Duodenal acidification stimulates gastric H\textsubscript{2}S release through upregulating mRNA expression of cystathionine gamma lyase

Seyyed Ali Mard\textsuperscript{*1}, Hajar Godarzinejad\textsuperscript{1}, Mahin Dianat\textsuperscript{2}

1. Physiology Research Center (PRC), Research Center for Infectious Diseases of Digestive System, Department of Physiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
2. Physiology Research Center (PRC), Department of Physiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Abstract

Introduction: It has been reported the alkaline response of pancreas to duodenal acidification is partly mediated through duodenal release of H\textsubscript{2}S, but till now the effect of duodenal acidification on gastric H\textsubscript{2}S release has not been investigated. Therefore, the present study designed to evaluate the effects of duodenal acidification on gastric H\textsubscript{2}S release and level of mRNA expression of cystathionine gamma lyase (CSE).

Methods: Twenty four rats were randomly assigned into 3 groups (8 in each). They were control, pH2- and pH3-treated groups. Under anesthesia, animals underwent midline laparotomy. Neutral isotonic saline or acidic isotonic solutions (pH=2 or 3) were injected in the duodenum 1 cm just below the pyloric sphincter. Ninety minutes after beginning the experiment, animals were sacrificed, stomachs ligated at lower esophageal sphincter and 2 ml saline infused in the stomach through pylorus and then gastric content was drained for measuring the pH. Two samples of gastric mucosal tissue were quickly snap-frozen and stored in liquid nitrogen for measuring the mucosal H\textsubscript{2}S concentration using ELISA kit and quantifying the mRNA expression of CSE by quantitative real-time PCR.

Results: Duodenal acidification with acidic solution (pH=2) increased the gastric release of H\textsubscript{2}S and upregulated mRNA expression of CSE in gastric mucosa. The gastric mucous content was significantly increased in response to duodenal application of acidic solutions with pH2 and 3.

Conclusion: Our findings indicated the stimulatory effect of duodenal acidification on gastric H\textsubscript{2}S release and mucous content is mediated through upregulation of CSE mRNA expression.

Keywords: Duodenal acidification; Cystathionine gamma lyase; H\textsubscript{2}S

Received: 13 Feb 2016
Accepted: 18 Apr 2016
*Correspondence to:
S.A. Mard
Tel: +98-91661185325
Fax: +98-6113362411

Email: alimard77@gmail.com; mard-sa@ajums.ac.ir

Introduction

The duodenal instillation of fat, acid, and hyperosmolar-solutions trigger a duodeno-gastric reflex that inhibits the gastric acid secretion (Lorenzo-Figures, 2004). This inhibitory response is triggered by both neural and humoral mechanisms and is important in the regulation of gastric acid secretion.
(Boron and Boulaep, 2012; Johnson, 2003). The major humoral factors released in response to acid, fat, and hyperosmolar solutions are secretin, cholecystokinin (CCK), somatostatin, and glucagon-like peptides known as enterogastrones (Boron and Boulaep, 2012; Johnson, 2003). The main physiological role of secretin and other acid-induced enterogastrones in response to entering the acidic chyme into duodenum is to neutralize it and protect the intestinal mucosa against gastric acid. The process of acid neutralization is mediated by inducing bicarbonate release from Brunner glands, biliary, and pancreatic ducts. In addition to induce such response in the duodenum, acid-induced enterogastrones stimulate a gastroprotective effect through promoting gastric mucous and bicarbonate secretion (Hall, 2010). Cystathionine gamma lyase [CSE] and cystathionine beta synthase [CBS] are two key enzymes involved on endogenous production of H$_2$S. They convert L-cysteine to hydrogen sulfide in mammalian body (Mard et al., 2015; 2016; Fiorucci et al., 2005). CBS and CSE are both expressed in the gastric mucosa in rat but the main enzyme for H$_2$S production is CSE (Mard et al., 2015; 2016; Fiorucci et al., 2005). It has been shown that bicarbonate secretion in response to duodenal acidification is partly mediated through H$_2$S in the rat duodenum (Ise et al., 2011). Recently, exogenous but not endogenous H$_2$S has been demonstrated to stimulate bicarbonate secretion in the rat stomach (Takeuchi et al., 2015). Moreover, it has been shown that gastric acid induces mucosal release of hydrogen sulfide in stomach by increasing gene and protein expressions of CSE (Mard et al., 2015). It has also been reported that the protective role of hydrogen sulfide on gastric mucosal barrier is mediated by stimulating mucous and bicarbonate secretion (Wallace, 2012).

As far as we are concerned, there is no report about the effect of duodenal acidification on gastric H$_2$S release. Therefore, the present study was designed to evaluate the effect of duodenal acidification on gastric H$_2$S release and on gene expression of CSE which is involved in endogenous synthesis of H$_2$S in stomach.

**Materials and methods**

**Animals**

Male Wistar rats (200-250 gr) were purchased from the animal house of Ahvaz Jundishapur University of Medical Sciences. The animals were fed on conventional diet and had free access to tap water. They were maintained under standard conditions of humidity, temperature (22±2°C) and 12 h light/dark cycle. The animals were deprived of food but not water overnight before intervention. All experiments were carried out in accordance with the regulations set by Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (APRC-94-18).

**Animal grouping and experimental procedures**

Twenty four adult male Wistar rats were randomly assigned into 3 groups (8 in each), including control, pH2-, and pH3-treated groups. Under a mixture of ketamine and xylazine anesthesia (60+15 mg/kg, i.p.), animals underwent midline laparotomy. Depth of anesthesia was checked throughout the experiment by 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 initial dose) was administered to maintain adequate anesthesia. Animal body temperature was measured with a rectal thermometer and maintained at 37°C using a homeothermic blanket control system (Harvard, UK). Thirty minutes after surgical operation, 0.5 ml of physiologic saline or acidic solutions were injected in the duodenum 1 cm below the pyloric sphincter. Control, pH3-and pH2-treated rats respectively received the same volume (0.5 ml) of neutral isotonic saline (pH=7), or acidic isotonic solution with pH 3 and pH 2. To make a 10 ml isotonic acidic solution with pH=1: 83μl of 37% HCl was added in to 9917 μl of isotonic saline solution and to make a 10 ml isotonic acidic solution with pH=2: 1 ml of above solution was added in to 9 ml isotonic saline solution. One ml of isotonic acidic solution with pH=2 was added to 9 ml isotonic solution to make isotonic acidic solution with pH=3.

Ninety minutes after the application of saline or acidic solutions in the duodenum, animals were sacrificed by an overdose of anesthetics, stomachs were ligated at lower esophageal sphincter and 2 ml of saline (pH7) infused into the stomach through the pylorus and then gastric content was drained for measuring the pH using a digital pH meter (iSTEK, Inc. South Korea). Then, the stomachs were
Duodenal acidification stimulates gastric H₂S release

**Physiol Pharmacol 20 (2016) 57-62**

removed, opened along the greater curvature, rinsed with physiological saline and pinned out in ice-cold saline. The gastric mucosal wall was determined using Perera et al. method (Perera et al., 2001). Briefly, the stomachs were washed with normal saline, and then the gastric wall mucous was scraped, and weighed. Two samples of gastric mucosal tissue were quickly snap-frozen and stored in liquid nitrogen for future analysis. The CSE mRNA expression was measured using quantitative real-time PCR and gastric H₂S release was assessed by ELISA kit.

**Quantitative real-time PCR**

The mRNA levels of the CSE, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real-time PCR (qRT-PCR) using a LightCycler® 480 System (Roche Diagnostics). Primers used were as follows:

**GAPDH** (5'-TGCTGGTGCTGAGTATGTCGTG-3' and 5'-CGGAGATGATGACCCTTTTGG-3', 101 bp); and **CSE** (5'-TGTGTGTGGCGTGTACCTG-3' and 5'-CCATCCATCCTCCTAGTG-3, 167 bp). All PCR amplifications were performed in duplicate reactions.
and in final volume of 20 µl containing 2 µl cDNA, 0.8 µl of forward and reverse primers, 10 µl of master mix SYBR green [TAKARA SYBR® Premix Ex Taq ™ II (Tli RNaseH Plus), Bulk], 6.4 µl ddH₂O using the following protocol: pre-incubation at 95 °C for 2 min to activate DNA Taq polymerase and 40 two-step cycles with denaturation at 95 °C for 20 s, and annealing/extension at 60 °C for 1 min. In addition, the no-template negative control (H₂O) was routinely included in every run. The melting curve was examined at the end of amplification process to ensure the specificity of PCR products. Expression level of CSE was normalized against GAPDH expression (internal calibrator for equal RNA template loading and normalization). Relative quantity of expression was calculated using comparative cycle of threshold (Ct) method with arithmetic formulae ($2^{-\Delta\Delta C_t}$).

### Determination of mucosal H₂S levels

To investigate the effect of duodenal acidification on gastric H₂S production/release, commercial enzyme-linked immunosorbent assay Kit (ABIN771902, antibodies-online, USA) was used following manufacturer’s instructions.

---

**Fig. 3**: Effect of duodenal acidification on mucous production in the rat stomach. C (Control rats): 0.5 milliliter neutral isotonic saline (pH=7) injected into duodenum 1 cm just below the pyloric sphincter; pH=2 and pH=3: 0.5 milliliter acidic solutions (pH=2, or 3) injected in the duodenum 1 cm just below the pyloric sphincter, respectively. Duodenal acidification with acidic solutions with pH2 and 3 significantly increased the gastric content of mucous compared with the control rats. **P<0.01 versus control group; Data are expressed as mean±SEM.**

**Fig. 4**: Effect of duodenal acidification on pH of the gastric effluents. C (Control rats): 0.5 milliliter neutral isotonic saline (pH=7) injected into duodenum 1 cm just below the pyloric sphincter; pH=2 and pH=3: 0.5 milliliter acidic solutions (pH=2, or 3) injected in the duodenum 1 cm just below the pyloric sphincter, respectively. Duodenal acidification did not affect the pH of the gastric effluents. Data are expressed as mean±SEM.
**Statistical analysis**

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

**Results**

**Effect of duodenal acidification with acidic solutions (pH=3, and 2) on mucosal release of H$_2$S in the stomach**

As shown in figure 1, analysis of ELISA results showed that duodenal acidification with 10 mM HCl (pH=2) significantly increased the gastric release of H$_2$S as compared with control rats (P<0.01). Application of the acidic isotonic solution with pH3 slightly but not significantly increased the gastric release of hydrogen sulfide.

**Effect of duodenal acidification with acidic solutions (pH=3, and 2) on mucosal mRNA expression of CSE in the stomach**

As illustrated in figure 2, analysis of qRT-PCR results showed that the level of mRNA expression of CSE in mucosal layer of the stomach in response to duodenal acidification with 10 mM HCl (pH=2) was significantly increased compared with control rats (P<0.01).

**Effect of duodenal acidification with acidic solutions (pH=3, and 2) on gastric mucous production and pH of gastric effluent**

As shown in figure 3, duodenal acidification with acidic solutions (pH=3, and 2) significantly increased the gastric content of mucous compared with the control rats (P<0.01). Injection of acidic isotonic solutions with pH=2, and 3 did not affect the pH of gastric effluent (Fig. 4).

**Discussion**

The entry of acidic chyme in the duodenum triggers the release of enterogastrones such as secretin and CCK. The enterogastrone response is aimed to protect the duodenal and gastric mucosa against acidic chyme. This protective phenomenon is mediated through increasing the duodenal and gastric bicarbonate secretions. Hydrogen sulfide has been reported to partly mediate the bicarbonate secretion in response to duodenal perfusion of 10 mM HCl (Johnson, 2003). The findings of the present study showed that that: (1) duodenal acidification with 10 mM HCl increased mucosal H$_2$S release and mucous content in the rat stomach; and (2) up-regulated mRNA expression of CSE in the gastric mucosa.

Recently, it has been shown that stimulation of gastric acid secretion (Mard et al., 2015) and mucosal acidification of the gastric lumen with 10 and 100 mM HCl induce mucosal H$_2$S release in the rat gastric mucosa (Mard et al., 2016). Moreover, it has been reported that duodenal perfusion of 10mM HCl induces H$_2$S release in the rat duodenum (Ise et al., 2011). The present study showed that the mucosal H$_2$S release in the rat stomach significantly increased in response to duodenal acidification. Therefore, these finding showed that mucosal and duodenum acidification stimulate mucosal H$_2$S release in gastric mucosa.

The results also demonstrated that duodenal acidification accompanied by increased mucosal release of H$_2$S in gastric mucosa, upregulated mRNA expression of CSE, the main involved enzyme in hydrogen sulfide synthesis in the rat gastric mucosa. Therefore, in accordance with current findings increased production of H$_2$S in response to duodenal acidification in gastric mucosa is largely mediated through up-regulating the mRNA expression of CSE. The current findings indicated that mRNA expression of CSE and H$_2$S release in gastric mucosa slightly but not significantly increased when mucosal tissue of duodenum exposed to the acidic solution with pH3. So, it is revealed that duodenal acidification with pH2 solution has a more potent effect on CSE gene expression and H$_2$S release in gastric mucosa than pH3, suggesting that duodenal acidification with 10 mM HCl solution is the threshold for triggering such
effects.
Hydrogen sulfide has been shown to increase the mucous secretion in the rat stomach (Wallace, 2012), that is consistent with present findings that the mucous production of the gastric wall and gastric H$_2$S release increased following duodenal acidification. It may be concluded that these effects could largely be mediated through increment of the H$_2$S release.

It has been shown that endogenous hydrogen sulfide does not increase bicarbonate secretion (Takeuchi et al., 2015). This study did not directly evaluate the gastric secretion of bicarbonate, but as shown in the results, the pH of gastric effluent was not affected by duodenal acidification in spite of increasing the gastric H$_2$S level. Therefore, the present results are in agreement with previous reports (Takeuchi et al., 2015).

What is the physiological significance of the present results? The results of the present study showed that the acid-induced enterogastrones in addition to inducing the gastric release of mucous as shown by the previous studies increased the gastric release of hydrogen sulfide by activation of CSE and upregulation of CSE mRNA expression. This response seems to protect the gastric mucosa against the acid. However, which enterogastrone(s) is involved in this response remains to be determined and therefore future studies are, prudenty, needed.

Conclusion
In conclusion, our findings showed that duodenal acidification increased the production of H$_2$S production in stomach through up-regulating mRNA expression of CSE in rats.

Acknowledgment
The authors would like to thankfully acknowledge the financial support of the Vice Chancellor of Research Affairs of Ahvaz Jundishapur University of Medical Sciences (Grant No. APRC-94-18). This paper was issued from M.Sc. thesis of Hajar Godarzinejad.

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

References