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Effect of sodium hydrosulfide on mRNA expression of prostaglandin E_2 receptors in response to mucosal acidification and distention-induced gastric acid secretion in rats

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ABSTRACT

Objective(s): Prostaglandins have been shown to mediate the gastro-protective effect of sodium hydrosulfide (NaHS) but effect of NaHS on mRNA expression of prostaglandin E_2 receptors (EP1, 3-4; EPs) has not been investigated. Therefore, this study designed to evaluate the effect of NaHS on mRNA expression of EPs receptors in response to mucosal acidification and distention-induced gastric acid secretion in rats.

Materials and Methods: Fasted rats were randomly assigned into 4 groups (n=6/group). They were control, and NaHS-treated groups. To evaluate the effect of NaHS on mucosal mRNA expression of EPs receptors, the gastric mucosa exposed to stimulated gastric acid output and mucosal acidification. The pylorus sphincter catheterized for instillation of isotonic neutral saline or acidic solution. Ninety min after beginning the experiments, animals sacrificed and the gastric mucosa collected to determine the pH, mucus secretion and to quantify the mRNA expression of EPs receptors by quantitative real-time PCR.

Results: present results showed that a) NaHS increased the mucus secretion, mRNA expression of EP3 and EP4 receptors in response to distention-induced expression; b) The mRNA expression of EP1 receptors increased while EP4 mRNA receptors decreased in response to mucosal acidification in NaHS-pretreated rats; and c) NaHS increased pH of gastric contents both in response to distention-induced gastric acid secretion and mucosal acidification.

Conclusion: NaHS behaves in a different manner. It effectively only increased the pH of gastric contents to reinforce the gastric mucosa against a highly acidic solution but modulated both acid and mucus secretion when the rate of acid increase in the stomach was slower.

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Introduction

It is well established that prostaglandins (PGs) protect the gastroduodenal mucosa against physiologic [gastric acid and pepsin] and non-physiologic irritants (1). The involved mechanisms by which PGs maintain the integrity of gastric mucosa are decreasing the gastric acid output and increasing the mucus and bicarbonate secretion as well as improving the gastric mucosal blood flow (2). The major PGs produced by the human and rodent gastric mucosa are PGE_2 and PGI_2 (3). Pharmacological studies have revealed that there are four PGE2 receptors, EP1-EP4 (3). PGE2 has dual effect on gastric acid secretion in rats (2). At low concentrations, inhibits acid secretion through activation of EP3 receptors while at high concentration, stimulates the gastric acid output by activating EP4 receptors. Both EP3 and EP4receptors are expressed by parietal cells (4). Activation of EP1 receptors of PGE2 in the rat's stomach leads to bicarbonate secretion (5).

The gastro-protective effect of the third gasotransmitter, hydrogen sulfide (H_2S), is well established (6). H_2S and its donor [NaHS] have been shown to protect the gastric mucosa through different pathways such as potentiating the gastric mucosal barrier [increasing mucosal blood flow, mucus, bicarbonate secretion, etc] and inhibiting the gastric acid output (6-8). It has been shown that NaHS through increasing the mucosal production of PGE2 protect the rat's gastric mucosa against water immersion stress-induced gastric lesions (9). Another research has been demonstrated that endogenous and exogenous H_2S increased the pH of gastric contents by

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upregulating the mucosal mRNA expression of sodium bicarbonate cotransporter1 [NBC1] in rats (10).

Therefore, previous reports have shown the effects of NaHS on mucosal production of PGE2 and bicarbonate as well as mucus secretion, but on the best of our knowledge its effect on mucosal mRNA expression of PGE2 receptors has not been investigated. Therefore, the present study designed to evaluate the effect of NaHS on mRNA expression of PGE2 receptors [EP1, EP3 and EP4) in gastric mucosa in rats. To determine this effect of NaHS, the gastric mucosa exposed to distention-induced gastric acid output and mucosal application of hydrochloric acid (pH=1).

Materials and Methods

Animals

Male Wistar rats (150-200 g) were supplied from the animal house of Ahvaz Jundishapur University of Medical Sciences. Animals were fed on conventional diets and tap water. They were maintained under standard conditions of humidity, temperature (22± 2 °C) and light/dark cycle (12 hr: 12 hr). All experiments were carried out in accordance with ethics committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1394.661).

Animal grouping and surgical procedures

Twenty-four rats were randomly assigned into 4 groups (6 per group). They were control (C), and NaHS-treated groups. Fasted rats were anesthetized with a mixture of ketamine and xylasine (60+15 mg/kg, IP). Depth of anesthesia was monitored throughout the experiment by the pedal withdrawal (toe pinch) reflex every 30-45 min. If the reflex was observed, a supplemental dose of anesthetics (1/3 of initial dose) was administered to maintain adequate anesthesia. Animal body temperature was controlled with a rectal thermometer and maintained at 37±0.5 ˚C by using a homoeothermic blanket control system (Harvard, Edenbridge,UK). Anesthetized rats underwent a midline laparotomy, and then both the stomach and duodenum were exposed. A polyethylene catheter [3 mm, OD (outer diameter)] was inserted into the stomach through the duodenum and held in place by a ligature around the pylorus. At the beginning of each experiment, the lumen of the stomach was gently rinsed with isotonic saline (pH=7, 37 ˚C) until gastric wash out was clear.

In the first set of experiments, to evaluate the effect of NaHS on mucosal mRNA expression of EPs receptors in response to distention-induced gastric acid secretion, 12 rats were assigned into control, and NaHS-treated rats. Thirty min after surgical operation, isotonic saline (1.5 ml/100 g of body weight; pH=7, 37 °C) (11) was instilled into the stomach to stimulate the gastric acid output. NaHS-treated rats received a single IP injection of NaHS (a H_2S donor) at 80 µg/Kg (7) along with gastric distention by isotonic saline.

In the second set of experiments, to evaluate the effect of NaHS on mucosal mRNA expression of EPs receptors in response to mucosal acidification, 12 rats were assigned into control, and NaHS-treated rats. 30 min after surgical operation, one ml of isotonic acidic solution (pH=1) (12) was instilled into the stomach of experimental groups. NaHS-treated rats were received a single IP injection of NaHS at 80 µg/Kg (7) along with the instillation of acidic solution (HCl; pH=1).

Ninety min after the instillation of isotonic saline or acidic solution, animals were sacrificed by an overdose of anesthetics, their stomachs were removed and gastric contents collected.

The pH of each sample was measured with a digital pH meter (isTEK; Inc, South Korea). After that, the stomachs were opened along the greater curvature, rinsed with physiological saline and pinned out in ice-cold saline. To determine the gastric mucus wall, the gastric wall mucus was scraped, weighed and presented as milligram. The mean of mucus content in control groups considered as 100%. Thirty mg of gastric mucosal tissues were quickly snap-frozen and stored in liquid nitrogen for mRNA analysis.

RNA extraction and cDNA synthesis

The total RNA was extracted from the frozen tissue samples using RNeasy Plus min Kit (Qiagen, QiagenGmBH, hilden, Germany). The purity and concentration of the extracted RNA was determined spectrophotometerically at 260 and 280 nm wave-length (Nanodrop thermoscientific S.N:D015). The cDNA was synthesized from 1 µg of the total RNA using QuantiTect reverse transcription Kit (Qiagen, QiagenGmBH, hilden, Germany) according to the manufacturer´s instruction.

Quantitative real-time PCR

The mRNA levels of prostaglandin E_2 receptors [EP1, EP3, and EP4], and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by quantitative real-time PCR (qRT-PCR) using a LightCycler® 96 System (Roche Diagnostics). The specific primers (Bioneer, Daejeon, South Korea) for measurement of target genes and GAPDH were used and the lengths for amplified products were as follows:
GAPDH (Forward: 5´-TGCTGGTGCTGAGTATGTCGTG-3 and 5´-GGAGATGATGACCCTTTTGG-3´, 101 bp); EP1(Forward: 5´-TGGCACAGATACAGGGGATG-3´and reverse: 5´-GTGGGACGTGAATCCAGAACT-3, 145 bp); EP3 (Forward: 5´-GTGTGTACTGTCCGTCTGCT-3´and reverse: 5´-TCAGGTTGTTCATCATCTGGCA-3,230 bp); and EP4 (Forward: 5´-GTTCTGGCAGAGACGGTTCA-3´ and reverse: 5´-AAGTTCTCAGCGAGGTGGTG-3´, 234 bp). All PCR amplifications were performed in duplicate reactions and in final volume of 20 µl containing 2 µl cDNA, 0.8 µl of specific primers, 10 µl of master mix SYBR green(TAKARA SYBR_ Premix Ex TaqTM II, Tli

RNaseH Plus, Bulk[TAKARA, BIO INC, Shiga, Japan]), 6.4 µl ddH2O using the following protocol: pre-incubation at 95 °C for 1 min to activate DNA Taq polymerase and 40 two-step cycles with denaturation at 95 °C for 15 sec, annealing at 54 °C for 30 sec, and extension at 72 °C for 30 sec. In addition, the no-template negative control (H_2O) was routinely run in every PCR. The melting curve was examined at the end of amplification process to ensure the specificity of PCR products.

Expression level of EPs receptors were normalized against GAPDH expression (internal calibrator for equal RNA template loading and normalization).

To determine the relative quantification of gene expression, comparative cycle of threshold (Ct) method with arithmetic formula ($2^{-\Delta\Delta Ct}$) was used.

Statistical analysis

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

Results

Effect of NaHS on mucosal mRNA expression of EPs receptors in response to distention-induced gastric acid output and mucosal acidification

As shown in Figures 1A and 3A, analysis of qRT-PCR results showed that NaHS treatment significantly increased the mucosal mRNA expression of EP1 receptors while decreased mRNA expression of EP4

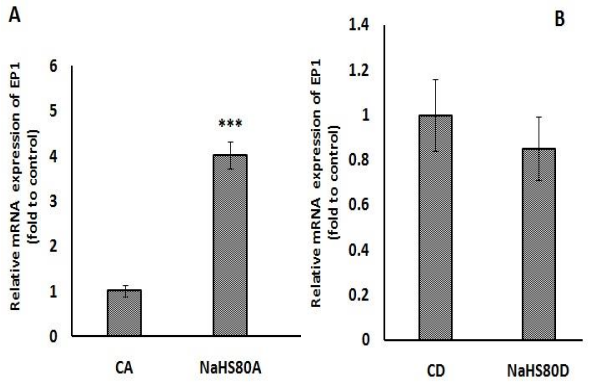

Figure 1. Effect of sodium hydrosulfide (NaHS) on mucosal mRNA expression of EP1 receptor of PGE2 in response to distention-induced gastric acid output and mucosal acidification. Analysis of qRT-PCR results showed that NaHS significantly increased the mucosal mRNA expression of EP1 receptors in response to mucosal acidification (Figure1A) while mRNA expression of EP1 was not affected in response to distention-induced gastric acid secretion in NaHS-treated rats (Figure1B). CA: control acid; and NaHS80A: animals received a single intraperitoneally injection of NaHS at 80 µg/kg along with mucosal instilattion of acid solution (pH=1); CD: control distention, and NaHS80D: animals received a single intraperitoneally injection of NaHS at 80 µg/kg along with stimulating the gastric acid output by gastric distention
***$P<0.001$ significant increase compared with the corresponding's control rats. Data are expressed as mean±SEM

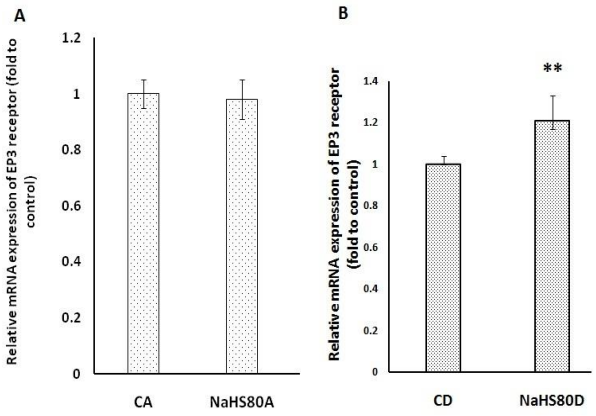

Figure 2. Effect of sodium hydrosulfide on mucosal mRNA expression of EP3 receptor of PGE2 in response to distention-induced gastric acid output and mucosal acidification. Analysis of qRT-PCR results showed that NaHS treatment significantly increased the mucosal mRNA expression of EP3 receptors in response to distention-induced gastric acid secretion (Fig.2B) while mRNA expression of EP3 was not affected in response to mucosal acidification in NaHS-treated rats (Fig.2A).**$P<0.01$ significant increase compared with control rats. Data are expressed as mean±SEM

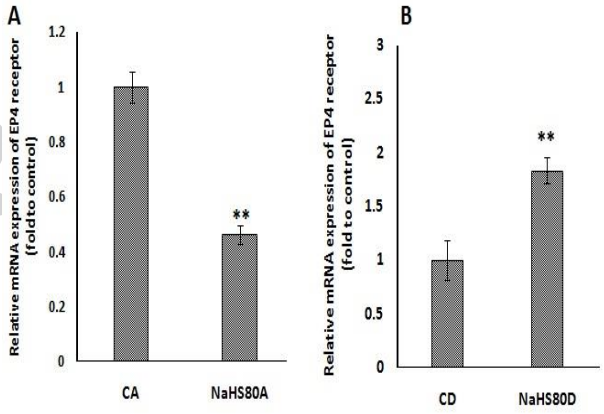

Figure 3. Effect of sodium hydrosulfide on mucosal mRNA expression of EP4 receptor of PGE2 in response to distention-induced gastric acid output and mucosal acidification. Analysis of qRT-PCR results showed that NaHS treatment significantly increased the mucosal mRNA expression of EP4 receptors in response to distention-induced gastric acid secretion (Figure 3B) while mRNA expression of EP4 was significantly decreased in response to mucosal acidification in NaHS-treated rats (Figure 3A).**$P<0.01$ significant versus corresponding's control groups. Data are expressed as mean±SEM

receptors in response to mucosal acidification. NaHS did not change the mRNA expression of EP3 receptor in response to mucosal acidification (Figure 2A). Figures 2B and 3B showed that the mucosal mRNA expression of EP3, and EP4 was significantly increased in response to distention-induced gastric acid secretion while mRNA expression of EP1 was not affected in NaHS-treated rats (Figure1B).

Effects of NaHS on gastric wall mucus production in response to distention-induced gastric acid output and mucosal acidification

As shown in Figure 4B, sodium hydrosulfide increased the gastric mucus production in response to distention-induced gastric acid output. NaHS did not affect the basal production of gastric mucus in response to mucosal acidification (Figure 4A).

Effects of NaHS on pH of gastric contents in response to distention-induced gastric acid output and mucosal acidification

As demonstrated in Figures 5A and 5B, sodium hydrosulfide significantly increased the pH of gastric contents both in response to mucosal acidification and distention-induced gastric acid output.

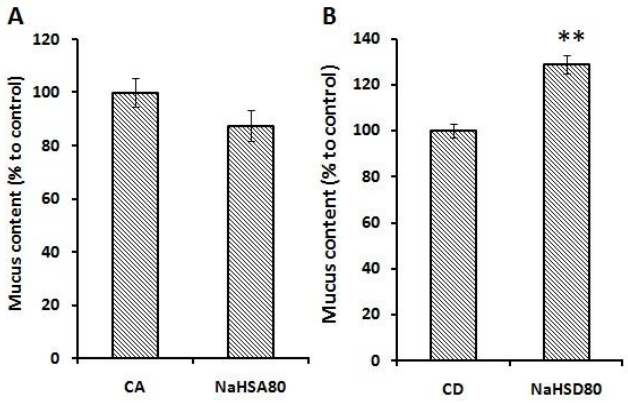

Figure 4. Effect of sodium hydrosulfide on gastric wall mucus production in response to distention-induced gastric acid output and mucosal acidification. Sodium hydrosulfide significantly increased the gastric mucus production in response to distention-induced gastric acid output (Figure 4B) while did not affect the basal production of gastric mucus in response to mucosal acidification (Figure 4A).**P<0.01significant versus corresponding's control group. Data are expressed as mean±SEM

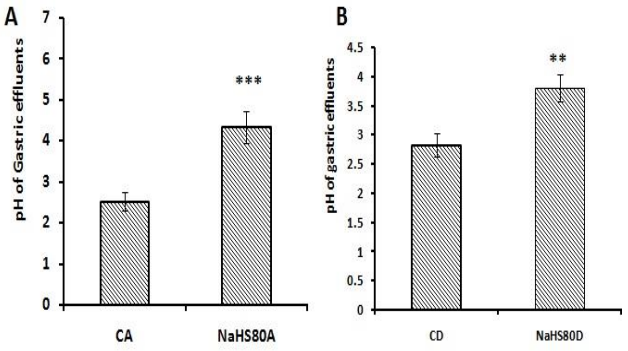

Figure 5. Effect of sodium hydrosulfide on pH of gastric contents in response to distention-induced gastric acid output and mucosal acidification. Sodium hydrosulfide significantly increased the pH of gastric contents in response to mucosal acidification (Figure 5A) and distention-induced gastric acid output (Figure 5B). **P<0.01, and ***P<0.001 significant versus corresponding's control groups. Data are expressed as mean±SEM

Discussion

The present study showed that a single administration of sodium hydrosulfide: 1. Increased the mucus secretion, and mRNA expression of EP3 as well as EP4 receptors of PGE2 in response to distention-induced expression; 2. Upregulated the mRNA expression of EP1 receptors of PGE2 while downregulated EP4 mRNA expression in response to mucosal acidification; and 3. Increased the pH of gastric contents both in response to distention-induced gastric acid secretion and mucosal acidification.

The current results showed that NaHS significantly increased the pH of gastric contents in response to mucosal application of acidic solution (pH=1). According to the real-time PCR results, this response can be resulted from dowregulation of EP4 receptors and at the same time upregulation of EP1 receptors in gastric mucosa. It is a general fact any increase in the pH of gastric content can be produce via decreasing the gastric acid output or increasing the gastric bicarbonate secretion and also simultaneous increase of $HCO3^-$ and decrease of HCl. Therefore, downregulating mRNA expression of EP4 receptors can be lead to decreasing EP4 protein receptors in parietal cell membrane which in turn inhibits the gastric acid secretion. Additionally, upregulating mRNA expression of EP1 receptors by NaHS could be the second reason for increasing the pH of gastric contents because of the activation of EP1 receptors results in HCO_3^-secretion by epithelial mucus cell (1, 5). Therefore, NaHS through increment the mucosal production of ligand [prostaglandin E2] as shown by Magierowski *et al* (9) and at the same time through modulating the receptor density of cell membrane (decrement EP4, while increment EP1 mRNA receptors) enhanced the pH of gastric contents.

The current findings showed that NaHS did not change the mucus content in response to mucosal acidification. It has been shown that activation of EP4 receptors on epithelial mucus cells by PGE2 stimulate the mucus secretion (5). Our qRT-PCR results showed that the mRNA expression of EP4 decreased in NaHS-treated rats. The present results also showed that the mucus content was slightly but not significantly decreased while the pH of gastric contents effectively and significantly increased in NaHS-treated rats. It was 2.52±0.23 in control rats while administration of NaHS at 80 µg/kg increased the pH to 4.33±0.38. Therefore, these results implied that when the gastric mucosa suddenly exposes to a highly acidic solution (pH=1), the preferred mechanism by which NaHS protected the gastric mucosa is neutralizing the entered acid. As results showed NaHS decreased the gastric acid output by downregulating mRNA expression of EP4and induced the bicarbonate secretion by upregulating mRNA expression of EP1. The final effect was a highly and effective increase of pH of gastric contents.

The results of the present study indicated that NaHS increased the rate of mucus secretion and enhanced the

pH of gastric contents in response to distention-induced gastric acid secretion. As shown in Figures 2B and 3B, the mRNA expression of EP3 and EP4 increased in NaHS-pretreated groups. EP3 receptors are expressed in the parietal cells of rat's stomach (2). It has been shown that the activation of EP3 receptor inhibits the gastric acid output (4). EP4 receptors are expressed by parietal (13) as well as mucus cells (2) in gastric mucosa. It has been shown that activation of EP4 receptors has two effects, stimulating the gastric acid output and inducing mucus secretion. In spite of parietal cells, mucus cells only expressed EP4 receptor mRNA (2). In contrast to mucosal acidification experiment, the rate of acid increase is slower in rats underwent gastric distention and provides much more time for NaHS to protect the gastric mucosa by two mechanisms: 1. Mucus production by upregulating EP4 mRNA receptors and inhibiting the gastric acid by upregulating EP3 mRNA receptors as evidenced by the present results. It also seems that the rate of mRNA expression of EP4 receptors was higher in epithelial mucus cell than in parietal cells because the results did not show the acid excitatory effect of EP4 receptors. On the other hand, the qRT-PCR and the pH findings together suggest that when EP3 receptors on parietal cell membranes activate due to the simultaneous increase of mRNA expression of EP3 as shown by current study and PGE2 as shown by a previous research (9), the membrane EP4 receptors inactivate or has lower affinity to PGE2. The present pH result was also support the activation of EP3 receptors rather than EP4 receptors in parietal cells.

However, this study aimed to evaluate the effect of NaHS on mucosal mRNA expression of EP1, EP3, and EP4 of PGE2 receptors but not their activation by NaHS. Thus, more studies need to exactly define the effect of NaHS on EPs activation. Whether the affinity and activation of EPs change by NaHS is also remained to be defined by further future experiments.

Conclusion

The results suggest NaHS increased the pH of the gastric contents in response to mucosal acidification through dowregulating mucosal mRNA expression of EP4, and upregulating EP1. The increased mucus and pH of gastric contents in NaHS-treated rats in response to distention-induced gastric acid output is mainly mediated through upregulating mucosal mRNA expression of EP3 and EP4 receptors.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgment

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