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A novel pathway for the production of H_2S by DAO in rat jejunum

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Key Messages

- This study reveals a novel pathway for the production of H_2S by D-Amino acid oxidase (DAO) in rat jejunum. D-cysteine may offer an attractive treatment for nutrition-related maladies, including a range of serious health consequences linked to undernutrition. The aim of this study was to investigate whether H_2S was generated from DAO in rat jejunum.
- The H_2S production was measured by the methylene blue assay. The expression of DAO was investigated by Western blotting and immunohistochemistry. The short-circuit current (*I*sc) was recorded using the Ussing chamber technique.
- The results show that H_2S can be generated from D-cysteine via DAO in rat jejunum.

Abstract

Background *Hydrogen sulfide (H_2S) is endogenously generated from L-cysteine (L-Cys) by the enzymes cystathionine-β-synthase (CBS) and cystathionine-γ-Lyase (CSE). Hydrogen sulfide is also produced from D-cysteine (D-Cys) by D-Amino acid oxidase (DAO).* ***Methods*** *The H_2S production was measured by the methylene blue assay. The expression of DAO was investigated by Western blotting and immunohistochemistry. The short-circuit current (Isc) was recorded using the Ussing chamber technique.* ***Key Results*** *The epithelium in rat jejunum possesses DAO, and generates H_2S. D-cysteine, originally used as a negative control for L-Cys, significantly increases the H_2S release, which is inhibited by I2CA, an inhibitor of DAO. In vitro study by Ussing chamber technique reveals that D-Cys decreases the Isc across the epithelium of the rat jejunum and enhances the Na^+-coupled L-alanine transport.* ***Conclusions & Inferences*** *A novel pathway for the production of H_2S by DAO exists in rat jejunum.*

Keywords *hydrogen sulfide, jejunum, D-cysteine, D-Amino acid oxidase, short-circuit current.*

INTRODUCTION

Beside nitric oxide (NO) and carbon monoxide (CO), hydrogen sulfide (H_2S) has been discovered recently as the third gasotransmitter. Hydrogen sulphide is produced from L-cysteine (L-Cys)by two pyridoxal 5′-phosphate (PLP)-dependent enzymes, cystathionine-β-synthase (CBS) and cystathionine-γ-Lyase (CSE), and PLP-independent 3-mercaptopyruvate sulfurtransferase (3MST).[1–5] 3-mercaptopyruvate sulfurtransferase produces H_2S from 3-mercaptopyruvate (3MP), an achiral α-keto acid, which is generated by PLP-dependent cysteine aminotransferase (CAT) from L-Cys and α-ketoglutarate (α-KG).[6–8] Recently, H_2S is also produced from D-cysteine (D-Cys), originally used as a negative control for L-Cys.[9] Hydrogen sulfide-producing pathway from D-Cys is distinct from the pathways involving L-Cys. Hydrogen sulfide from D-Cys is

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generated by D-Amino acid oxidase (DAO).[10,11] Enzymes producing H_2S from L-Cys are expressed in many tissues.[1–3,5,10,12–14] Unlike the L-Cys pathways, the D-Cys pathway operates predominantly in the cerebellum and the kidney.[9,15] Interestingly, our present study identified a novel pathway for production of H_2S in rat small intestine.

METHODS

Experimental animals

Adult male Wistar rats (180–240 g) were purchased from the Animal Centre of Shandong University (Jinan, China). Rats were allowed free access to water but were fasted overnight before the experiments.

All experimental procedures were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals of Shandong University, and the study was approved by the Medical Ethics Committee for Experimental Animals, Shandong University, China (number ECAESDUSM 2013023).

Measurement of H_2S

The tissue H_2S production rate was measured as described previously.[3] In brief, rats were sacrificed by dislocation of the cervical spine. Jejunum (mucosa and submucosa, 0.1 g) was homogenized in ice-cold 50 mmol/L potassium phosphate buffer (pH = 6.8) with a polytron homogenizer. Homogenate (0.5 mL) was preincubated at 37 °C with or without inhibitor for 5 min in the outside slot of a 20-mL reaction vial. A piece of filter paper (0.5 × 1.5 cm) soaked with zinc acetate (1%; 0.5 mL) was put into the internal slot of the vial. The vial was flushed with a slow stream of nitrogen gas for 20 s before L-Cys or D-Cys (10 mmol/L final concentration) and pyridoxal 5′-phosphate (2 mmol/L final concentration) were added. The vial was then capped with an airtight serum cap and bathed at 37 °C. After 90 min, trichloroacetic acid (TCA; 50%; 0.5 mL) was added into the outside slot to stop the reaction. In the next 60 min, evolved H_2S was captured by the zinc acetate solution as zinc sulfide. The filter paper and zinc acetate solution was then removed into a tube where *N*,*N*-dimethyl-p-phenylenediamine sulfate (20 mmol/L; 0.5 mL) in 7.2 mol/L HCl and FeCl3 (30 mmol/L; 0.4 mL) in 1.2 mol/L HCl were added into. Twenty minutes later, absorbance at 670 nm was measured with a microplate reader (Spectra Max 190; Molecular Devices, Sunnyvale, CA, USA). A standard curve was generated with known concentrations of sodium hydrogen sulfide (NaHS).

Western blotting

Homogenized rat jejunum mucosa (about 0.1 g) was centrifuged (10 800 *g*) at 4 °C for 20 min. The lysate was separated by 10% SDS-PAGE gels. After electrophoresis, the separated proteins were transferred onto the PVDF membrane and blocked with 5% nonfat dried milk in Tris-HCL-buffered saline (TBS) containing 0.2% Tween-20 for 2 h at room temperature. Subsequently, the membranes were incubated with the appropriate primary antibody overnight at 4 °C (DAO:sc-26077, goat polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA, USA; final dilution 1 : 1000). Following three washes with TBST, the membrane was incubated with secondary antibody (ZB2301, rabbit anti-goat IgG horseradish peroxidase-conjugated antibodies, ZSGB biology; final dilution 1 : 20 000) for 1 h followed by three washes. The target protein was detected with chemiluminescence method (ECL amersham hyperfilm; Beyotime, Haimen, China).

Immunohistochemistry

Rat jejunum was fixed were fixed in 4% paraformaldehyde overnight and embedded in paraffin wax. The tissues were then sectioned (3–4 μm slices). After dewaxing and hydration, antigen retrieval was performed by boiling slices for 30 min in 10 mmol/L sodium citrate buffer (pH 6.0). Samples were blocked with 3% BSA (IHC grade) and incubated with primary antibody at 4 °C overnight. Primary antibody used was goat anti-DAO (1 : 200 dilution; Santa Cruz Biotechnology). As negative control, sections were incubated with a solution that did not contain the primary antibodies. Stainings were developed with a 3,3′-diaminobenzidine.

Measurement of short-circuit current (I_{sc})

The I_{sc} was measured using the Ussing chamber technique. Tissue preparations were according to that described previously.[16] In brief, adult male Wistar rats were sacrificed by dislocation of the cervical spine. Jejunum were cut into 1.5-cm-long pieces. These pieces were cut along the longitudinal axis and washed with cold Krebs' solution. The serosa and muscularis propria were stripped away to obtain a mucosa–submucosa preparation. The mucosa–submucosa preparations were bathed on both sides with 5 mL Krebs' solution, pH 7.4, gassed with 95% O_2, and 5% CO_2 (exposed area of 0.5 cm^2). The Krebs' solution was maintained at 37 °C during the experiments by circulation through a reservoir. The tissue was continuously voltage-clamped to zero potential difference by the application of external current, with compensation for fluid resistance (VCC MC4; Physiologic Instruments, San Diego, CA, USA). Baseline values of the electrical parameters were determined as a mean value over the 3 min immediately prior to drug administration. The tissue was equilibrated to these conditions for 30 min to stabilize I_{sc} prior to the addition of drugs. The change in the I_{sc} (ΔI_{SC}) was determined as the difference in values before and after stimulation.

Experimental agents

The composition of Krebs' solution was (in mmol/L): NaCl 120.6; KCl 5.9; $CaCl_2$ 2.5; KH_2PO_4 1.2; $MgCl_2$ 1.2; $NaHCO_3$ 15.4; glucose 11.5. For immunohistochemical experiments, phosphate-buffered saline was used containing (mmol/L): NaCl 135; KCl 2.7; KH_2PO_4 1.5; K_2HPO_4 8. For Western blot experiments, TBS was used containing (mmol/L): Tris 50; NaCl 150. The pH was adjusted to 7.4 with HCl. Tetrodotoxin (TTX) was from Ruifang (Dalian, China) and dissolved in citrate buffer. L-Cys and D-Cys were purchased from Sigma (St. Louis, MO, USA). Pyridoxal 5-phosphate and dimethyl aniline hydrochloride were from Aladdin (Shanghai, China). Zinc acetate, $FeCl_3$, and TCA were from Damao Chemical Reagent Company (Tianjin, China).

Statistical analysis

All data are expressed as means ± SEM of n animals. One-way ANOVA followed by *t*-tests was used to evaluate the significance

of differences between groups. $p < 0.05$ was considered significant.

RESULTS

A H_2S generation pathway from D-Cys exists in intestine epithelium

To determine whether the rat jejunum mucosa generates detectable amounts of H_2S, we performed a methylene blue assay to measure the H_2S level in jejunum mucosa homogenates. The results showed that the biosynthesis of H_2S was significantly increased after incubation of tissue homogenates with L-Cys, the CBS/CSE substrate (Fig. 1A). Notably, in the presence of D-Cys, the production of H_2S was dramatically increased, which was reduced by I2CA, an inhibitor of DAO (Fig. 1A–C). Of note, I2CA did not affect the H_2S production from L-Cys. These findings suggest that a H_2S generation pathway from D-Cys exists in intestine epithelium.

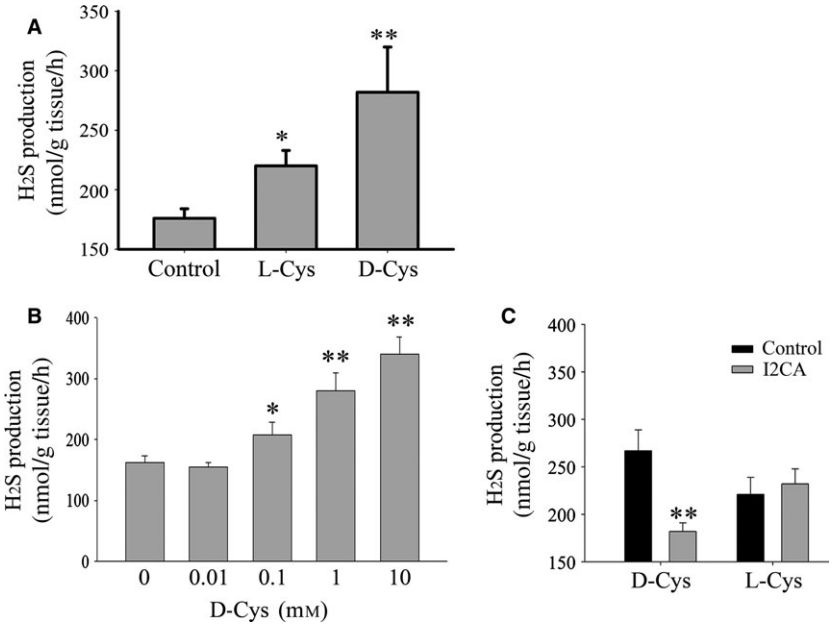

Figure 1 The production of H_2S from D-Cys in intestine epithelium. Rat jejunum mucosa homogenate produced H_2S under basal conditions (control). Incubation of rat jejunum mucosa homogenate with either L-Cys or D-Cys caused a significant increase in the H_2S production compared with basal values (A). D-Cys dose-dependently increased the production of H_2S rat jejunum mucosa (B). The DAO inhibitor indole-2-carboxylate (I2CA) significantly inhibited the D-Cys, but not L-Cys-induced increase in the H_2S production (C, $^{**}p < 0.01$). Data represent the mean ± SEM from 6 or 8 rats. $^{*}p < 0.05$; $^{**}p < 0.01$ *vs* control, 0 or D-Cys.

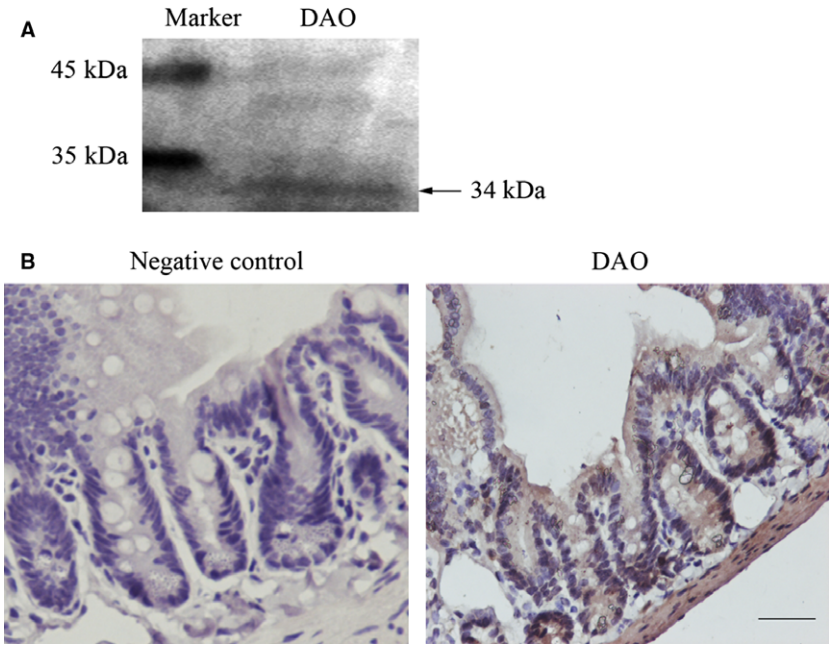

Figure 2 A DAO-derived H_2S generation pathway exists in intestine epithelium. Representative Western blot analysis for DAO in rat small intestine (A). Immunochemistry for DAO in rat jejunum (B). DAO was detected in absorptive cells near the tips of the villi and crypt cells of the jejunum. Immunoreactivity for DAO was also observed in smooth muscle cells of the jejunum. Results illustrated are from a single experiment and are representative of three different specimens, scale bar: 100 μm.

DAO that produces H_2S from D-Cys was localized to intestine epithelium

Next, we sought to determine if DAO was expressed in the small intestine. The results showed that DAO was expressed in the rat small intestine, which was localized to jejunum epithelium. D-Amino acid oxidase staining was also observed in the smooth muscle cells of the jejunum (Fig. 2).

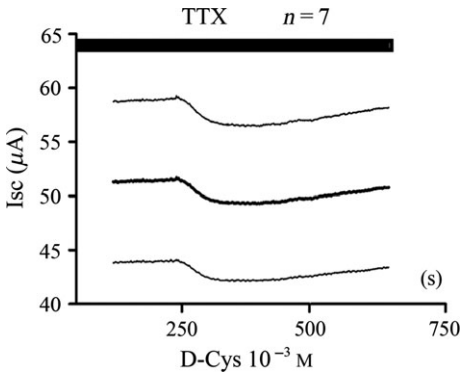

Figure 3 D-Cys causes a decrease in I_{sc} across the epithelium of the rat jejunum. D-Cys induced a rapid decline and subsequent recovery process in I_{sc}.

D-Cys causes a decrease of I_{sc} in rat jejunum

To investigate whether H_2S generated from D-Cys is functional, rat jejunum mucosa transepithelial I_{sc} was measured. In all experiments, the tissues were pretreated with TTX (10^{-5} mol/L at the serosal side), which blocks the propagation of action potentials. D-cysteine induced a rapid decline and subsequent recovery process in I_{sc} (Fig. 3), the pattern of which is similar to that of L-Cys (A. Xiao, J. Li, T. Liu, Z. Liu, C. Wei, X. Xu, Q. Li, J. Li, unpublished data).

D-Cys enhances the L-alanine absorption in rat jejunum *in vitro*

The change in I_{sc} represents the sodium ion transport coupling with L-alanine across epithelium. L-alanine (2×10^{-2} mol/L) induced an increase in I_{sc} (Fig. 4A). The serosal administration of D-Cys (10^{-3} mol/L) enhanced the L-alanine-evoked I_{sc} (Fig. 4B–D), which is a similar pattern as L-Cys (A. Xiao, J. Li, T. Liu, Z. Liu, C. Wei, X. Xu, Q. Li, J. Li, unpublished data).

DISCUSSION

Hydrogen sulfide is synthesized in the GI tract[17] and it produces various physiological and pathophysiological effects in gut, ranging from gastrointestinal motility, sensory to secretion.[18] Our previous studies have

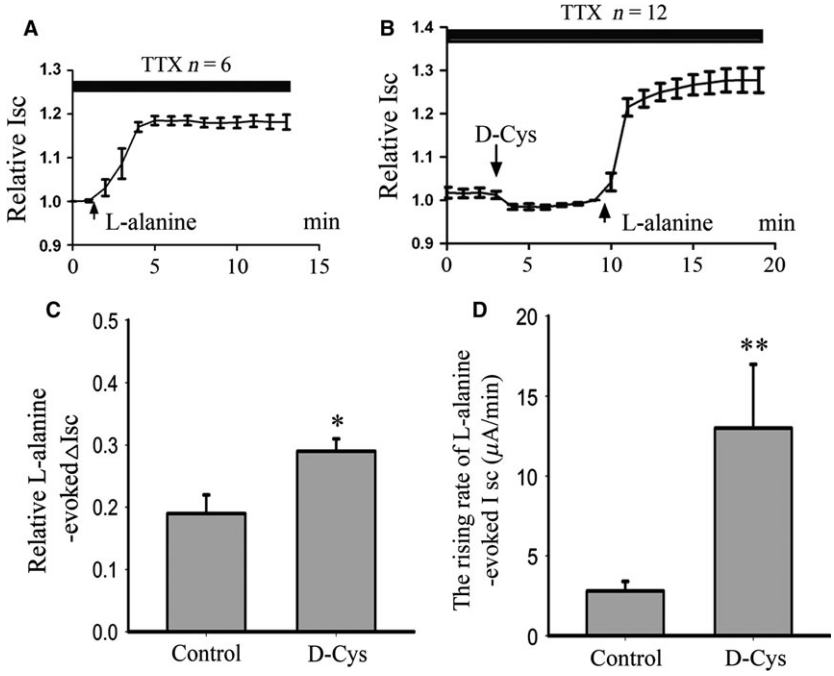

Figure 4 D-Cys enhances the L-alanine transport in rat jejunum. L-alanine (2×10^{-2} mol/L) induced an increase in I_{sc} (A). The change in I_{sc} represents the sodium ion transport coupling with L-alanine across epithelium. The serosal administration of D-Cys (10^{-3} mol/L) enhanced either the amplititude or the rate of L-alanine-evoked I_{sc} rise (B–D). $^*p < 0.05$; $^{**}p < 0.01$ *vs* Control.

demonstrated that both H_2S-generating enzymes, CBS and CSE, are distributed in rat small intestine epithelium (A. Xiao, J. Li, T. Liu, Z. Liu, C. Wei, X. Xu, Q. Li, J. Li, unpublished data). In the present study, we identified a novel pathway of H_2S production from D-Cys in rat small intestine epithelium.

H_2S is produced by DAO from D-Cys in rat jejunum mucosa

In gut, the previous findings, including our own, have demonstrated that CBS and CSE are expressed in over 90% of sub-mucous and myenteric neurons in human, guinea pig colon,[19] and rat small intestine.[20] In the present study, we identified that, besides the two key enzymes generating H_2S, DAO is also a primary enzyme responsible for the production of H_2S in rat jejunum mucosa.

There are at least two possible sources of D-Cys, namely, racemase-induced chiral change in L-Cys and absorption from food. D-cysteine is easily absorbed through the gastrointestinal tract.[21] L-cysteine is an excitotoxin comparable in potency to other excitatory amino acids.[22] In contrast, the subcutaneous administration of D-Cys does not cause excitotoxic damage to the brain.[23] Therefore, systemic application of D-Cys may be less toxic than that of L-Cys. The administration of D-Cys may provide a safety source of H_2S via a novel pathway with DAO.

Hydrogen sulfide stimulates basolateral epithelial K^+ channels in rat distal colon.[24] In practically all living cells, K^+ channels play a pivotal role for the generation and stabilization of a hyperpolarized membrane voltage. In epithelia, Na^+-dependent solute transporters are fueled by the chemical gradient of Na^+ and by the hyperpolarized membrane voltage.[25,26] The activation of basolateral K^+ channels[27] has been shown to enhance Na^+-coupled transport. In the present study, D-Cys induced a rapid decline and subsequent recovery process in I_{sc}, the pattern of which is as same as L-Cys (Fig. S1). We propose that basolateral K^+ channels may involve in D-Cys-evoked change in I_{sc}.

Of note, as similar as the role of H_2S from L-Cys in nutrient absorption from small intestine (A. Xiao, J. Li, T. Liu, Z. Liu, C. Wei, X. Xu, Q. Li, J. Li, unpublished data), H_2S from D-Cys also increased the absorption of nutrients.

In summary, our study unveils a novel pathway of H_2S production in small intestine. D-cysteine may offer an attractive treatment for nutrition-related maladies, including a range of serious health consequences linked to undernutrition.

FUNDING

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CONFLICTS OF INTEREST

None.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site:

Figure S1 The DAO inhibitor indole-2-carboxylate (I2CA) significantly inhibited the D-Cys, but not L-Cys-induced change in I_{sc}.