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Sodium hydrosulfide upregulates mRNA and protein expression of TGF-α in gastric mucosa in experimental model of stimulated gastric acid secretion in rats

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ABSTRACT

Objective(s): Transforming growth factor alpha (TGF-α) has been shown to modulate the gastric acid secretion. Therefore, the aim of the present study was to investigate the effect of sodium hydrosulfide (NaHS) on TGF-α expression in gastric mucosa in rats.

Materials and Methods: Eighteen rats were randomly divided into 3 groups (6 per group). To determine the effect of NaHS on gene and protein expression of TGF-α in gastric mucosa in response to gastric acid, the acid output induced by gastric distension. At the end of experiment, rats were euthanized by anesthetics, and gastric effluents, in addition to mucosa were collected to measure the pH of gastric effluents and to quantify protein and gene expression of TGF-α.

Results: The stimulated gastric acid upregulated expression levels of TGF-α in gastric mucosa. These levels were higher in animals pretreated with NaHS.

Conclusion: TGF-α upregulatory effect of sodium hydrosulfate implied that TGF-α is involved in the acid inhibitory effect of NaHS.

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Introduction

Transforming growth factor α (TGF-α) is a polypeptide with 50 amino acids (1), which has a 30% homology with epidermal growth factor (EGF), and functionally operates like this protein through binding to the EGF-R (receptor). This protein is produced and secreted by parietal cells of gastric fundus, which has an important suppressor of acid secretion (2). It may act locally through autocrine or paracrine route (3). Several biological functions have been documented for this protein including suppressing acid production, secretion, and protective effect against ethanol- and aspirin-induced damage (4). TGF-α preserves gastric mucosal integrity by advancing migration and proliferation of cells. Additionally, the inhibitory effect of TGF-α on the release of histamine from entero-chromaffin-like cells has been reported (5).

EGF, and TGF-α have been demonstrated to be the most important mitogenic agents for special types cells in the gastrointestinal tract both *in vivo* and *in vitro*. In addition, these proteins are suggested to regulate the mucosal blood flow, gastro intestinal activity, and mucus production and also conserve gastric and colonic mucosa versus insult. It has been shown that the protein and gene expression levels of TGF-α increased dramatically following acute mucosal damage (6).

Of interest, several researches showed that following gastrointestinal injury, TGF- α precursors increase, indicating that these precursors may be responsible for biological activities (6, 7). Other studies indicated that chronic administration of aspirin is associated with a significant increase in TGF-α concentration, which in turn reduced aspirin-induced gastric damage (8, 9). These reports showed that TGF-α may be involved in gastric adaptation to long term application of non-steroidal anti-inflammatory drugs (6).

Many reports have shown that hydrogen sulfide (H_2S) has many beneficial effects in gastrointestinal system (10-12). The enzymatic and non-enzymatic pathways are both responsible for production of H_2S. Cystathionine gamma lyase (CSE) is the main enzyme involved in H_2S production in rat's gastric mucosa while cystathionine beta synthase (CBS) is responsible for H_2S production in the brain of rat (13). TGF-α has been shown to increase the mRNA expression of CBS in brain astrocytes (14). Additionally, it has been reported that

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the damaged neurons produce TGF-α (15).

The protective effects of NaHS against gastric injury and its involved mechanisms were defined in part, but the exact mechanism(s) remained to be explained. Therefore, the aim of this study was to evaluate the effect of NaHS on mRNA and protein expression of TGF-α in response to distension-induced gastric acid secretion in rats.

Materials and Methods

Animals

Male rats [Wistar; weighing 200–250 g] purchased from the animal house of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, and were fed with a conventional diets and tap water *ad libitum*. They were maintained under standard conditions of humidity, temperature (20 to 24 ˚C), and 12-hr light–dark cycle. Animals were deprived of food, but not water, overnight before experiments. All experiments were performed in accordance with ethics committee of Ahvaz Jundishapur University of Medical Sciences (RDC-94-9307).

Experimental procedures

Animals were anesthetized by a single intrapritoneally injection of ketamine and xylazine [60+15 mg/kg, (IP)] (16). Their body temperature was controlled with a rectal thermometer and kept at 36.5-37.5 ˚C by using a homoeothermic blanket control system (Harvard, Edenbridge, UK). At the next step, a midline laparotomy was performed to expose the stomach. Afterwards, the stomach was cannulated with a polyethylene catheter [outer diameter: 3 mm] through the duodenum. Finally, the stomach was gently rinsed with physiologic (pH: 7, 37 ˚C) (13).

Animal grouping

Eighteen rats were randomly assigned into 3 experimental groups, each consisting of six rats. They were:

- Control (C): one milliliter of neutral physiologic saline [37 ˚C] was instilled into the stomach 30 min after ending the surgery.
- Distention (D): the stomach was distended by neutral physiologic saline [1.5 ml/100 g of body weight, pH: 7, 37 ˚C] (11) 30 min after ending the surgery to stimulate acid output.
- NaHS+distention (NaHS+D): Thirty minutes after surgical operation, this group of rats received a single intraperitoneally injection of sodium hydrosulfate [a H_2S donor at 80 μg/kg] (7) in concomitant to induction the acid output as mentioned above. NaHS was prepared freshly and dissolved in isotonic saline.

At the end of experiment (90 min after the instillation of neutral physiologic saline), rats were euthanized by anesthetics, after that the gastric effluents were totally collected and the gastric acid output was measured as explained later. Following the collection of gastric effluents, the stomachs were resected, opened along the greater curvature, washed with neutral physiological saline and pinned out in ice-cold saline. Mucosal samples were collected, weighed, and immediately snap-frozen, then stored at -80 ˚C for molecular evaluations.

RNA extraction and cDNA synthesis

Mucosal tissue RNA was extracted from the frozen tissues using Qiagen Extraction kit (RNeasy Plus min Kit, Qiagen GmBH, hilden, Germany). The purity and concentration of the extracted RNA was determined using a Nanodrop (Thermoscientific S.N:D015). At the next step, complementary DNA (cDNA) was synthesized from 1 μg of the total RNA using Qiagens QuantiTect reverse transcription Kit (Qiagen GmBH, hilden, Germany) according to the manufacturer's instruction (17).

Semi-quantitative real-time PCR

The expression levels of TGF-α gene, and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured by semi-quantitative real-time PCR (qRT-PCR) using a LightCycler® 96 System (Roche Diagnostics). The following primers (Bioneer, Daejeon, South Korea) used for measurement of TGF- α and GAPDH. GAPDH (Forward primer: 5′-TGCTGGTGCTGAGTATGTCGTG-3' and reverse primer 5′-CGGAGATGATGACCCTTTTGG-3′); and TGF-α (Forward primer: 5′-GACAGCTCGCTCTGCTAG -3′and reverse primer: 5′- CACCACTGCCAGGAGATC- 3'). All reactions were performed in duplicate. The final volume for each reaction was 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, TliRNaseH Plus, Bulk[TAKARA, BIO INC, Shiga, Japan]), and 6.4 μl distilled water. The reactions were performed using the following protocol: pre-incubation at 95 ˚C for 60 sec 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. The level of TGF-α expression was normalized against house- keeping gene expression (16).

Protein extraction

Total proteins was extracted from frozen tissues using Radio-immunoprecipitation assay buffer [RIPA buffer; (25mM Tris-HCl pH 7.4, 1.0% NP-40 or Triton X-100 1%, SDS 0.1%, Sodium deoxycholate 0.5%, 150 mM NaCl, 1 mM EDTA and dd-H_2O)] containing protease inhibitor cocktail (complete mini, Roche, Indianapolis, IN, USA). At the next step, protein obtained using RIPA buffer, from stomach samples, were resuspended in 1% SDS. Bradford assay and SDS–polyacrylamide gel electrophoresis were used to determine the total recovery and integrity of protein fractions (17).

Western blotting analysis

The extracted proteins were separated by SDS-PAGE on 12% acrylamide gels and then transferred onto a nitrocellulose membrane. Following that, the blocking

step of membranes was performed using 5% non-fat dry milk dissolved in tris-buffered saline with 0.1% Tween 20 (TBST, pH: 7.6) for 6 hr. At the next step, the blocked membranes were incubated overnight at 4 ˚C with anti-TGF-α antibody (mouse monoclonal, dilution 1:200; Abcam [ab80562], USA), or anti-beta actin antibody (mouse monoclonal, dilution 1:5000; Abcam [ab20272], USA). After 5 times washing 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 using a chemiluminescence western blotting system. The expression level of TGF-α protein was semi-quantified by specific software (Image J) and the values were normalized to β-actin as a housekeeping protein.

Quantification of the acid output

As mentioned earlier, the gastric effluents were collected, and their pH was determined with an autotitrator pH meter (Radiometer, Copenhagen; Denmark) by automatic potentiometric titration to pH=7 with 0.01 N NaOH, and the acid output was reported as $mEqH^{+}$ (18).

Statistical analysis

Data are expressed as mean±standard error of means. One-way analysis of variance (ANOVA) and Tukey's tests were used to analysis the data. $P<0.05$ was considered significant.

Results

Sodium hydrosulde upregulated protein and gene expression of TGF-α in response to stimulated gastric acid output

Molecular evaluations revealed that pretreatment with sodium hydrosulfide (NaHS) significantly increased the expression level of protein and gene of TGF-α in response to distension-stimulated gastric acid secretion in comparison with control group (*P<0.05*). The highest expression levels of TGF-α were observed in NaHS-

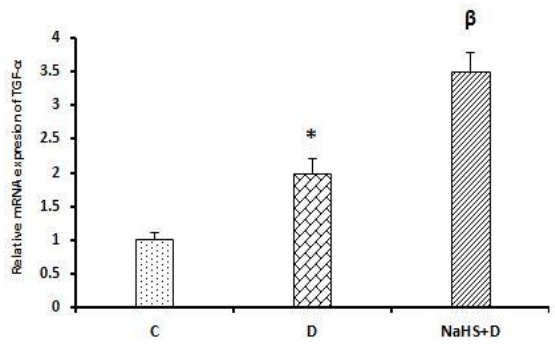

Figure 1. NaHS effect on gene expression of TGF-α in response to distension-induced gastric acid output in rats stomach. Data are expressed as mean±SEM. ∗*P<0.05* significant increase compared with the control group and $^{\beta}P<0.01$ significant difference compared to distention group. TGF-α: Transforming growth factor-alpha; NaHS: Sodium hydrosulfide; C: Control; D: Distention

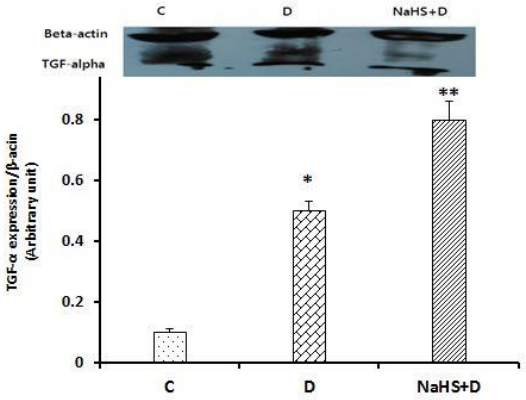

Figure 2. NaHS effect on protein expression of TGF-α in response to distension-induced gastric acid output in rats stomach. Results presented as mean±SEM. ∗*P<0.05* significant increase compared with the control group and ∗∗*P<0.01* significant difference compared to distention group. TGF-α: Transforming growth factor-alpha; NaHS: Sodium hydrosulfide; C: Control; D: Distention

treated rats (*P<0.01*). As shown in Figures 1, and 2, the level of mRNA and protein expression of TGF-α in NaHS+D group were higher in comparison with distention group (*P<0.01*).

Discussion

The present study showed that 1.mRNA and protein expression of TGF-α were upregulated following stimulated gastric acid secretion in mucosal layer of the stomach in rats; and 2. These upregulatory effects were higher than in animals pretreated with NaHS. The present study showed the excitatory action of stimulated acid output on gene and protein expression of TGF-α. This result showed that gastric acid acted as an inducer for TGF-α expression.

It has been shown that water immersion restraint stress (WIRS) increases the gastric acid output (19). Another study showed that the expression of TGF-α increases following WIRS (20). Therefore, these findings together showed that the level of TGF-α increases by stimulated acid output. Exogenous TGF-α has been revealed to inhibit histamine-induced gastric acid output in pylorus-ligated rats (20). In addition, an *in vitro* study also showed that exogenous TGF-α inhibited the gastric acid output in response to histamine in guinea pig (21).

The increment of TGF-α level following the stimulation of acid output as shown by the present study and TGF-α effect on gastric acid secretion as shown by previous studies implied an important physiological message that, TGF-α could be a significant regulator for gastric acid secretion. The upregulatory action of NaHS on protein and gene expression of TGF-α was the major finding of the present study. The antisecretory and mucosal protective effects of NaHS in the rat stomach are well established (12, 22, 23). It has been shown that both endogenous and exogenous hydrogen sulfide inhibits distention-induced gastric acid secretion via a

NO-dependent pathways (12). Besides, it acted via TGF-α in rodents (24). These findings together suggest that there is a common signaling pathway for mediating the effect of TGF-α and hydrogen sulfide on gastric acid secretion.

As shown in the present study, the expression of TGF-α in animals pretreated with sodium hydrosulfide was more than that in un-treated rats. The previous work of the authors showed that the acid output in rats given sodium hydrosulfide in response to stimulated acid output was lower than that in un-treated rats (12). Therfore, these results proposed that TGF-α could be a mediator for anti-secretory effect of NaHS. The stronger mucosal NO response to NaHS-treated rats, as shown by authors in a previous work, may reflect the effect of NaHS on TGF-α levels.

Moreover, it is observed that TGF-α suppresses acid secretion through stimulatory effect on somatostatin (25). Taken together, it has been shown that it inhibits acid secretion either directly by affecting the parietal cells or indirectly by suppressing the histamine releasing (26) in which the later mechanism may be carried out via stimulatory effect of NO on D cells, which secrete somatostatin (27). As stated previous, some effects of TGF-α on stomach include stabilizing the gastric mucosal integrity, increasing mucosal blood flow, and reducing gastric acid secretion. Other findings indicated that H_2S increased $HCO3^-$ secretion in stomach through several mediators such as NO, prostaglandins and capsaicin-sensitive sensory neurons (10).

Conclusion

The results of the current research showed that the protein and gene expression levels of TGF-α in mucosal tissue of the rat's stomach increase by distention-induced gastric acid output. These responses are potentiated by the administration of NaHS. These findings suggested that TGF-α plays a mediatory role in the acid inhibitory effect of NaHS.

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