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Evidence that D-cysteine protects mice from gastric damage via hydrogen sulfide produced by D-amino acid oxidase

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ABSTRACT

Hydrogen sulfide (H_2S) is a signaling molecule in the gastrointestinal tract. H_2S production can derive from D-cysteine via various pathways, thus pointing to a new therapeutic approach: delivery of H_2S to specific tissues. This study was designed to evaluate the concentration and effects of H_2S (generated by D-amino acid oxidase [DAO] from D-cysteine) in the gastric mucosa and the protective effects against ethanol-induced lesions in mice. Mice were treated with L-cysteine or D-cysteine (100 mg/kg *per os*). Other groups received oral L-propargylglycine (cystathionine γ-lyase inhibitor, 100 mg/kg) or indole-2-carboxylate (DAO inhibitor), and 30 min later, received D- or L-cysteine. After 30 min, 50% ethanol (2.5 mL/kg, *per os*) was administered. After 1 h, the mice were euthanized and their stomachs excised and analyzed. Pretreatment with either L-cysteine or D-cysteine significantly reduced ethanol-induced lesions. Pretreatment of D-cysteine- or L-cysteine-treated groups with indole-2-carboxylate reversed the gastroprotective effects of D-cysteine but not L-cysteine. Histological analysis revealed that pretreatment with D-cysteine decreased hemorrhagic damage, edema, and the loss of the epithelium, whereas the administration of indole-2-carboxylate reversed these effects. D-Cysteine also reduced malondialdehyde levels but maintained the levels of reduced glutathione. Furthermore, pretreatment with D-cysteine increased the synthesis of H_2S. Thus, an H_2S-generating pathway (involving D-cysteine and DAO) is present in the gastric mucosa and protects this tissue from ethanol-induced damage by decreasing direct oxidative damage.

1. Introduction

Hydrogen sulfide (H_2S) participates in the homeostasis of numerous systems serving as a mediator of various physiological or pathological processes, and novel therapies are being developed to suppress H_2S production or to enhance its availability [1,2]. H_2S is a signaling molecule involved in signal transduction in the nervous system, in the circulatory system, and in many organs [3] including the gastrointestinal (GI) tract, which is a major site of H_2S production [2]. This molecule is a neuromodulator [4] and an endogenous regulator of acute inflammation [5,6] and pain [7,8]. It has also been demonstrated that H_2S contributes to the maintenance of gastric mucosal integrity after damage by various aggressive agents [9–11]. Furthermore, H_2S participates in ulcer repair, regulates gastric mucosal blood flow, and contributes to GI motility [9,12,13].

Until recently, researchers had assumed that cystathionine γ-lyase (CSE) and cystathionine β-synthetase (CBS)—two pyridoxal 5′-phosphate-dependent enzymes—are the only enzymes producing endogenous H_2S and that the only substrate for the production of endogenous H_2S is L-cysteine, a sulfur-containing amino acid derived from food sources [14]. Nevertheless, it was demonstrated that H_2S is also synthesized in mammalian cells by two other enzymes: cysteine aminotransferase (CAT) and 3-

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mercaptopyruvate sulfurtransferase (3MST) [3,15]. The gastric mucosa expresses both CSE and CBS, which can drive H_2S synthesis [9], but studies on the CAT- and 3MST-generated H_2S in the GI tract are scarce [16].

Recent research showed that H_2S production can also be based on D-cysteine [17,18]. The pathway of H_2S production from D-cysteine is different from the pathways involving L-cysteine. D-Cysteine is metabolized by D-amino acid oxidase (DAO) to an achiral 3-mercaptopyruvate, which is also produced by CAT from L-cysteine in the presence of α-ketoglutarate [19]. D-Cysteine is less toxic than L-cysteine and represents a new therapeutic approach: delivery of H_2S to specific tissues [17]. The D-cysteine-dependent pathway is present predominantly in the brain and kidneys, and in the latter, the synthesis of H_2S from D-cysteine is more prevalent than the synthesis from L-cysteine; this situation protects the renal cortex more efficiently [17,18]. In a recent study [20], researchers reported a novel pathway for the production of H_2S by DAO in the rat small intestine, indicating that DAO-produced H_2S (from D-cysteine) may be important for GI functions and integrity. Nonetheless, there are no reports about the levels and effects of H_2S generated by DAO from D-cysteine in the gastric mucosa or about the protective effects in the stomach. Thus, in this study, we evaluated the effect of H_2S (produced by DAO from D-cysteine) on ethanol-induced gastric mucosal lesions in mice.

2. Methods

2.1. Reagents

D-Cysteine, L-cysteine, indole-2-carboxylic acid (I2CA), L-propargylglycine (PAG), and ethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). The volume of substances given is 0.5 ml/25 g of weight. All other reagents were of analytical grade and were acquired from standard commercial suppliers. When appropriate, these reagents were dissolved in saline before use.

2.2. Animals

Mice (Swiss strain, 25–30 g) of both sexes were obtained from the Vivarium Sector of the Center for Research on Medicinal Plants, Federal University of Piauí. The animals were maintained in cages in the laboratory at a temperature of 22 °C ± 1 °C in a 12-h light/12-h dark cycle. The mice had free access to drinking water and a standard pellet diet. The experimental groups contained 5–6 animals each. The animals were deprived of food for 18–24 h before the experiments but had free access to water. All animal treatments and surgical procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and were approved by the local ethics committee (Protocol Nº 068/14).

2.3. Effects of D-cysteine on ethanol-induced gastric damage

The animals were initially pretreated with vehicle (0.9% saline), D-cysteine (100 mg/kg), or L-cysteine (100 mg/kg) orally [21]. After 30 min, 50% ethanol (0.5 mL per 25 g) was administered by gavage. One hour after ethanol administration, the mice were euthanized, and stomach samples were collected for biochemical assays and macroscopic and histopathological analyses of the gastric mucosa [10]. Gastric damage was measured using a computer planimetry program (ImageJ software; National Institutes of Health, Bethesda, MD, USA). Samples of the stomach were fixed in 10% formalin immediately after their collection for subsequent histopathological assessment. Unfixed samples were weighed, frozen, and stored at −80 °C until quantification of reduced glutathione (GSH) [22] and malondialdehyde (MDA) concentrations [23].

2.4. The role of H_2S generated by DAO from D-cysteine in ethanol-induced gastric damage

To determine this role, mice were treated orally with I2CA, a DAO inhibitor, or received L-propargylglycine (an inhibitor of CSE; 50 mg/kg). All doses and the route of administration were chosen according to the studies by Medeiros et al. [10] and Xiao et al. [21] and previous studies of our research group. After 30 min, the mice received D-cysteine (100 mg/kg, by gavage) or L-cysteine (100 mg/kg, by gavage). Thirty minutes later, gastric damage was induced by intragastric instillation of 50% ethanol (0.5 mL per 25 g). One hour later, gastric damage was quantified as described above. Finally, a sample of the corpus region of each stomach was fixed in 10% formalin for subsequent histopathological assessment, and other full-thickness pieces of the gastric corpus were weighed, frozen, and stored at −70 °C until measurement of GSH and MDA concentrations.

2.5. Histological evaluation of the gastric damage

For this purpose, the glandular stomach was fixed in a 10% neutral-buffered formalin solution for 24 h. After that, the samples were transferred to a 70% solution of alcohol, sectioned, and embedded in paraffin. Four-micrometer-thick slices were deparaffinized, stained with hematoxylin and eosin, and then examined under a light microscope by an experienced pathologist who was blinded to the treatment. The tissue samples were assessed according to previously described criteria [24] and were assigned scores on the following parameters: epithelial cell loss (a score of 0–3), edema in the upper mucosa (a score of 0–4), hemorrhagic damage (a score of 0–4), and the presence of inflammatory cells (a score of 0–3), yielding a maximal possible score of 14.

2.6. Quantification of H_2S in gastric tissue

Isolated gastric samples were obtained by the previously described procedure, and were used to determine the levels of H_2S by the method described in Qi et al. [25] and Gu et al. [26]. This method allows the indirect quantification of H_2S levels in tissues by the use of an antioxidant buffer diluted in the sample of interest. This buffer contains sodium salicylate, ascorbic acid, and NaOH, which convert all H_2S into the S_2^- ion, which can be measured by a sulphide ion microelectrode. Using this protocol, the gastric samples were weighed and homogenized in diluted antioxidant buffer. The homogenate was inserted into the sulphide ion microelectrode (Lazar Research Laboratories, Los Angeles, CA, USA) connected to a pH meter (model 6230M, Jenco Instruments, San Diego, CA, USA) and the levels of S_2^- were measured following the manufacturer's recommendations. The standard solutions for the calibration curve were prepared using NaHS as the sulphide donor.

2.7. The glutathione assay

GSH content in stomach tissues as non-protein sulfhydryls was estimated according to the method described by Sedlak and Lindsay [22]. A glandular segment from each stomach was homogenized in 5 mL of an ice-cold 0.02 M EDTA solution (1 mL per 100 mg of tissue). Aliquots (400 μL) of the tissue homogenate were mixed with 320 μL of distilled water and 80 μL of 50% (w/v) trichloroacetic acid in glass tubes. The samples were then centrifuged at 3000 × *g* for 15 min, and the resulting supernatants (400 μL) were mixed with 800 μL of Tris-HCl buffer (0.4 M, pH 8.9), followed by addition of 20 μL of 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB; 0.01 M). After