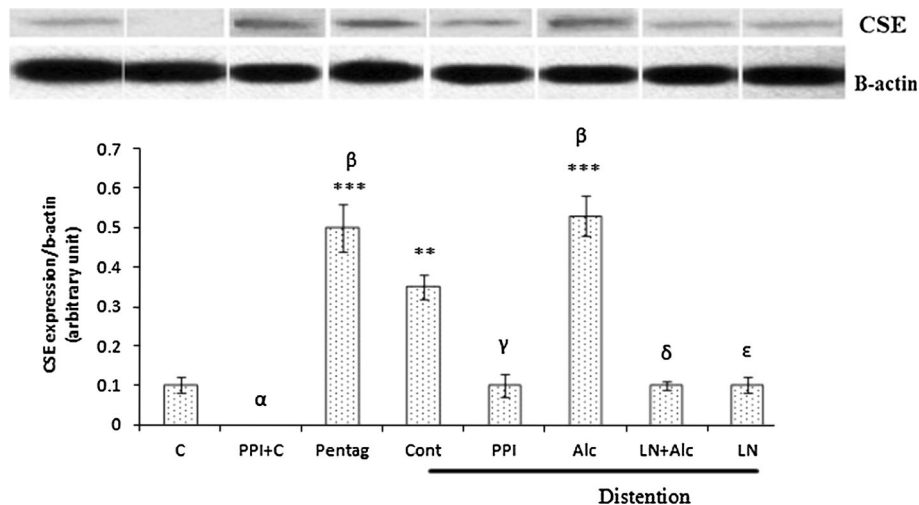


**Fig. 4** Effects of distention (saline and 15 % alcohol solutions) and pentagastrin-induced gastric acid secretion on protein expression of cystathionine gamma lyase (CSE) in gastric mucosa. Analysis of western blot results showed that distention and pentagastrin-induced gastric acid secretion upregulated protein expression of CSE. Pantoprazole and L-NAME inhibited this increase. $^{\alpha}P < 0.001$ significant decrease compared with control group; $^{***}P < 0.001$ and $^{**}P < 0.01$ versus control rats; $^{\beta}P < 0.05$ significant increase compared with distention-induced gastric acid secretion group; $^{\gamma}$ and $^{\varepsilon}P < 0.01$ significant decrease compared with distention-induced gastric acid secretion group; and $^{\delta}P < 0.01$ significant decrease compared with distention-induced gastric acid secretion using 15 % alcohol solution group. The protein levels were normalized to β-actin. Results are expressed as mean ± SEM

these findings showed that increased production of $H_2S$ in response to basal and stimulated gastric acid secretion is largely mediated by up-regulating mRNA expression of CSE.

The western blot results showed that protein expression of CSE increased in response to distention (both by isotonic saline and by 15 % alcohol solutions) and pentagastrin-induced gastric acid secretion. Therefore, these findings revealed that gastric acid, in addition to up-regulation of the gene expression of CSE, increased its protein

expression; they also revealed that up-regulation of protein expression of CSE in response to stimulated gastric acid secretion is another mechanism causing the mucosal increase of $H_2S$. These findings also revealed that pantoprazole pretreatment restored the increased level of protein expression of CSE in response to distention-induced gastric acid secretion to the control level and completely inhibited the basal level of protein expression of CSE in control rats. This again shows that both stimulated and basal gastric acid secretion upregulated protein synthesis of CSE.
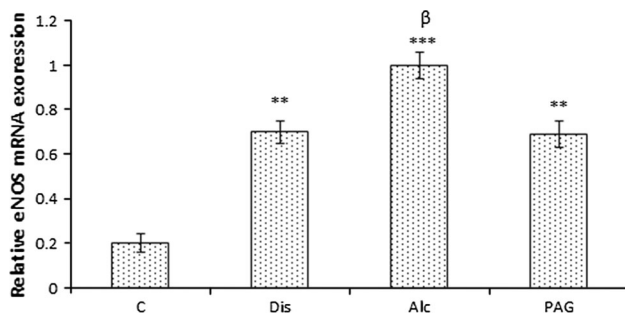
**Fig. 5** Effects of distention (by saline and 15 % alcohol solutions)-induced gastric acid secretion on mRNA expression of endothelial nitric oxide synthase (eNOS) in the gastric mucosa in rats. Analysis of qRT-PCR results showed that distention-induced gastric acid secretion upregulated mRNA expression of eNOS. **$P < 0.01$ and ***$P < 0.001$ versus control rats; $^\beta P < 0.05$ significant increase compared with distention-induced gastric acid secretion group. Results are expressed as mean ± SEM

Therefore, these findings indicated that basal production of $H_2S$ is partly mediated through the stimulatory effect of basal acid secretion on mRNA and protein expression of CSE.

The results also showed that L-NAME administration inhibits CSE protein expression in response to distention (both by isotonic saline and by 15 % alcohol solutions)-induced gastric acid secretion. Inhibition of protein expression of CSE but not its mRNA by L-NAME treatment implied that protein expression of CSE is activated in the presence of nitric oxide. Consistent with these results, NO has been established to act as a signaling molecule and a regulator of mRNA translation [17, 18]. These results together showed that translation of mRNA of CSE is activated by NO [18].

These findings showed that levels of mRNA and protein expression of eNOS increased in response to distention-induced gastric acid secretion by isotonic and by 15 % alcohol solutions. This finding is in agreement with previous work which showed that stimulated gastric acid increased NO release [15]. Our results showed that acid inhibition by pantoprazole pretreatment inhibited the increased level of protein expression of eNOS in response to distention-induced gastric acid secretion. Therefore, these results indicated that NO production and release depend on gastric acid. Our findings also showed that gastric acid in addition to activation of eNOS, as reported elsewhere [15], up-regulated gene and protein expression of eNOS, as shown by this study.

These findings also showed that acid-induced $H_2S$ release and CSE protein expression were inhibited by L-NAME treatment whereas PAG pretreatment did not affect
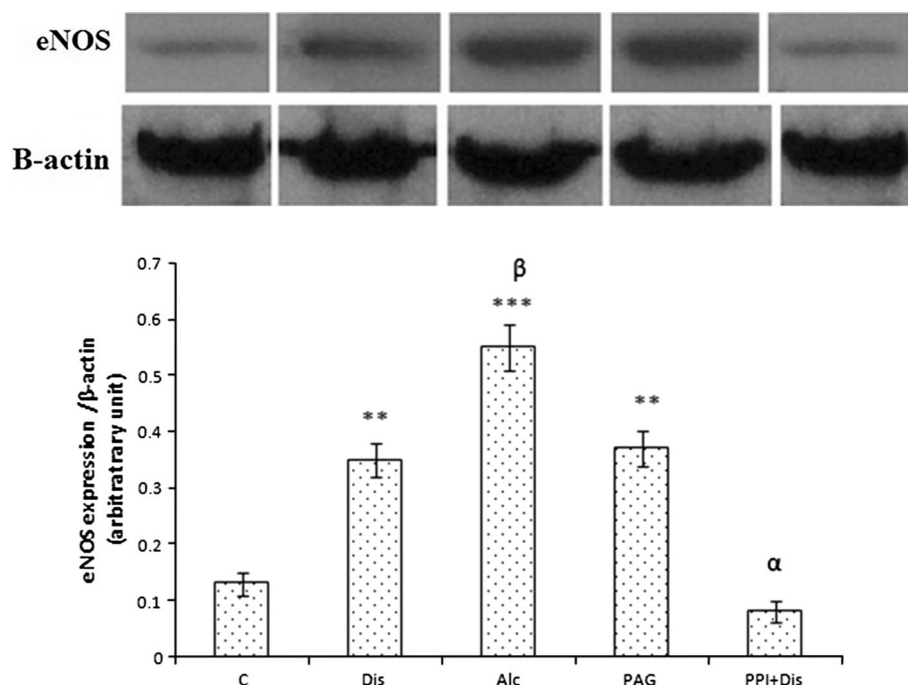


**Fig. 6** Effect of distention (by saline and 15 % alcohol solutions)-induced gastric acid secretion and propargylglycine on protein expression of endothelial nitric oxide synthase (eNOS) in the gastric mucosa in rats. Analysis of western blot results showed that distention-induced gastric acid secretion upregulated protein expression of eNOS. Pantoprazole pretreatment but not propargylglycine inhibited the increased level of protein expression of eNOS in response to distention-induced gastric acid secretion. **$P < 0.01$ and ***$P < 0.001$ versus control rats; $^\beta P < 0.05$ significant increase compared with distention-induced gastric acid secretion group; and $^\alpha P < 0.01$ significant decrease compared with distention-induced gastric acid secretion group. Results are expressed as mean ± SEM. The protein levels were normalized to $^\beta$-actin

the increased level of protein expression of eNOS in response to distention-induced gastric acid secretion. As shown in Fig. 6, there is no difference between the level of protein expression of eNOS in response to distention-induced gastric acid secretion in PAG-treated and untreated rats, which verified that NO production depends on gastric acid but not on hydrogen sulfide, implying that CSE expression and $H_2S$ production are activated by NO. $H_2S$ and its precursor, NaHS, have been shown to increase NO release from vascular endothelium and duodenal lumen in rats [3, 19] whereas our results showed a reverse effect, NO induced hydrogen sulfide release in gastric mucosa in rats. These findings together indicate there is an interaction between these two signaling molecules.

This study also showed that after acid inhibition by pantoprazole pretreatment, levels of protein expression of eNOS and CSE returned to control levels. Therefore, these results strongly showed that gastric acid, by up-regulating protein expression of eNOS and its activity, increased mucosal CSE protein synthesis and $H_2S$ release. In the other words, to produce mucosal $H_2S$, the presence of NO is necessary but NO is produced irrespective of the presence of hydrogen sulfide.

As shown in Figs. 5 and 6, eNOS expression when acid secretion was induced by 15 % alcohol solution increased significantly compared with distention-induced gastric acid secretion using isotonic solution. This response might have occurred because of the increased permeability of the mucous gel layer to gastric acid as a result of application 15 % alcohol solution [2]. In addition, more eNOS expression in the 15 % alcohol solution group probably led to more CSE expression and $H_2S$ production in these rats than in distention (saline)-induced gastric acid secretion.

As already indicated, the findings of this study showed that luminal acidity induced by pentagastrin and gastric distention upregulated eNOS and CSE expression. A possible underlying mechanism of this response may be the effect of luminal acidity on sensory afferent neurons. Luminal acidity has been shown to activate CGRP afferent neurons which, in turn, activate the vascular bed via a nitric oxide-dependent pathway [20]. It has also been shown that activation of CGRP neurons by capsaicin increased mRNA expression of eNOS in the gastric mucosa in rats [21]. The nitric oxide donor sodium nitroprusside has been shown to increase the activity and expression of CSE [22]. Consistent with a previous finding [22], our results also showed that nitric oxide increased the activity and expression of CSE. Therefore, these results together suggest that luminal acidity by activation of CGRP neurons upregulated eNOS and CSE expression.

As suggested in Fig. 7, exposure of gastric lumen to acid activates sensory afferent nerves underlying the epithelium (CGRP afferent neurons). Activation of these neurons

results in release of CGRP that can induce nitric oxide production in both mucosal vascular endothelium and nitrergic nerves which, in turn, can affect the mucosal epithelial cells and lead to $H_2S$ generation.

The results of this study showed that CBS and CSE are expressed in the gastric mucosa of rats. Consistent with these results, it has been shown that both CBS and CSE are expressed in the gastric mucosa in rats, but the main enzyme responsible for $H_2S$ production is CSE [9]. There is evidence that CBS is a constitutive enzyme and that its expression and activity are not affected by acetylsalicylic acid or ethanol [9, 23]. Our results are also in agreement with previous findings [9, 17] that mRNA and protein expression of CBS did not change in response to distention and pentagastrin-induced gastric acid secretion. Therefore, these findings indicated that the rate of CBS production in gastric mucosa is independent of changes in intra-gastric pH. Taken together, these observations suggest that CBS is a constitutive enzyme.

What is the physiological significance of these results? After this study we concluded that under physiological conditions any increase in gastric acid secretion or any decrease in intra-gastric pH is a result of increased mucosal $H_2S$ production whereas in non-physiological situations, for example NSAID-induced gastritis, the increased acid output was not associated with $H_2S$ release, as shown by Fiorucci et al. [9]. These findings also show that mucosal defensive factors are active in response to physiological irritants such as gastric acid. In conclusion, our findings showed that basal and stimulated gastric acid secretion increased mucosal $H_2S$ production by up-regulating mRNA and protein expression of CSE in the gastric mucosa of rats. This response is mediated by the involvement of nitric oxide.
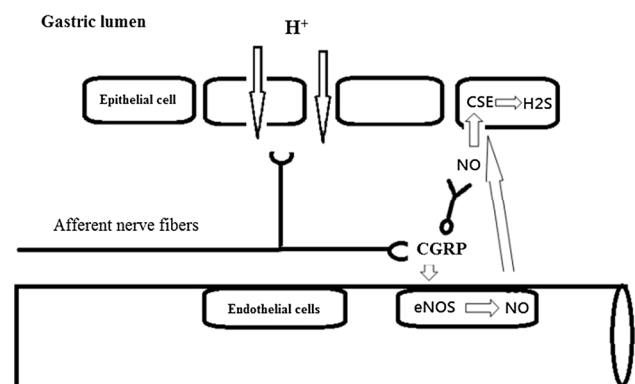


**Fig. 7** Possible mechanisms underlying upregulation of eNOS and CSE in response to distention and pentagastrin-induced gastric acid and the effect of endogenous NO in the regulation of this phenomenon

**Compliance with ethical standards**

**Conflict of interest** All authors declare that they have no conflicts of interest.

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