Protective effects of DIDS against ethanol-induced gastric mucosal injury in rats

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The compound 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) is an efficient anion exchanger inhibitor that can block the activities of anion exchanger 2 (AE2), which plays an indispensable role in gastric acid secretion. DIDS also has potent anti-oxidative and anti-apoptosis activities. This study aimed to investigate the effect of DIDS on ethanol-induced mucosal damage in rats and to evaluate the underlying mechanisms that mediate the action of the compound. The rats received 1 ml of absolute ethanol or saline orally. DIDS [50 mg/kg intravenous (i.v.)] was given 5 min before ethanol administration. Gastric lesions were evaluated macroscopically, microscopically, and electron microscopically at 60 min after ethanol challenge. Gastric myeloperoxidase (MPO) activity, malonyldialdehyde (MDA) level, prostaglandin E2 (PGE2) synthesis, and cyclooxygenase-2 (COX-2) expression were assessed. For the evaluation of the effect of DIDS on gastric acid secretion, histamine-stimulatory gastric acid secretion was examined with or without pretreatment of DIDS (50 mg/kg; i.v.). Ethanol-induced gastric lesions were characterized by increasing gastric MDA level, MPO activity, and COX-2 expression, and decreasing PGE2 synthesis. It was found that DIDS significantly reduced the extent of gastric mucosal damage and reversed tissue MDA level and MPO activity. DIDS further enhanced the expression of COX-2 and reversed the decrease of PGE2. Our results suggested that DIDS is beneficial in rat model of gastric injury through mechanisms that involve inhibiting inflammatory cell infiltration and lipid peroxidation and up-regulating the COX-2/PGE2 pathway.

Keywords DIDS; gastric injury; MPO; MDA; COX-2; PGE2

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Introduction

The gastric mucosa is constantly exposed to potentially noxious substances, hydrochloric acid, and proteolytic enzymes, but the stomach usually remains its structural and functional integrity because of tight intercellular junctions and secretion of bicarbonate to neutralize the gastric acid [1]. However, a variety of agents can induce gastric injury if they overwhelm gastric defenses. Oral consumption of excessive alcohol results in acute hemorrhagic lesions, mucosal edema, epithelial exfoliation, and inflammatory cell infiltration in the stomachs of humans and experimental animals [2,3].

The molecular mechanism of ethanol-induced gastric injury has not yet been completely elucidated. In addition to a toxic diet, several factors including generation of oxygen-derived free radicals, lipid peroxidation, gastric acid back diffusion, genetic damage, submucosal vasodilatation, release of inflammatory mediators, and decreased prostaglandin content are proposed to be involved in the pathway [4,5]. In particular, the effects of oxidative stress [6,7] and decreased prostaglandin level [8] on gastric lesions have been extensively studied.

The compound 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), a classic inhibitor of anion transport, inhibits anion exchange across the plasma membrane of cells and regulates intracellular pH, which, in the stomach, is related to gastric acid secretion [9]. In addition to inhibiting anion transporter activity, DIDS also has potent anti-oxidative and anti-apoptosis activities. DIDS protects oxidative lung injury through inhibiting production of superoxide anions in red blood cells [10]. It also protects stress-induced gastric lesions and prevents reperfusion arrhythmias in rats [11]. It rescues neuronal death under various stimuli such as hypoxia and removal of growth factors [12,13]. However, the mechanisms for these effects are not completely clear.
In the present study, we aimed to investigate the effect of DIDS on gastric acid secretion in rats. We also investigated the effect of DIDS on ethanol-induced gastric injury in rats and to explore the underlying mechanisms that mediated the action of this compound.

**Materials and Methods**

**Animals**

Male Sprague–Dawley rats (240–260 g; n = 5–7 per group) were purchased from the Experimental Animal Center, Shanghai Jiaotong University School of Medicine (Shanghai, China). Rats were housed under standardized conditions of light and temperature and were deprived of food for 24 h before the experiments, but allowed free access to tap water. Animals were divided into five groups: animals were treated with (i) saline instead of DIDS, (ii) histamine, (iii) DIDS and histamine, (iv) ethanol, (v) DIDS and ethanol. All experimental procedures described were approved by the Experimental Animal Research Committee of School of Medicine, Shanghai Jiaotong University.

**Gastric secretion study**

The acid–base content of gastric secretions was determined according to previous reports [14,15]. In brief, 15 min after subcutaneous injection of histamine (5 mg/kg) or 60 min after oral treatment of 1 ml of ethanol, each rat was anesthetized with an intraperitoneal injection of urethane (1.25 g/kg). The whole stomach was exposed through a midline laparotomy and clamped at the pyloric and gastroesophageal junction prior to removal. DIDS [50 mg/kg; intravenous (i.v.)] was administered 5 min prior to the administration of histamine or ethanol. The stomachs were then immersed in 5 ml of physiological saline solution at room temperature, opened along the lesser curvature, and rinsed of their contents. The gastric contents were collected and centrifuged. The gastric acid in the supernatants was determined by titration with 0.1 M NaOH, pH 7.0. The gastric output was expressed as the amount acid produced per gram of stomach (μEq/g).

**Animal model for gastric injury**

Under light urethane anesthesia, gastric hemorrhagic damage was induced by intragastric administration of 1 ml of absolute ethanol into each rat [16]. Control animals received 1 ml of physiological saline. DIDS (50 mg/kg; i.v.) was administered 5 min prior to the administration of ethanol. Animals were killed 60 min after the administration of ethanol. The stomach of each animal was removed, opened along the greater curvature, and rinsed with physiological saline. Gastric mucous samples were taken for macroscopic, histological examination, electron microscopic observation, and for the measurement of malonyldialdehyde (MDA) level and myeloperoxidase (MPO) activity.

**Determination of gastric damage**

For histological evaluation, gastric sections were stained with hematoxylin and eosin, and observed under a light microscope. Each section was scored histologically on a 0–3 scale according to the previously described method [17] (epithelial exfoliation, mucosal hemorrhage, glandular damage, and neutrophil infiltration were scored from 0 to 3, where 0 represents none, 1 represents mild, 2 represents moderate, and 3 represents severe). The maximum total score was 12. For electron microscopic examination, small pieces (about 2 mm³) of the gastric corpus were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 1 day. The specimens were then fixed in 1% osmium tetroxide, dehydrated through a graded ethanol series, and embedded in Epon 618 (TAAB Laboratories Equipment, Berks, UK). Ultra-thin sections of the gastric mucosa were stained with lead citrate and uranyl acetate, and then examined under a transmission electron microscope (Philip CM-120, Eindhoven, Netherlands) at 100 kV.

**Measurement of MDA level**

Gastric samples were homogenized with final concentration of 5% trichloroacetic acid and boiled at 95°C for 80 min. After cooling, samples were centrifuged at 800g for 10 min at 4°C. The supernatant was removed and concentrations of thiobarbituric acid-reactive substances were measured and calculated by the absorbance coefficient of the malondialdehyde–thiobarbituric acid complex [18]. Lipid peroxide levels were expressed in terms of MDA equivalents (in nmol/g).

**Measurement of MPO level**

Gastric mucosa was homogenized in phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide. The homogenized samples were subjected to two cycles of freezing and thawing. MPO activity was determined by adding 0.2 ml of o-dianisidine hydrochloride and 0.0005% hydrogen peroxide into 4 ml buffer containing 0.2 ml tissue sample using an MPO activity measurement kit (Jiancheng Biology, Nanjing, China). The change in absorbance at 460 nm for each sample was
recorded on a spectrophotometer [19]. Gastric MPO activity is an indicator of neutrophil accumulation and is expressed in units per gram tissue.

Determination of PGE2 formation using enzyme immunoassays

The gastric mucosa was weighed, minced with scissors, and homogenized at 4°C in PBS buffer. Homogenates were centrifuged at 13 400 g for 10 min, and the supernatants were subjected to a PGE2 assay using a PGE2 Monoclonal Enzyme Immunoassay Kit (BPB Biomedicals, Franklin, USA), according to the manufacturer’s instructions [20,21].

Gastric mucosal immunohistochemistry for cyclooxygenase-2 (COX-2)

Paraffin-embedded gastric tissue was selected from each experimental case. Four-micrometer thick sections were dewaxed and rehydrated using xylene and alcohol. Before immunostaining, antigen retrieval was performed by immersing sections in a 10-mM concentration of citrate buffer (pH 6.0) and boiling in a pressure cooker for 15 min followed by cooling at room temperature for 20 min. Samples were then incubated for 10 min in 3% H2O2 to block endogenous peroxidase activity. Sections were then incubated overnight at 4°C with rabbit monoclonal antibodies against COX-2 (dilution 1:200; Thermo Fisher Scientific, Fremont, USA). HRP-conjugated goat anti-rabbit secondary antibodies (GeneTech, Shanghai, China) were used as a secondary detection system. The peroxidase reaction was developed using diaminobenzidine tetrachloride as chromogen [22,23]. Immunoreactivity in gastric mucosa was graded by intensity score (0–3: 0, negative staining; 1, weakly staining; 2, moderate staining; 3, intense staining).

Statistical analysis

Results are expressed as mean ± SEM. The macroscopic and microscopic lesion scores and other parameters were analyzed using ANOVA. P < 0.05 was regarded as significant differences. All statistical procedures were performed with the SPSS statistical analysis package.

Results

Macroscopic and microscopic evaluation of gastric lesions

In rats treated with absolute ethanol, multiple erosions and severe bleeding were developed in the glandular stomach 60 min after ethanol administration. In contrast, pretreatment with DIDS 5 min before ethanol administration significantly inhibited the gastric lesions. The protective effects of DIDS were also confirmed histologically. Ethanol administration resulted in significant epithelial desquamation, hemorrhage, and inflammatory cell infiltration in the mucosa. In the DIDS pretreated group, the epithelial desquamation was largely prevented. The glands showed a moderate degree of injury. Slight inflammatory cell infiltration and limited hemorrhagic regions were observed. Electron microscopic examination was used to evaluate the protective effects of DIDS on parietal cells and other epithelial cells. The results showed that ethanol administration resulted in severe damage to parietal cells and DIDS pretreatment prevented the damage. We did not find the differences in the severity of injury between parietal cells and other epithelial cells. The micrographs further confirmed the protection by DIDS against gastric injury (Figs. 1, 2 and Table 1).

DIDS-reduced stimulated gastric acid secretion

According to previous reports [14,15], gastric secretion was collected 15 min or 60 min after pylorus ligation and the acid–base content was evaluated. Histamine and absolute ethanol, respectively, induced a significant and moderate increase in gastric acid secretion that was reversed by pretreatment of DIDS (Fig. 3).

Effects of DIDS on gastric mucosal MDA level and MPO activity in rats exposed to ethanol

Ethanol administration caused a significant increase in gastric MPO activity (2.26 ± 0.75 U/g) compared with the control group (0.37 ± 0.08 U/g). Pretreatment with DIDS prevented the increase in gastric MPO activity (0.75 ± 0.24 U/g) [Fig. 4(A)]. The ethanol administration increased gastric MDA, a product of tissue lipid peroxidation (8.37 ± 2.94 nmol/g protein) when compared with controls (1.52 ± 0.40 nmol/g protein). MDA level was also prevented by DIDS treatment (2.96 ± 0.94 nmol/g protein) [Fig. 4(B)].

Involvement of the COX-2/PGE2 system in DIDS-induced gastric protection

The involvement of the COX-2/PGE2 system in the protective effects of DIDS was studied. Rats were pretreated with DIDS before ethanol administration. As shown in Fig. 5, COX-2 was very slightly expressed in the normal gastric mucosa. Ethanol increased the COX-2 expression, which was diffuse over the gastric mucosa. The expression of COX-2 was further increased in DIDS pretreated animals. To assess whether DIDS induced PGE2 release,
the gastric mucosa obtained from the same experimental animals were used to measure PGE2 levels. PGE2 was decreased (−46%) in gastric mucosa stripped from the injured stomachs compared with tissue from normal rats (1.56 ± 0.34 and 2.87 ± 1.03 ng/mg protein, respectively). DIDS increased PGE2 levels compared with the ethanol untreated group (3.33 ± 1.08 ng/mg protein) (Fig. 6).

**Discussion**

Exogenous compounds, mainly acetylsalicylic acid, non-steroid anti-inflammatory drugs and high levels of ethanol cause erosive lesions in the gastrointestinal mucosa. Acute ethanol challenge induces oxidative stress, acid back diffusion, DNA damage, increases xanthine oxidase activity, and malondialdehyde levels, and decreases prostaglandin content in gastric mucosal cells. Ethanol increases superoxide anion and hydroxyl radical production and lipid peroxidation in the gastric mucosa [5].

Ethanol-induced tissue damage in rodents is a useful model for evaluation of ethanol-induced pathologic changes in gastric mucosa [24]. Ethanol-induced gastric mucosal lesions can be produced reliably by intragastric administration of varying amounts (0.5–2 ml) of concentrated (50–100%) solutions of ethanol [25]. In our experiment, the amount of ethanol was 1 ml, and the...
concentration was 100%. Our results showed that 1 ml of absolute ethanol stimulated the secretion of gastric acid, and DIDS inhibited the ethanol-induced acid secretion in rats. DIDS inhibited gastric acid secretion because it could efficiently inhibit the $Cl^-/HCO_3^-$ exchange activity of AE2, which was located on the plasma membrane of parietal cells, supply $Cl^-$ influx for its apical secretion and extrude $HCO_3^-$. This resulted in acidification of the cytosol and allows apical proton secretion [28]. Our data indicated that DIDS was a potent gastric antisecretory agent. Because gastric acid back diffusion was one of the important factors involved in the ethanol-induced gastric mucosal injury, the protective properties of DIDS appeared to be partly related to gastric acid inhibition.

Neutrophils often migrate toward inflammatory sites in the earlier phases of inflammation and play an important role in host defenses [29]. But, neutrophils generate reactive oxygen radicals and oxygen radical-mediated lipid peroxidation, which may be potentially harmful to normal tissue [30]. It has recently been proposed that neutrophil and oxygen radical-dependent lipid peroxidation may be important prime events that lead to mucosal injury induced by non-steroidal anti-inflammatory drugs [31]. In the present experiment, we evaluated injury based on epithelial desquamation, mucosal hemorrhage, glandular damage, and neutrophil infiltration and found that DIDS protected against gastric injury for all four aspects. Among these four aspects, neutrophil infiltration in gastric mucosa was kept at grade 1 (mild level), suggesting that the inhibition of neutrophil infiltration is an important mechanism for the effects of DIDS. Ethanol administration caused an increase in mucosal MPO activity and MDA level, and these effects were abolished by pretreatment of DIDS. This also indicated that the effect of the compound was associated with the inactivation of neutrophils and the decrease of lipid peroxidation.

PGE2 is the most abundant gastrointestinal prostaglandin and it regulates functions of the gut, including

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The gastric injury was scored using a scale ranging from 0 to 3 described in the materials and methods. E, ethanol; D, DIDS.

Fig. 2 Electron microscopic examination of gastric mucosa Electron microscopic examination of the gastric mucosa in control, untreated ethanol and DIDS pretreated groups. (A) Control group micrographs depict normal parietal cell. (B) Untreated ethanol group micrographs indicate severe damaged parietal cell. (C) The micrographs of the group pretreated with DIDS represent a slightly damaged parietal cell. Magnification, × 7400.

Table 1 Microscopic score of gastric lesions in each experimental animal
motility and secretion. PGE2 has also been shown to exert a protective action on the stomach through the activation of EP receptors. According to previous studies, prostaglandins were synthesized mainly through two cyclooxygenase (COX) isoforms, COX-1 and COX-2. COX-1 appears to be responsible for the production of PG that is physiologically important for homeostatic functions, such as maintenance of the mucosal integrity and mucosal blood flow. Under physiological conditions, prostanoid synthesis depends upon the availability of arachidonic acid and the COX-1 activity. While COX-2 is not constitutively expressed in most of the tissues but is dramatically up-regulated during inflammation and injury. We found that exposure to ethanol produced a significant fall in PGE2 generation in the gastric mucosa despite overexpression of COX-2. This result might be explained by other reports in which the authors described that in the presence of oxidative damage, the PGs could be converted into products of oxidation such as 8-iso-PGF2alpha. In addition, oxidative stress could inhibit COX activity, thus reducing PG levels. In our study, this fall in PGE2 level was counteracted by DIDS, indicating that PGE2 were responsible for the putative beneficial effects of DIDS in ethanol-induced mucosal injury. On the other hand, recent reports had indicated that PGE2 was related to the up-regulation of COX-2. Thus, DIDS-enhanced synthesis of PGE2 might be related to the over-expression of COX-2. Our results were in agreement with recent reports that demonstrated that the suppression of COX-2 activity by rofecoxib aggravated mucosal damage indicating that COX-2/PGE2 were involved in inhibition of neutrophil activation and attenuation of the oxidative stress in the gastric mucosa.

In parietal cells, AE2 mediates the exchange of Cl⁻/HCO₃⁻ in basolateral membrane and compensates for luminal H⁺ pumping while providing Cl⁻ for apical secretion. Gastric acid secretion across the apical membrane of the parietal cell is closely related to the AE2 activity. DIDS inhibited the stimulating gastric acid secretion by blocking the activity of AE2 and protected against ethanol-induced gastric injury partly through inhibiting ethanol-induced gastric acid secretion and via inhibition of neutrophil infiltration and lipid
peroxidation. The protective effects of DIDS were also related to the overexpression of COX-2 and the maintenance of PGE2 level.

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