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Effect of garlic sulfur compounds on neutrophil infiltration and damage to the intestinal mucosa by endotoxin in rats

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ABSTRACT

We investigated the protective effects of garlic sulfur compounds (GSCs), specifically, diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS), on endotoxin-induced intestinal damage. Wistar rats received by gavage 0.125 or 0.025 mmol/kg body wt of each GSC or the vehicle (corn oil; 2 mL/kg body wt) every other day for 2 weeks before being injected with endotoxin (ip, 5 mg/kg body wt). Control rats were administered corn oil and were injected with sterile saline. Rats were killed at 18 h after injection. Both doses of DAS suppressed endotoxin-induced neutrophilia, serum levels of sICAM-1 and CINC-1, cellular CD11b on neutrophils, and intestinal contents of ICAM-1, CINC-1, TNF-alpha, and IL-1beta ($p < 0.05$). DADS suppressed endotoxin-induced intestinal contents of ICAM-1, TNF-alpha, and IL-1beta at both doses, but only suppressed the serum sICAM-1 level and cellular CD11b on neutrophils at the low dose ($p < 0.05$). DATS did not ameliorate the endotoxin-induced serum level of sICAM-1 or CINC-1 but suppressed intestinal IL-1beta at both doses. The low but not the high dose of DATS also ameliorated the intestinal contents of ICAM-1 and TNF-alpha ($p < 0.05$). All GSCs reversed endotoxin-induced neutrophil infiltration and damage in the intestine, and the order of the effects of these GSCs to normalize intestinal morphology was DAS > DADS > DATS.

1. Introduction

Neutrophils are the most abundant leukocytes in peripheral blood and are found at inflammatory sites during acute inflammation. These cells are one of the most important first-line defenses in the host response to bacterial infection. It is well known that neutropenia increases the risk of opportunistic infection and causes major clinical consequences in conditions such as in patients receiving chemotherapy. By contrast, however, overactivation of the transmigration of neutrophils into tissues has been recognized to result in tissue damage under acute inflammatory conditions such as found in trauma, sepsis, and cardiac infarction because the activated neutrophils can release cytotoxic molecules, such as proteases and reactive oxygen species, that are harmful to host tissue (Basset et al., 2003; Grisham and Neil Granger, 1988; Smith, 1994).

During an acute inflammatory condition, the number of neutrophils in peripheral blood increases. The activated neutrophils adhere to endothelial cells of the blood vessel wall. The interaction between several adhesion molecules on the cell surface of both cell types is an early step in the transmigration of neutrophils through the blood wall into the insulted tissue to perform their phagocytotic ability. CD11b/CD18 on neutrophils and intercellular adhesion molecule-1 (ICAM-1) on endothelium have been reported to play important roles in this interaction (Witko-Sarsat et al., 2000; Wang and Doerschuk, 2002; Stadnyk et al., 2005). After transmigrating through the blood vessel wall, neutrophils are subsequently recruited to the inflammatory site by a gradient of soluble chemoattractants such as interleukin-8 (IL-8) in humans and cytokine-induced neutrophil chemoattractant-1 (CINC-1) in mice (Furie and Randolph, 1995). In addition, in mucosal systems such as the intestine, activated neutrophils also interact with epithelial cells via the above-mentioned adhesion molecules and have been suggested to be a mechanism for mucosal damage under inflammatory conditions (Madara, 1997; Okada et al., 1998; Beck-Schimmer et al., 2001). IL-1β and tumor necrosis factor-α (TNF-α) are two potent mediators that induce the expression of

Abbreviations: CINC, cytokine-induced neutrophil chemoattractant-; DADS, diallyl disulfide; DAS, diallyl sulfide; DATS, Diallyl trisulfide; GSCs, garlic sulfur compounds; ICAM, intercellular adhesion molecule-; IL, interleukin; ip, intraperitoneal; MAb, monoclonal antibody; MFI, mean fluorescence intensity; MPO, myeloperoxidase; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

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these adhesion molecules and chemokines (Calkins et al., 2002; Ohira et al., 2003). Consequently, inhibition of the expression and activity of proinflammatory cytokines, as well as inhibition of the interaction between neutrophils and endothelial cells, has been suggested as an anti-inflammation strategy (LaRosa and Opal, 2008; Leung and Panaccione, 2008; Stefanelli et al., 2008).

Garlic (*Allium sativum*) is one of the most popular spicy food materials that is also used for prophylactic and medicinal purposes worldwide. Garlic oil is reported to possess an anti-inflammatory effect via an inhibitory effect on the production of mediators for inflammation, such as eicosanoids, proinflammatory cytokines, and NO, especially as a result of the regulation of monocyte/macrophage activity (Chang et al., 2005; Chang and Chen, 2005). Previously, we demonstrated that, *in vitro*, the migration activity of neutrophil-like cells toward a concentration gradient of IL-8 was suppressed by garlic oil in a dose-dependent manner (Shih et al., 2010). It has also been shown that garlic oil can ameliorate endotoxin-induced small intestinal damage and apoptosis *in vivo* (Chiang et al., 2006). Very recently, we reported that the anti-inflammatory effect of garlic oil was associated with suppressed neutrophil infiltration into tissue and with lowered levels of certain soluble and cellular adhesion molecules generated under inflammatory conditions (Kuo et al., 2011). These data suggested the use of garlic oil as an anti-inflammatory agent; however, it remains to be clarified which component of garlic oil provides such effects.

It has been reported that the major action of garlic is attributed to its sulfur-containing compounds (Touloupakis and Ghanotakis, 2010). In the present study, we aimed to compare the protective effect of three major garlic sulfur compounds (GSCs) in garlic oil, namely, diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS), on endotoxin-induced intestinal damage and their association with levels of soluble and cellular adhesion molecules. We were also interested in studying how the intake of these GSCs affects the endotoxin-induced elevation of local cytokines in the intestine and their association with neutrophil infiltration into the intestinal mucosa.

2. Materials and methods

2.1. Reagents

DAS and DADS were purchased from Fluka Chemical (Buchs, Switzerland). DATS was purchased from LKT Laboratories (St. Paul, MN, USA). Ficoll-Paque™ Plus was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), phenylmethylsulfonyl fluoride was purchased from Roche (Indianapolis, IN, USA), OCT (22-oxacalcitriol) medium (tissue freezing medium) was purchased from Sakura Finetek (Torrance, CA, USA), rat CINC-1 ELISA kits and rat ICAM-1 ELISA kits were purchased from R&D Systems Inc. (Minneapolis, MN, USA), rat IL-1β ELISA kits and rat TNF-α ELISA kits were purchased from Biosource International Inc. (Camarillo, CA, USA), FITC-conjugated mouse anti-rat CD11b monoclonal antibody (MAb) and RPE-conjugated mouse anti-rat CD18 MAb were purchased from AbD Serotec (Kidlington, UK), and protein assay kits were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Endotoxin and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Animals and Experimental Procedure

Four-week-old weanling male Wistar rats were purchased from the National Animal Breeding and Research Center (Taipei, Taiwan). The animals were kept under a 12-h light–dark cycle at an ambient temperature of 23 °C and were given free access to water and standard rat feed (Rodent Diet 5001; Purina Mills, Richmond, IN, USA). The rats were allowed to adapt to the environment for 1 week after their arrival before the experiment started. Animals were randomly assigned to eight groups and received by gavage DAS, DADS, or DATS (0.025 or 0.125 mmol/kg body wt) or the vehicle (corn oil; 2 ml/kg body wt) every other day for 2 weeks. The doses of GSCs used in the study were calculated in accordance with the above-mentioned study carried out in our laboratory previously (Chiang et al., 2006; Kuo et al., 2011) in which 10 and 50 mg/kg of garlic oil were found to prevent endotoxin-induced neutrophil infiltration in the small intestine.

During the 2 weeks of treatment, the animals were housed in metabolic cages and were given free access to water and a powdered diet (Rat Diet 5012; Purina Mills). Endotoxin was injected 15 days after the first administration of the GSCs or vehicle. The ip injection of endotoxin from *Salmonella typhimurium* (5 mg/kg body wt) was carried out at 24 h after the final administration of GSCs. The rats' food supply was withdrawn followed the injection. The control rats, which had received corn oil for 2 weeks, were injected (ip) with the same volume of sterile saline. Immediately before and after the injection, blood samples were withdrawn from the lateral tail vein for measurement of soluble adhesion molecules and CINC-1. The rats were killed by carbon dioxide euthanasia at 18 h after the injection. Blood was collected, and the intestine was immediately removed. Organs including liver, spleen, kidney, and cervical lymph nodes were then removed and weighed. Housing conditions and experimental procedures were in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*, and all protocols were approved by the ethical committee for animal experimentation of Chung Shan Medical University, Taichung, Taiwan.

2.3. Neutrophil Isolation and Flow Cytometric Analysis of CD11b and CD18 Expression

Peripheral neutrophils were isolated with a standard density gradient separation method by using commercially available separation media as described elsewhere (Kuo et al., 2011). The neutrophils were then washed and resuspended in phosphate-buffered saline (PBS) at a final density of 1×10^6/100 μl for flow cytometry analysis of CD11b/CD18 expression. Viability determined by trypan blue dye exclusion and morphological investigation showed sample yields of >95% neutrophils with >95% viability. The expression of CD11b and CD18 on rat neutrophils was analyzed by using FITC-conjugated mouse anti-rat CD11b MAb and RPE-conjugated mouse anti-rat CD18, respectively, in accordance with the manufacturer's instructions and with a FACScan Calibur system (Becton Dickinson, NJ, USA). Cells from the control group that did not stain with antibody were used as a negative control. Ten thousand cells were analyzed in each sample. Data were analyzed with commercially available software (WinMDI2.8) and are expressed as mean fluorescence intensity (MFI).

2.4. Preparation of the Intestinal Tissue Samples

Immediately after the intestine was removed, the ileum segment (defined as the intestinal segment of 20 cm proximal to the cecum) was irrigated with cold PBS (pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride to remove the intestinal contents and was separated into three segments as described elsewhere for the analysis of TNF-α, IL-1β, CINC-1, and ICAM-1; activity analysis of myeloperoxidase (MPO); and histological analysis (Kuo et al., 2011).

2.5. Biochemical Analysis of Blood Samples and Intestinal Tissue

Levels of serum soluble intercellular adhesion molecule (sICAM)-1 and mucosal ICAM-1 were determined by using rat ICAM-1 ELISA kits. Levels of serum and mucosal CINC-1 were analyzed by use of rat CINC-1 ELISA kits. Levels of mucosal TNF-α and IL-1β were analyzed by rat IL-1β and TNF-α ELISA kits, respectively, in accordance with the manufacturer's instructions and with a micro-plate reader (VersaMax; Molecular Devices Ltd., UK). Protein assays were performed by using Bio-Rad protein assay kits.

2.6. Enzymatic Assay of Myeloperoxidase

Recruited neutrophils in intestinal mucosa were evaluated by measuring MPO activity as described by Bradley et al. (1982) with some modifications. The intestinal mucosa collected was homogenized in lysis buffer (0.5% [w/v] hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer, pH 6.0) at 1:20 (w/v). Homogenized samples were frozen and thawed three times followed by centrifugation at 20,000*g* for 15 min at 4 °C. MPO activity in the supernatants was analyzed spectrophotometrically at a wavelength of 460 nm with a UV/visible spectrophotometer (U-3000, Hitachi, Japan) with *o*-dianisidine as a substrate and is expressed as units per gram of tissue.

2.7. Histologic Analysis of Intestinal Integrity

The distal ileum fixed in 10% neutral buffered formalin was embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin to evaluate the destruction of the villus architecture of the mucosa.

2.8. Statistical Analysis

The data are expressed as means ± SDs and were analyzed by one-way analysis of variance. Student's *t*-test was used to detect differences in means between the control group and the endotoxin-injected rats. Duncan's multiple-comparison test was used to detect differences among the means of the endotoxin-injected groups.

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P values < 0.05 were considered significant. All statistical analyzes were performed with commercially available software (SPSS 12 for Windows; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Animal Characteristics

Compared with vehicle (corn oil), the administration of DAS, DADS, and DATS did not significantly affect either body weight gain or food intake in the rats before endotoxin injection, which suggested that the doses of the GSCs used did not affect normal growth (Table 1). In rats not previously administered GSCs, the injection of endotoxin significantly elevated ratios of liver weight, spleen weight, and cervical lymph node weight to body weight compared with that of the saline-injected controls. None of the tested GSCs significantly affected the endotoxin-induced organ hypertrophy (Table 1).

Compared with the saline-injected control group, rats injected with endotoxin showed elevated peripheral neutrophil counts (Fig. 1, $p < 0.05$). The elevated blood neutrophil count induced by endotoxin tended to be reversed in rats pretreated with the GSCs, and DAS was shown to be significantly effective ($p < 0.05$) in a dose-dependent manner.

3.2. Serum Concentrations of sICAM-1 and CINC-1

We found no significant difference in the basal levels of sICAM-1 or CINC-1 in serum of rats administered DAS, DADS, and DATS (Fig. 1). Compared with rats injected with saline, the injection of endotoxin significantly elevated serum levels of sICAM-1 and CINC-1 ($p < 0.05$). Although all tested GSCs ameliorated the endotoxin-induced elevation of serum sICAM-1 levels, such an effect was only statistically significant in rats pretreated with low doses of DAS and DADS and the high dose of DAS (Fig. 1A). DAS also had an inhibitory effect on the endotoxin-induced elevation of the serum CINC-1 level in a dose-dependent manner; however, such effect was not found for either DADS or DATS (Fig. 1B).

3.3. Cellular CD11b/CD18 Level on Neutrophils

When compared with the saline-injected control rats, endotoxin significantly elevated the expression of CD11b and CD18 on peripheral neutrophils (Fig. 2). Compared with the vehicle-pretreated endotoxin-injected group, pretreatment with DAS significantly suppressed the cellular level of CD11b and CD18 on neutrophils in a dose-dependent manner, whereas the low but not the high doses of DADS and DATS showed a significant suppressive effect on CD11b expression (Fig. 2A and B). DADS suppressed the cellular level of CD18 on neutrophils in a dose-dependent manner but to a lesser extent than did DAS. DATS did not significantly affect the endotoxin-induced elevation of CD18 levels on neutrophils (Fig. 2C and D).

3.4. Content of CINC-1, ICAM-1, TNF-α, and IL-1β in the Intestinal Mucosa

Compared with saline-injected control rats, rats injected with endotoxin had significantly elevated levels of both CINC-1 and ICAM-1 in the intestinal mucosa ($p < 0.05$) (Table 2). Pretreatment with DAS significantly lowered mucosal ICAM-1 and CINC-1 contents induced by endotoxin in a dose-dependent manner. The low and the high dose of DADS also significantly lowered the endotoxin-induced elevation of ICAM-1 and CINC-1 in the intestinal mucosa. DATS was significantly effective at lowering the endotoxin-induced elevation of mucosal ICAM-1 at the low but not the high dose. DATS did not significantly affect the endotoxin-induced elevation of mucosal CINC-1 content.

In saline-injected control rats injected with endotoxin, local levels of TNF-α and IL-1β were significantly elevated ($p < 0.05$) (Table 2). Local TNF-α content induced by endotoxin injection was significantly suppressed to a similar level by all GSCs tested except for the high dose of DATS (Table 2). By contrast, all tested doses of GSCs significantly suppressed the endotoxin-induced elevation of local IL-1β content (Table 2).

3.5. Neutrophil Infiltration of Intestinal Mucosa

Neutrophil infiltration was determined by analyzing MPO activity spectrophotometrically in ileum mucosa. The activity of this enzyme in intestine was significantly elevated in endotoxin-injected rats compared with that of the saline-injected controls ($p < 0.05$; Fig. 3). Pretreatment with any of the GSCs at both low and high doses significantly reduced MPO activity in the intestine; however, the high dose of DATS had a significantly weaker effect than did high doses of DAS or DADS ($p < 0.05$).

3.6. Morphologic Analysis of the Intestinal Mucosa

The integrity of the intestinal mucosa was impaired by the injection of endotoxin; areas of ulceration and overt breaches of the epithelial barrier were apparent, with exposure of the connective tissue. The height of the villi was shorter than in the controls, and some villi were even fused together in the ileum from endotoxin-injected rats (Fig. 4). In general, endotoxin-induced mucosal changes were less severe in rats pretreated with any of the GSCs. In rats pretreated with a low dose of DAS, the mucosal integrity was very similar to that of the controls. In rats pretreated with the high

Table 1
Effect of GSCs on food intake, body weight gain, organ weights, and neutrophil count of rats.[x]

	Control	Endotoxin	Endotoxin + DAS-L	Endotoxin + DAS-H	Endotoxin + DADS-L	Endotoxin + DADS-H	Endotoxin + DATS-L	Endotoxin + DATS-H
Body wt gain (g)[y]	97.7 ± 7.9	97.7 ± 7.9[a]	95.3 ± 11.3[a]	105.7 ± 11.2[a]	104.1 ± 12.8[a]	95.7 ± 9.2[a]	99.4 ± 8.0[a]	105.1 ± 11.6[a]
Food intake (g/24 h)[y]	24.2 ± 3.3	24.2 ± 3.3[a]	26.3 ± 5.8[a]	24.0 ± 3.9[a]	25.7 ± 2.4[a]	23.8 ± 4.6[a]	26.2 ± 3.7[a]	26.3 ± 2.6[a]
Liver wt/body wt (%)[z]	3.97 ± 0.31	4.47 ± 0.35[*,a]	4.27 ± 0.39[a]	4.42 ± 0.38[a]	4.39 ± 0.38[a]	4.55 ± 0.41[a]	4.48 ± 0.33[a]	4.50 ± 0.41[a]
Spleen wt/body wt (%)[z]	0.34 ± 0.03	0.43 ± 0.06[*,a]	0.38 ± 0.06[a]	0.42 ± 0.06[a]	0.38 ± 0.04[a]	0.39 ± 0.04[a]	0.37 ± 0.05[a]	0.43 ± 0.06[a]
Cervical lymph nodes (%)[z]	0.046 ± 0.01	0.068 ± 0.022[*,a]	0.053 ± 0.017[a]	0.064 ± 0.008[a]	0.051 ± 0.008[a]	0.066 ± 0.017[a]	0.069 ± 0.028[a]	0.061 ± 0.022[a]
Neutrophil count (cell/mL blood x 10^5)[z]	13.2 ± 4.7	26.3 ± 3.7[*,b]	15.4 ± 6.6[a]	12.1 ± 3.2[a]	18.6 ± 3.2[ab]	18.5 ± 5.0[ab]	20.2 ± 4.9[ab]	21.7 ± 9.3[ab]

[ab]Endotoxin-injected groups not sharing the same superscript letter are significantly different ($p < 0.05$).
[x] Values are the mean ± SD for six rats per group. L and H refer to the low and high dose, respectively.
[y] Determined before the administration of endotoxin. Both Control and Endotoxin groups were treated with vehicle and thus the data were combined.
[z] Determined at 18 h after the injection of endotoxin or saline.
[*] Significantly different from the control group ($p < 0.05$).

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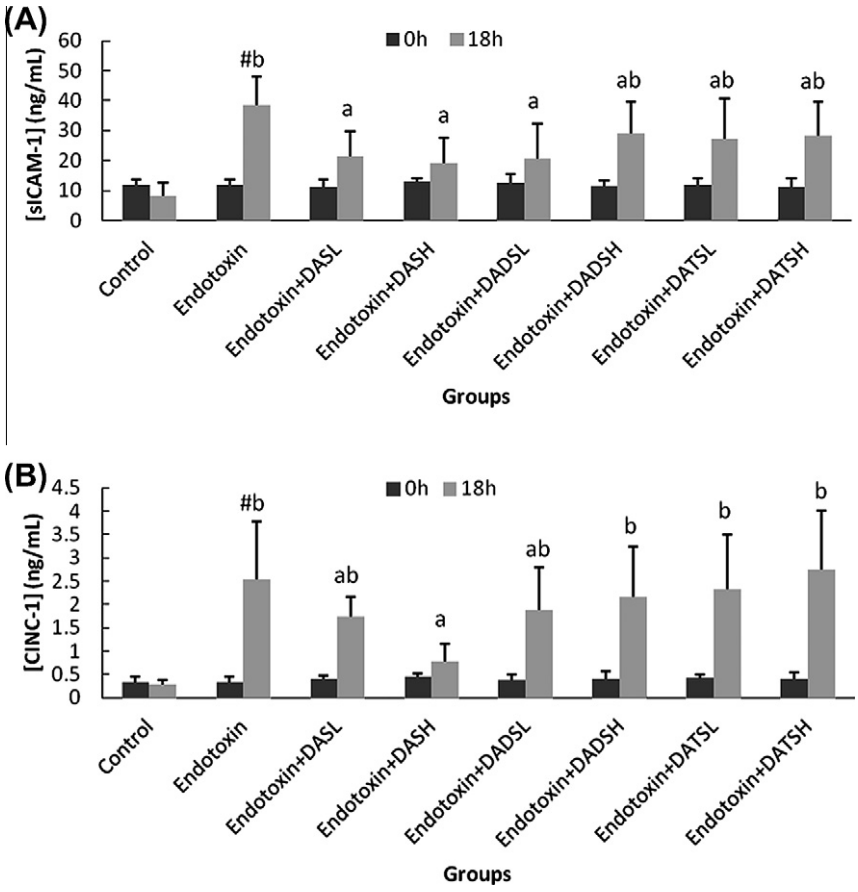

Fig. 1. Effect of GSCs on serum levels of sICAM-1 (A) and CINC-1 (B) of rats injected with endotoxin. Rats received by gavage 0.025 mmol/kg DAS, DADS or DATS (DAS-L, DADS-L or DATS-L, respectively), 0.125 mmol/kg DAS, DADS, or DATS (DAS-H, DADS-H, or DATS-H, respectively), or the vehicle (corn oil) for 2 weeks and were then injected with endotoxin from *S. typhimurium* (i.p., 5 mg/kg body weight). Control rats were pretreated with vehicle followed by the injection with saline. Sample was collected before and at 18 h after injection. Data are mean ± SD for six rats per group. *Significantly different from the control group ($p < 0.05$). [a,b]Groups not sharing the same superscript letter are significantly different ($p < 0.05$).

dose of DAS, the density of the villi was slightly lower than that of the low-dose samples; however, the height of the villi remained similar to that of the controls. Mucosa from rats pretreated with DADS had shorter and thinner villi with less density compared with the mucosa from rats pretreated with DAS. Furthermore, even though the epithelial barrier was largely repaired in mucosa pretreated with DATS compared with that from the endotoxin-injected and vehicle-pretreated rats, the mucosa of rats pretreated with low or high doses of DATS had minor and major swollen lamina propria, respectively, accompanied by the shortest villi with the least density among mucosa from DAS-, DADS-, and DATS-pretreated animals. In accordance with the morphology of the ileum, DAS appeared to be the most effective at protecting against mucosal injury induced by endotoxin, whereas DATS was the least effective.

4. Discussion

The present study demonstrated that endotoxin induced neutrophil infiltration that reflected by the elevated MPO activity in the small intestine and damaged the integrity of the small intestinal mucosa. This was showed to be associated with an increased blood neutrophil count, increased serum levels of the neutrophil chemokine CINC-1, and increased serum levels of sICAM-1, which is similar to what is found in certain clinical inflammatory conditions (Waage et al., 1991; Ito et al., 2001; Shapiro et al., 2010). On the peripheral neutrophils isolated from endotoxin-injected rats, increased cellular levels of CD11b and CD18 show the activation of neutrophils by endotoxin (Witko-Sarsat et al., 2000). These findings suggest an association between these serum factors and the transmigration of neutrophils in endotoxin-injected rats. Because ICAM-1 and CINC-1 are two major determinants of neutrophil recruitment in inflammatory lesions (Madara, 1997; Furie and Randolph, 1995; Leeuwenberg et al., 1992), and IL-1β and TNF-α are known to be two potent mediators of the endotoxin-induced expression of CINC-1 and ICAM-1 (Calkins et al., 2002; Ohira et al., 2003), it was as expected that local levels of ICAM-1, CINC-1, TNF-α, and IL-1β would be elevated in the small intestinal mucosa in the endotoxin-injected rats in the present study.

Garlic contains characteristic sulfur compounds that contribute to its pharmacologic activities. We previously demonstrated the effectiveness of garlic oil (composed of roughly 40% DATS and 40% DADS and minor amounts of many other volatile compounds including 4% DAS) at ameliorating endotoxin-induced neutrophil infiltration and damage in the intestine at doses of 10 and 50 mg/kg (Kuo et al., 2011). These doses of garlic oil are equivalent to doses of 0.023 and 0.112 mmol/kg of DATS or 0.027 and 0.137 mmol/kg of DADS, or 0.0035 and 0.0175 mmol/kg of DAS, respectively. It has been suggested that the number of sulfur atoms in GSCs is important for certain biological functions of the compounds (Wu et al., 2002; Tsai et al., 2005). Therefore, in the present study, we administered the same molarities of DAS, DADS, and DATS to rats, namely, 0.025 and 0.125 mmol/kg. The data pre-

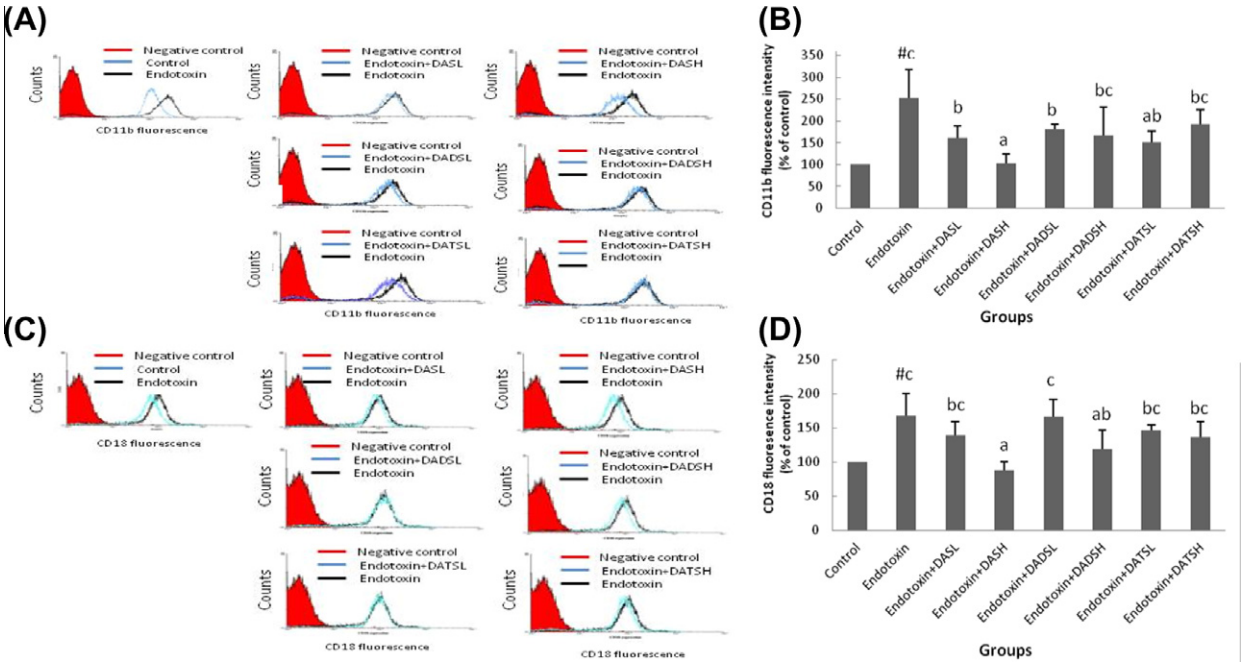

Fig. 2. Effect of GSCs on cellular levels of CD11b and CD18 on neutrophil isolated from rats injected with endotoxin. (A) Fluorescence intensity of peripheral neutrophils stained with FITC-conjugated anti-rat CD11b MAb and determined by flow cytometry. (B) Mean fluorescence intensity of CD11b. (C) Fluorescence intensity of peripheral neutrophils stained with RPE-conjugated anti-rat CD18 MAb and determined by flow cytometry. (D) Mean fluorescence intensity of CD 18 was calculated and expressed as a percentage of the control group. Rats received by gavage 0.025 mmol/kg DAS, DADS or DATS (DAS-L, DADS-L or DATS-L, respectively), 0.125 mmol/kg DAS, DADS, or DATS (DAS-H, DADS-H, or DATS-H, respectively), or the vehicle (corn oil) for 2 weeks and were then injected with endotoxin from *S. typhimurium* (i.p., 5 mg/kg body weight). Control rats were pretreated with vehicle followed by the injection with saline. Blood was collected and neutrophils isolated at 18 h after injection. Data are mean ± SD for six rats per group. $^{\#}$Significantly different from the control group ($p < 0.05$). ^{ab}Endotoxin-injected groups not sharing the same superscript letter are significantly different ($p < 0.05$).

Table 2
Content of ICAM-1, CINC-1, TNF-α, and IL-1β in ileum mucosa of control rats or endotoxin-injected rats who did or did not receive GSCs.

pg/mg protein	Control	Endotoxin	Endotoxin + DAS-L	Endotoxin + DAS-H	Endotoxin + DADS-L	Endotoxin + DADS-H	Endotoxin + DATS-L	Endotoxin + DATS-H
ICAM-1	2474 ± 833	4544 ± 1492^{*,d}	1641 ± 635^{ab}	1280 ± 693^{a}	1843 ± 680^{ab}	2944 ± 944^{bc}	2249 ± 445^{abc}	3355 ± 1422^{cd}
CINC-1	13.2 ± 5.0	39.8 ± 9.9^{*,d}	22.1 ± 3.7b^{c}	13.8 ± 5.0^{a}	23.5 ± 8.2^{bcd}	17.0 ± 2.6^{ab}	31.4 ± 4.8^{d}	26.0 ± 6.5^{cd}
TNF-α	12.1 ± 3.1	29.3 ± 6.5^{*,b}	19.0 ± 5.9^{a}	18.4 ± 6.3^{a}	16.6 ± 7.7^{a}	16.6 ± 3.2^{a}	16.5 ± 4.6^{a}	23.5 ± 8.8^{ab}
IL-1β	36.8 ± 15.6	118.6 ± 31.0^{*,e}	48.5 ± 17.4 ^{ab}	62.4 ± 8.1^{bc}	39.9 ± 17.1^{ab}	83.0 ± 15.8^{cd}	35.1 ± 3.4^{a}	93.1 ± 14.2^{d}

Values are the mean ± SD for six rats per group and were determined at 18 h after the injection of endotoxin or saline. L and H refer to the low and high dose, respectively.
^{abcd}Endotoxin-injected groups not sharing the same superscript letter are significantly different ($p < 0.05$).
* Significantly different from the control group ($p < 0.05$).

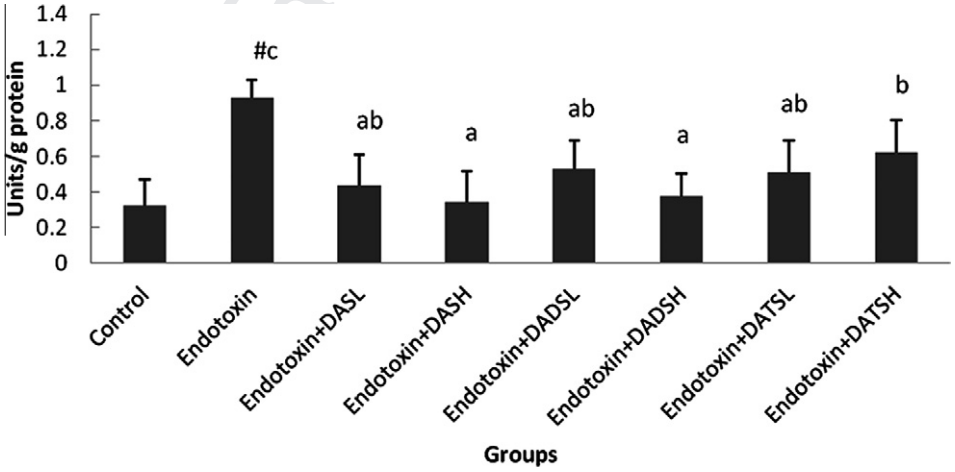

Fig. 3. The effect of GSCs on MPO activity in ileum prepared from rats injected with endotoxin. Rats received by gavage 0.025 mmol/kg DAS, DADS or DATS (DAS-L, DADS-L or DATS-L, respectively), 0.125 mmol/kg DAS, DADS, or DATS (DAS-H, DADS-H, or DATS-H, respectively), or the vehicle (corn oil) for 2 weeks and were then injected with endotoxin from *S. typhimurium* (i.p., 5 mg/kg body weight). Control rats were pretreated with vehicle followed by the injection with saline. Sample was collected at 18 h after injection. Data are mean ± SD for six rats per group. ∗Significantly different from the control group ($p < 0.05$). ^{a,b}Groups not sharing the same superscript letter are significantly different ($p < 0.05$).

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sented here show that all three GSCs tested reversed the endotoxin-induced neutrophil infiltration and damage to the intestine, which is consistent with our previous findings with garlic oil (Kuo et al., 2011). Furthermore, the present study demonstrated that regardless of dose, the order of the effects of the tested GSCs to normalize the intestinal morphology was DAS > DADS > DATS, even though all three GSCs had a similar inhibitory effect on endotoxin-induced neutrophil infiltration at the low dose. We interpret such results to be at least partly due to the fact that these compounds possess different cytotoxic effects and may affect intestinal epithelial cells to a different extent.

GSCs are known for their antiproliferative and proapoptotic effects in many cell types, including a variety of cancer cells (Musk et al., 1997; Hosono et al., 2005; Jakubíková and Sedlák, 2006; Prager-Khoutorsky et al., 2007; Yu et al., 2009; Kim et al., 2011). In certain tumor cells, the order of cytotoxicity of these three compounds has been reported to be DAS < DADS < DATS (Jakubíková and Sedlák, 2006). Why allyl sulfides contain more sulfur atoms have higher cytotoxic activity remains unclear, but their differential reactivity to the protein sulfhydryls is likely an explanation. Prager-Khoutorsky et al. (2007) demonstrated that treating cultured NIH-3T3 mouse fibroblasts with low, subtoxic levels of allicin, the precursor of the GSCs tested in the present study, leads to rapid depolymerization of cytoplasmic and spindle microtubules and arrests cell division and concluded that allicin interferes with microtubule assembly by modifying SH-groups in tubulin. Similarly, Hosono et al. (2005) found that in HCT-15 and DLD-1 human colon cancer cell lines, DATS at 10 mM reacts with Cys-12b and Cys-354b of beta-tubulin to form S-allylmercaptocysteine, which disrupts the microtubule network and inhibits cell growth and pro-

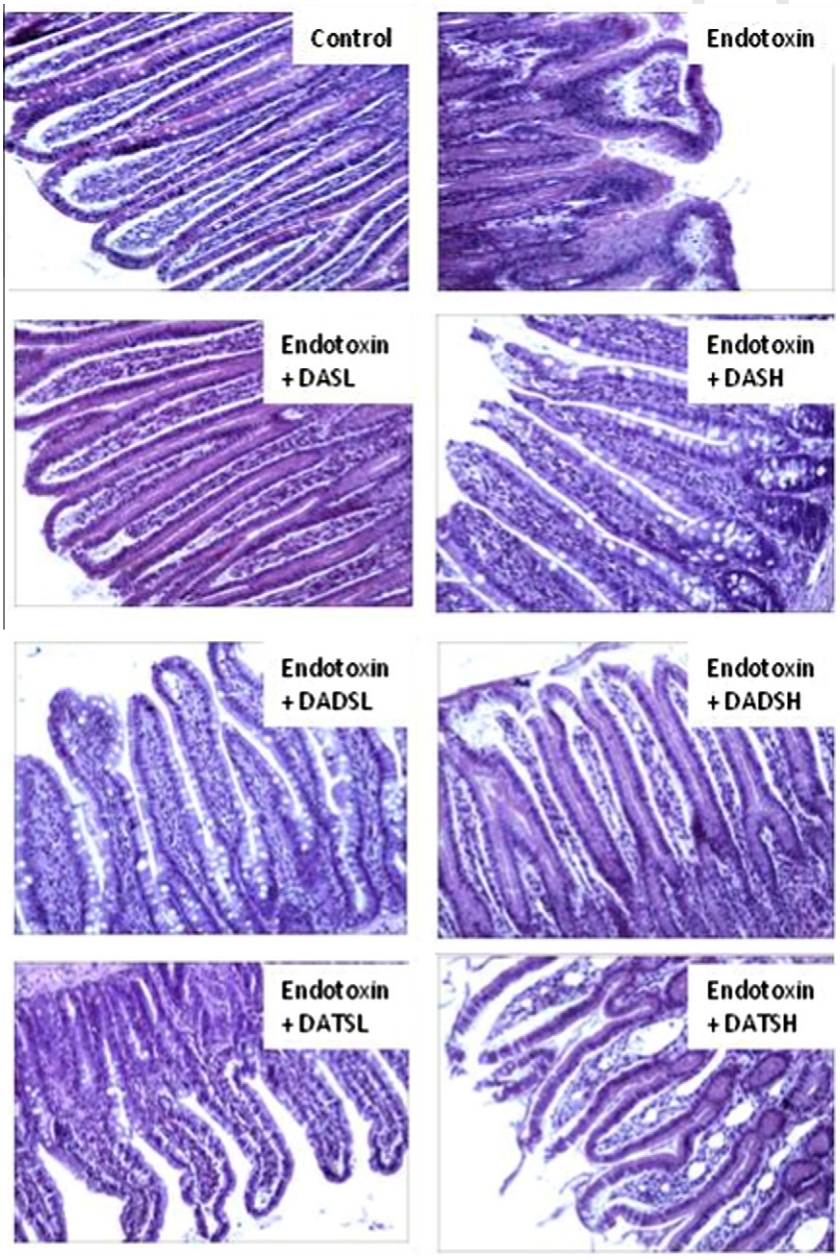

Fig. 4. Cross-sections of ileum stained with hematoxylin and eosin. Rats received by gavage 0.025 mmol/kg DAS, DADS or DATS (DAS-L, DADS-L or DATS-L, respectively), 0.125 mmol/kg DAS, DADS, or DATS (DAS-H, DADS-H, or DATS-H, respectively), or the vehicle (corn oil) for 2 weeks and were then injected with endotoxin from *S. typhimurium* (i.p., 5 mg/kg body weight). Control rats were pretreated with vehicle followed by the injection with saline. Sample was collected at 18 h after injection. Original magnification ×100.

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liferation. In contrast, these authors did not note DADS or DAS to possess such a tubulin modification activity, even at the higher concentration of 100 mM. In normal cells DAS exhibits less cytotoxicity and genotoxicity than does DADS (Musk et al., 1997). Because intestinal epithelial cells have a high turnover rate in the body, direct contact of GSCs via the enteral route may well affect the renewal of these cells, which was reflected by the changed height, thickness, and density of villi in the present study. To our knowledge, this is the first report to demonstrate that GSCs with a different number of S atoms differentially affect the morphology of the intestinal epithelium *in vivo*. The use of such compounds should therefore be cautioned to avoid any potential deleterious effects.

Although the present study demonstrated that DATS was the least effective at inhibiting levels of systemic inflammatory indicators, sICAM-1 and CINC-1, in serum compared with DAS and DADS, it is not likely that this was due to its cytotoxic effect on the intestinal epithelium. The present study did not find any effect of the three GSCs, including DATS, on either the normal growth of rats or the basal level of soluble adhesion molecules or CINC-1, which are indicators of systemic inflammation. Nevertheless, a possible interaction between endotoxin and DATS could not be excluded. Although various garlic preparations have been investigated for their inhibitory effect on proinflammatory cytokine proteins in various *in vivo* and *in vitro* models, studies of the effects of single GSCs on cytokine production are rare. Lee et al. (2009) showed a potential anti-inflammatory effect of DAS on joint inflammation induced by monosodium urate crystals and IL-1b, which is consistent with what was found in our endotoxin-induced model in the intestine. On the other hand, it has been reported that *in vitro* cytotoxic activity of macrophages induced by endotoxin can be further enhanced by pretreatment with DATS (Feng et al., 1994). The possible synergic effect of endotoxin and DATS may explain our finding that DATS at the high dose, although suppressing neutrophil infiltration in the intestine, only marginally reversed local ICAM-1, CINC-1, and TNF-alpha content at the inflammation site. In addition, it is also possible that the abnormal morphology of the mucosa in DATS-treated animals facilitates endotoxin-induced intestinal damage.

CD11b/CD18 on neutrophils is a major membrane molecule that interacts with endothelial cells before their transmigration (Witko-Sarsat et al., 2000; Wang and Doerschuk, 2002; Stadnyk et al., 2005). The present study found that at the low dose, GSCs tested had a similar inhibitory effect on the level of membrane CD11b/CD18 of neutrophils and the MPO activity in the intestine. However, only DAS, and not DADS or DATS, had a further improved suppressive effect at the high dose. On the other hand, both DAS and DADS but not DATS further ameliorated neutrophil infiltration in the intestine at the high compared with the low dose. We interpreted such findings to mean that the transmigration of neutrophils also relies on the severity of inflammation at the local site. The present study showed that both the high and the low dose of DAS and DADS reversed the elevated contents of ICAM-1 and CINC-1 induced by endotoxin and also the contents of TNF-alpha and IL-1beta. These results are consistent with the morphological findings. Since DAS represents only a minor part while DATS and DADS represent the predominant part of garlic oil compositions, the results of the present study that DAS was protective but DATS and DADS were harmful for normalizing intestinal morphology when provided at high dose help to interpret the findings by Kuo et al. (2011) who reported that the protective effect of garlic oil at low dose was offset when provided at high dose.

In conclusion, oral pretreatment with GSCs at doses of 0.025 and 0.125 mmol/kg, which did not affect the normal growth or basal level of systemic inflammatory indexes, showed a protective effect on endotoxin-induced neutrophil infiltration in the small intestine and appeared to be associated with an ameliorated inflammatory condition in the intestine and CD11b/CD18 level on neutrophils. Among all three tested GSCs, DAS was the most protective, whereas DATS was the least effective compound. The possible cytotoxic effect of DATS on intestinal epithelial cells, which may offset its protective effect on endotoxin-induced intestinal damage, warrants further investigation.

Conflict of Interest

The authors declare that there are no conflict of interest.

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