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ORIGINAL ARTICLE

Endogenous Hydrogen Sulfide Is an Anti-inflammatory Molecule in Dextran Sodium Sulfate-Induced Colitis in Mice

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Abstract
Background Endogenous hydrogen sulfide (H_2S) is increasingly being recognized as an important gaseous physiological mediator. Accumulating evidence shows the functions of H_2S in various models of disease, but rarely in colitis. In this study, we investigated the role of endogenous H_2S in a dextran sodium sulfate (DSS)-induced colitis model.
Methods Acute colitis was induced using 8% DSS in male BALB/c mice. The mRNA expression of cystathionine γ-lyase (CSE), the primary synthetase of H_2S in the gastrointestinal tract, and cystathionine-β-synthetase (CBS) was measured by real-time RT-PCR. The amount of H_2S in the colonic mucosa was measured by gas chromatography. Colitis severity was evaluated clinically, histologically, and biochemically under the condition of co-treatment with DL-propargylglycine (PAG), an irreversible CSE inhibitor, and sodium sulfide (Na_2S), an H_2S donor.
Results The mRNA expression levels of CSE and CBS, and the H_2S content in the colonic mucosa were increased with time after DSS administration. The disease activity index, which was determined by weight loss, stool consistency, and intestinal bleeding, increased after DSS administration. PAG significantly enhanced the increase in the disease activity index scores. PAG also significantly increased tissue-associated myeloperoxidase activity and thiobarbituric acid-reactive substances in the inflamed mucosa. Moreover, Na_2S counteracted these effects of PAG.
Conclusions Taken together, the results indicated that the inhibition of endogenous H_2S generation caused the deterioration of DSS-induced colitis. We conclude that physiological H_2S might act as an anti-inflammatory molecule in colitis.

Keywords Hydrogen sulfide · Dextran sodium sulfate · Inflammation · Colitis · Lipid peroxidation

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Introduction

Hydrogen sulfide (H_2S) is a colorless, water-soluble gas with the unpleasant smell of rotten eggs. Like nitric oxide (NO) and carbon monoxide (CO), H_2S has been recognized not only as a pollutant but also as an important gaseous physiological mediator [1]. H_2S is synthesized endogenously from L-cysteine in various mammalian tissues by two enzymes: cystathionine-β-synthetase (CBS) and cystathionine-γ-lyase (CSE). CBS is the predominant enzyme in brain, nervous system, liver, and kidney. CSE is expressed mainly in liver and in both vascular and non-vascular smooth muscles. Low levels of CSE are also detectable in the gastrointestinal tract of rodents [2]. It has been reported that H_2S mediates vasodilation and analgesic action by opening K^+_{ATP} channels [2]. The involvement of H_2S in inflammation has been studied in various disease models. Evidence is accumulating to show that H_2S exerts anti-inflammatory effects at low concentrations [3–7],

whereas some papers demonstrate the pro-inflammatory effects of H_2S [8–13]. H_2S can upregulate anti-inflammatory genes such as heme oxygenase-1 [14, 15], which leads to the production of CO, another anti-inflammatory gaseous mediator [16–19]. Furthermore, low levels of H_2S can upregulate the endogenous antioxidant systems [20] and can neutralize reactive species [21].

The aim of this study is to elucidate the involvement of H_2S in colitis, and to provide new insights into the pathogenesis of inflammatory bowel disease and into therapeutic approaches to it. We examined the change in the expression levels of H_2S-generating enzymes and the amount of H_2S in the inflamed colonic mucosa of mice. The effects of a donor and an inhibitor of H_2S on colitis and oxidative stress were also investigated.

Materials and Methods

Chemicals

All chemicals were prepared immediately before use. DL-propargylglycine and sodium sulfide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Thiobarbituric acid (TBA) and 3,3′,5,5′-tetramethylbenzidine were obtained from Wako Pure Chemical (Osaka, Japan). 1,1,3,3-Tetramethoxy propane was obtained from Tokyo Kasei (Tokyo, Japan). All other chemicals were of reagent grade.

Experimental Procedures

Six-week-old male BALB/c mice weighing 18–20 g were purchased from Shimizu Experimental Animals (Osaka, Japan). The mice were caged individually in a room kept at 18–24°C with 40–70% relative humidity and a 12-h light/dark cycle. They were allowed free access to food and drinking water. The mice were fed rodent diet CE-2 (Nihon Clea, Tokyo, Japan) during their 1-week acclimatization. Then, acute colitis was induced by 8.0% (w/v) DSS (Wako Pure Chemical) solution in the drinking water for 7 days. The intake of DSS solution was monitored throughout the experiments and was found to be unchanged among the experimental groups. PAG and Na_2S were dissolved in physiological saline. The mice were randomized into groups receiving either or both PAG (1, 10 mg/kg) and Na_2S (1, 10 μmol/kg) or physiological saline only, by intraperitoneal injection on each of the 7 days. Body weight, stool consistency, and blood in stool of each mouse were assessed on days 0, 3, 5, and 7. The mice were then killed on day 7 and their colons were removed for macroscopic and histological examination. The colon was resected and measured between the ileocecal junction and the proximal rectum, close to its passage under the pelvisternum, and then the colonic mucosa was scraped off and used for biochemical assay and RNA isolation. The maintenance of the animals and the experimental procedures performed on them were carried out in accordance with National Institutes of Health (NIH) guidelines for the use of experimental animals. All procedures were approved by the Animal Care Committee of Kyoto Prefectural University of Medicine (Kyoto, Japan).

Real-Time RT-PCR for *Cystathionine γ-Lyase* (CSE) and *Cystathionine-β-Synthetase* (CBS)

Mice were killed after DSS administration for 0–7 days, and then colonic mucosa was collected and used for RNA isolation. The expression of mRNA for CSE and CBS was quantified by real-time PCR. Total cellular RNA was extracted by the acid guanidinium phenol chloroform method using an Isogen kit (Nippon Gene, Tokyo, Japan). The concentration of RNA was determined by absorbance at 260 nm in relation to absorbance at 280 nm. RNA was stored at −70°C until reverse transcription was performed. An aliquot (1 μg) of extract RNA was reverse-transcribed into first-strand cDNA at 42°C for 40 min, using 100 U/ml reverse transcriptase (Takara Biochemicals, Shiga, Japan) and 0.1 μM of oligo(dT)-adapter primer (Takara) in a 20-μl reaction mixture. Real-time PCR was carried out with a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using the DNA-binding dye SYBER Green I for the detection of PCR products. The reaction mixture (RT-PCR kit, Code RRO43A; Takara) contained 12.5 μl Premix Ex Taq, 2.5 μl SYBER Green I, custom-synthesized primers, ROX reference dye, and cDNA (equivalent to 20 ng total RNA) to give a final reaction volume of 25 μl. The primers were as follows: for mouse *cystathionine γ-lyase*, sense primer 5′-GATGGCGGTGGCTCGTT-3′ and antisense primer 5′-GGTAGCCCAGGATAAACAACCTT-3′; for mouse *cystathionine-β-synthetase*, sense primer 5′-TGAACCAGACGGAGCAAACA-3′ and antisense primer 5′-CCAGGACTGTCGGGATGAAG-3′; and for *β-actin*, sense primer 5′-TGTCCACCTTCCAGCAGATGT-3′ and antisense primer 5′-AGCTCAGTAACAGTCCGCCTAGA-3′. The PCR settings were as follows: initial denaturation of 10 s at 95°C was followed by 40 cycles of amplification for 5 s at 95°C and 31 s at 60°C. The PCR products were quantified using standard DNA for each purified PCR product of the reverse-transcribed RNA. Relative expression was calculated as the density of the product of the respective target gene divided by that of *β-actin* from the same cDNA.

Measurement of H_2S Content in the Colonic Mucosa

Mice were killed after DSS administration for 0–7 days. The amount of H_2S in the colonic mucosa was measured by

gas chromatography (Biogas H_2S Analyzer BAS-3000; Mitleben R&D Associates, Osaka, Japan). Briefly, each homogenate of the colonic mucosa with 1 ml of saline was poured into a 10-ml vial with septum after vigorous stirring, and then 1,000 μl of pure water was added. H_2S gas was released by adding 500 μl of 100% trichloroacetic acid into each vial with a syringe through the septum, and then incubated at 40°C for 30 min after vigorous stirring. Then, 500 μm of head space gas was removed from the reaction tube and applied to the BAS-3000 port.

Evaluation of Colitis Severity

The disease activity index (DAI) was determined by scoring changes in body weight, blood in stool, and stool consistency, as described previously [22]. Occult bleeding was tested using a commercial kit based on the detection of the peroxidase activity of heme in the stool (Occult Blood Slide 5 Shionogi; Shionogi & Co., Osaka, Japan). The DAI score has been shown to correlate well with histological measures of inflammatory and crypt damage. We used five grades of weight loss (0, no loss or weight gain; 1, 1–5% loss; 2, 5–10% loss; 3, 10–20% loss; 4, >20% loss), three grades of stool consistency (0, normal; 2, loose; and 4, diarrhea), and three grades of occult blood (0, normal; 2, occult blood-positive; and 4, gross bleeding). The combined scores were then divided by three to obtain the final DAI scores. The colon was placed on a nonabsorbent surface and measured with a ruler. The entire colon was divided into three segments (proximal, middle, and distal), and a part of each segment was fixed in 10% neutral buffered formalin. After fixation, the specimens were embedded in paraffin, divided into 7-μm sections, and stained with H&E stain.

Measurement of MPO Activity and TBA-Reactive Substances

Tissue-associated myeloperoxidase (MPO) activity was measured by an alternative method of Grisham et al. [23] as an index of neutrophil accumulation. Two milliliters of mucosal homogenate was centrifuged at 20,000 × *g* for 15 min at 4°C to pellet the insoluble cellular debris. The pellet was then rehomogenized in an equivalent volume of 0.05 mol potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide. The samples were centrifuged at 20,000 × *g* for 15 min at 4°C, and the supernatants were saved. MPO activity was assessed by measuring the H_2O_2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine. One unit of enzyme activity was defined as the amount of MPO that altered the absorbance for 1.0 min under the conditions of 655 nm and 25°C.

The concentration of thiobarbituric acid (TBA)-reactive substances in the colonic mucosa was measured using the method of Ohkawa et al. [24] as an index of lipid peroxidation. After the experiments, the colonic mucosa was collected and homogenized with 1.5 ml of 10 mmol/l potassium phosphate buffer (pH 7.8) containing 30 mmol/l KCl in a Teflon Potter–Elvehjem homogenizer. The level of TBA-reactive substances in the mucosal homogenates was expressed as nanomoles of malondialdehyde per milligram of protein using 1,1,3,3-tetramethoxypropane as the standard. Total protein in the tissue homogenates was measured by the method of Lowry [25].

Statistical Analysis

All data are presented as means ± SEM. Comparisons of groups of data were performed using one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's post-tests. All analyses were performed using GraphPad Prism 5.01 for Windows (GraphPad Software, La Jolla, CA, USA). A p value of less than 0.05 was considered significant.

Results

CSE, CBS mRNA Expression, and H_2S Content in the Inflamed Mucosa

After DSS administration, mice presented with acute colitis including the symptoms of loose stool, melena, and severe weight loss.

The relative expression level of mRNA for CSE and CBS in the colonic mucosa increased from day to day after DSS administration. The CSE and CBS mRNA expression was significantly higher on day 7 than on day 0 (Fig. 1a). The expression level of CSE mRNA was more than 200 times larger than that of CBS mRNA on day 7.

The amount of H_2S in the colonic mucosa significantly increased from 0.02797 ± 0.002292 nmol/mg tissue, a basal concentration, to 0.06468 ± 0.009493 nmol/mg tissue 7 days after DSS administration (Fig. 1b).

Effects of PAG and Na_2S on DAI Scores, Colonic Length, and Histology

Colonic length decreased significantly after DSS administration for 7 days. The colons of the PAG (1, 10 mg/kg)-treated mice were significantly shorter than those of the vehicle-treated mice. The colon shortening after DSS administration was not affected by Na_2S at either dose of 1 or 10 μmol/kg (Fig. 2).

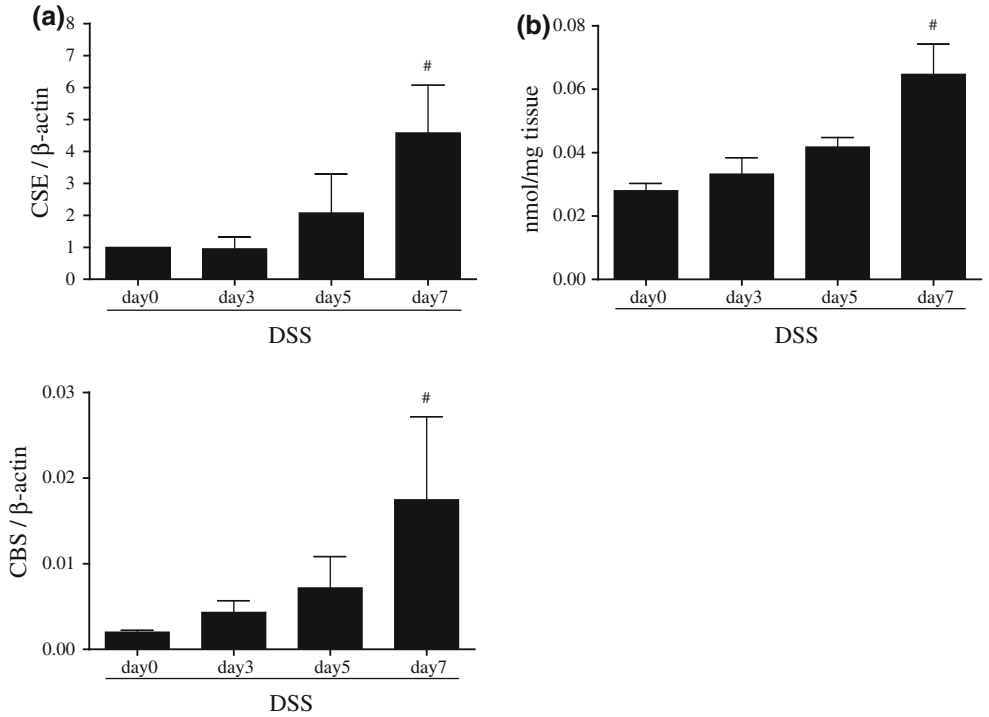

Fig. 1 Chronological relative expression level of mRNA for CSE and CBS (**a**) and the amount of H_2S (**b**) in the colonic mucosa of mice administered 8% DSS. Each value indicates the mean ± SEM for 5–7 mice. # $p < 0.05$ when compared to day 0

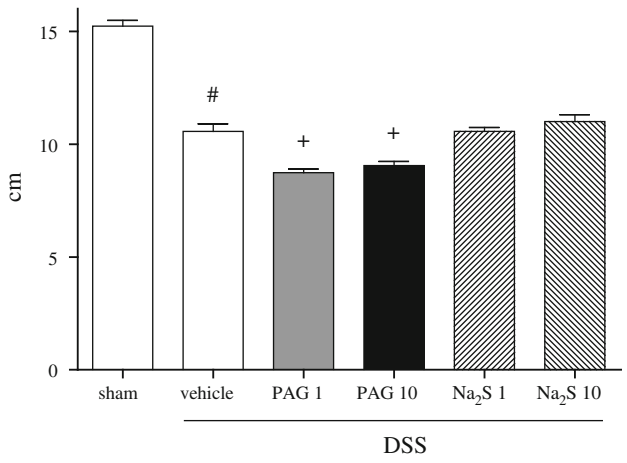

Fig. 2 Colon length after DSS administration for 7 days with or without PAG or Na_2S. Each value indicates the mean ± SEM for 5–7 mice. # $p < 0.05$ when compared to the sham group, and + $p < 0.05$ when compared to the vehicle-treated group

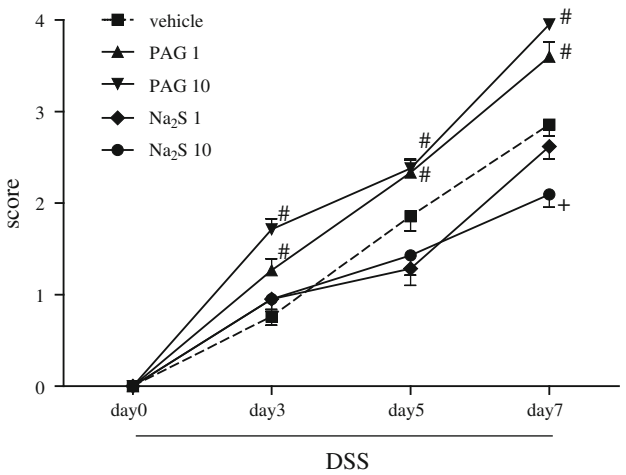

Fig. 3 The effect of PAG or Na_2S on DAI scores. Each value indicates the mean ± SEM for 7 mice. #, + $p < 0.05$ when compared to the vehicle-treated group

DSS administration elevated the DAI scores day by day. The DAI scores were higher in the PAG-treated mice than in the vehicle-treated mice (significant on days 3, 5, and 7 at both 1 and 10 mg/kg of PAG). In contrast, the DAI scores were lower in the Na_2S-treated mice than in the vehicle-treated mice (significant on day 7 at 10 μmol/kg of Na_2S) (Fig. 3). Treatment with PAG or Na_2S did not affect the DAI scores in sham mice (data not shown).

Therefore, we used PAG and Na_2S at concentrations of 10 mg/kg and 10 μmol/kg, respectively, in the following experiments.

The DAI scores were significantly higher in the PAG-treated mice than in the vehicle-treated mice on day 7. The increase in the DAI scores by PAG was significantly counteracted by co-treatment with Na_2S (Fig. 4).

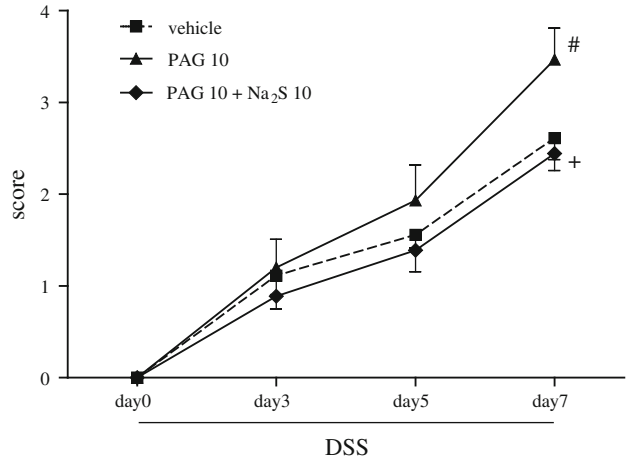

Fig. 4 The effect of PAG with or without Na_2S on DAI scores. Each value indicates the mean ± SEM for 7 mice. # $p < 0.05$ when compared to the vehicle-treated group, and + $p < 0.05$ when compared to the PAG-treated group

Figure 5 shows typical histological appearances in the PAG-treated mice and in the PAG + Na_2S-treated mice. Large areas of epithelial crypt loss, ulceration, and neutrophil infiltration throughout the colonic mucosa were observable in the colon of the vehicle-treated mice. PAG deteriorated these findings of colitis. In contrast, co-treatment with Na_2S resulted in smaller erosions with fewer neutrophils.

Effects of PAG and Na_2S on MPO Activity and TBA-Reactive Substances

Tissue-associated MPO activity in the colonic mucosa increased after DSS administration for 7 days. MPO activity was significantly higher in the PAG-treated mice (4.157 ± 1.564 mU/mg protein) than in the vehicle-treated mice (0.5966 ± 0.0819 mU/mg protein). MPO activity was significantly lower in the PAG + Na_2S-treated mice (0.7013 ± 0.2756 mU/mg protein) than in the PAG-treated mice. The increase in MPO activity by PAG was negated by co-treatment with Na_2S (Fig. 6a).

Levels of TBA-reactive substances, which serve as an index of lipid peroxidation, in the colonic mucosa 7 days after DSS administration were significantly higher in the PAG-treated mice (11.07 ± 2.129 nmol/mg protein) than in the vehicle-treated mice (5.285 ± 0.2810 nmol/mg protein). Levels of TBA-reactive substances were significantly lower in the PAG + Na_2S-treated mice (5.342 ± 0.2871 nmol/mg protein) than in the PAG-treated mice. The increase in TBA-reactive substances by PAG was negated by co-treatment with Na_2S (Fig. 6b).

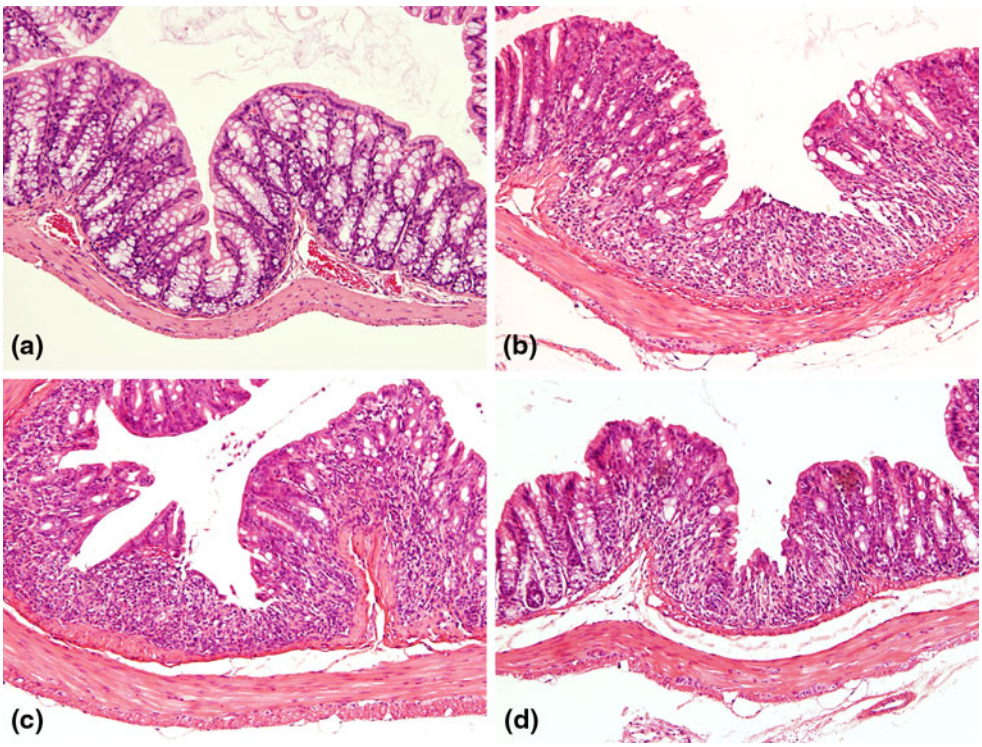

Fig. 5 Histological findings (hematoxylin and eosin (H&E) staining) of the colonic mucosa in a sham (**a**), a vehicle-treated (**b**), a PAG-treated (**c**), and a PAG + Na_2S-treated (**d**) mouse. Loss and shortening of crypts, mucosal erosions, inflammatory cell infiltration, and goblet cell depletion are seen in (**b–d**), those are induced by DSS

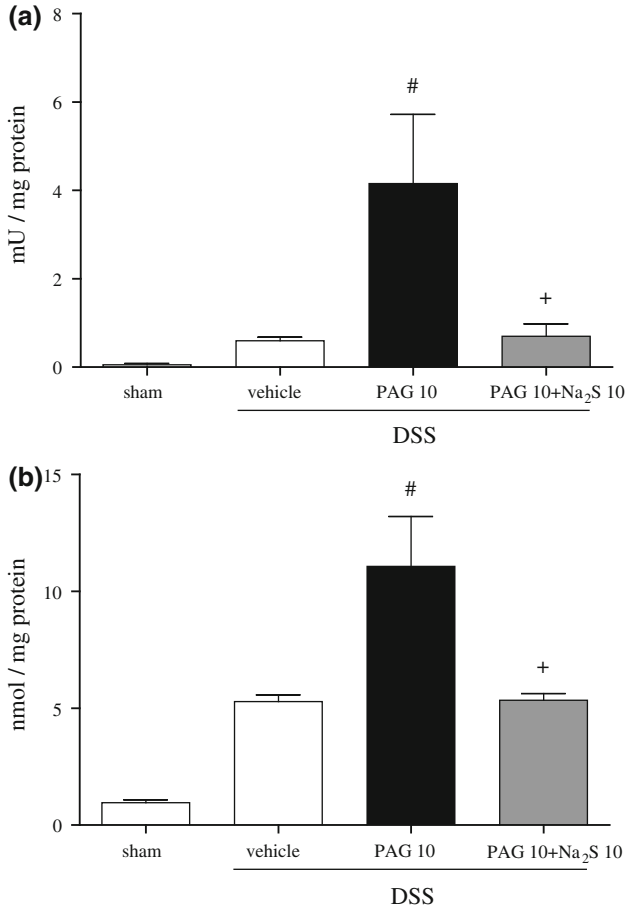

Fig. 6 The effect of PAG with or without Na_2S on neutrophil accumulation expressed as myeloperoxidase (MPO) activity (**a**) and lipid peroxide concentration expressed as thiobarbituric acid (TBA)-reactive substances (**b**) in the colonic mucosa of mice administered 8% DSS. Each value indicates the mean ± SEM for 5–7 mice. # $p < 0.05$ when compared to the vehicle-treated group, and + $p < 0.05$ when compared to the PAG-treated group

Discussion

The present study showed that the mRNA expression levels of CSE and CBS, H_2S-generating enzymes, and H_2S content in the colonic mucosa increased with time after DSS administration. Both CSE and CBS mRNA expression were significantly elevated after development of colitis, however, the expression level of CSE mRNA was more than 200 times larger than that of CBS mRNA. These results suggest that CSE may operate as the primary synthetase of H_2S in the colonic mucosa in this model. Several lines of evidence suggest that ulcerative colitis could be associated with excessive bacterial production of H_2S in the colon [26]. Moreover, DSS contains a non-absorbable carbohydrate-bound sulfate that can be metabolized to H_2S by sulfate-reducing bacteria. However, the hypothesis that endogenous sulfide production by bacterial flora contributes to the pathogenesis of inflammatory bowel disease is controversial. Furne et al. [27] showed that treatment with dextran sulfate did not increase the release of H_2S in feces, and scavenging excessive intraluminal H_2S using bismuth did not affect the dextran sulfate-induced colitis. However, our data strongly suggest that H_2S is produced in the colonic mucosa, although the origin of H_2S is not determined.

In our study, colitis severity was assessed by the DAI scores and histology. By each assessment, inhibiting the endogenous H_2S-synthesis by PAG significantly deteriorated DSS-induced colitis. Na_2S, an H_2S donor, negated the effect of PAG. In addition, we showed that PAG treatment significantly increased MPO activity and TBA-reactive substances in DSS-induced inflamed colonic mucosa, and that these increases were significantly negated by co-treatment with Na_2S.

Many investigators, including us, have hypothesized that neutrophil-mediated inflammation is involved in the development of DSS-induced colonic mucosal injury. Three lines of evidence support this hypothesis: (1) colonic mucosal endothelial intercellular adhesion molecule 1 (ICAM-1) expression is enhanced at an early stage in the inflammatory cascade of DSS-induced colitis [28]; (2) selective depletion of neutrophils by monoclonal antibody RP-3 suppresses colitis in rats [29]; and (3) immunoneutralization of ICAM-1 on endothelial cells significantly attenuates colonic mucosal injury and neutrophil accumulation in rats [30]. The present study has shown that MPO activity, an index of tissue-associated neutrophil accumulation, significantly increases in the colonic mucosa by inhibiting endogenous H_2S generation, and this increase is significantly inhibited by co-treatment with an H_2S donor. Histological findings also indicated that inhibiting H_2S-synthesis by PAG enhanced leukocyte infiltration and caused severe colitis. These results indicate that the inhibition of neutrophil accumulation by H_2S may be one of the protective factors helping to decrease DSS-induced colitis. The inhibitory effect of H_2S against neutrophil accumulation is also supported by previous reports, in which H_2S inhibited neutrophil-endothelial interaction. Fiorucci et al. [4] showed that H_2S suppresses nonsteroidal anti-inflammatory drug-induced gastropathy by suppressing leukocyte adherence via opening K^+_{ATP} channels. Zanardo et al. [5] demonstrated that H_2S inhibited neutrophil adhesion/activation in carrageenan-induced paw edema.

We showed that PAG significantly enhanced the increase in TBA-reactive substances, an index of lipid peroxidation, in the DSS-induced inflamed colonic mucosa, and this increase was significantly inhibited by supplementing H_2S with Na_2S. Polyunsaturated fatty acids of cell membranes are degraded by lipid peroxidation, with subsequent disruption of membrane integrity, suggesting that lipid peroxidation mediated by oxygen radicals is an important cause of damage

to and destruction of cell membranes [31, 32]. Our data support another recent finding in our laboratory, in which colonic TBA-reactive substances were markedly reduced by treatment with Mn-superoxide dismutase and a synthetic vitamin E analogue in the same DSS colitis mouse model; this suggests that induction of lipid peroxidation is an early critical event in this experimental inflammatory bowel disease model [33]. Although we demonstrated the anti-oxidant activity of H_2S in the present study, it was uncertain whether this activity might result from its anti-inflammatory activity or from its direct anti-oxidant activity. Previous reports have demonstrated the cytoprotective effects of H_2S, which may be related to the neutralization of various reactive species including oxyradicals [34], peroxynitrite [21], and hypochlorous acid [35]. H_2S can also upregulate the endogenous antioxidant system [20].

Our conclusion is that endogenous H_2S may act as an anti-inflammatory molecule by preventing neutrophil accumulation and anti-oxidant ability. Further research is needed, because the molecular mechanisms underlying sulfide's cellular signaling or interactions between H_2S and NO/CO are poorly understood.

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