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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.