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Suppression of inducible nitric oxide synthase expression and amelioration of lipopolysaccharide-induced liver injury by polyphenolic compounds in *Eucalyptus globulus* leaf extract

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ABSTRACT

Eucalyptus leaf extract (ELE) is rich in hydrolyzable tannins. We examined the effects of ELE and its constituents on lipopolysaccharide (LPS)-induced liver injury in mice. Mice fed a diet supplemented with 1% ELE were intraperitoneally administered LPS. Six hours later, the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were significantly lower in the ELE-supplemented mice than in the controls; LPS-induced hepatic inducible nitric oxide synthase (iNOS) expression was also suppressed. ELE lowered LPS-stimulated iNOS expression in cultured RAW 264.7 macrophages. Furthermore, the aglycones of hydrolyzable tannins, gallic acid (GA) and ellagic acid (EA), inhibited iNOS induction to a greater extent than did ELE (15-fold higher). When mice were fed a 1% GA or EA diet, the increase in the serum ALT and AST activities and hepatic iNOS expression in response to the LPS challenge were significantly attenuated. Thus, hydrolyzable tannins in ELE ameliorate LPS-induced liver injury.

1. Introduction

Lipopolysaccharide (LPS) is derived from the outer membrane of Gram-negative bacteria, and spontaneous exposure to small doses of LPS is a normal event that may be important for mucosal antigen sampling in the gut. LPS is a potent activator of macrophages and stimulates the production of proinflammatory mediators and proteins, such as inducible nitric oxide synthase (iNOS), tumour necrosis factor-α, interleukin-6, and cyclooxygenase-2 (Caradonna et al., 2000; Medvedev, Kopydlowski, & Vogel, 2000). These LPS-induced responses may be important defence mechanisms against invading Gram-negative bacteria. However, these responses are exaggerated in the presence of excess amounts of nitric oxide (NO) radicals and may cause serious inflammation (Morrison et al., 1994), owing to increased translocation of LPS through the gut, which can be caused by a number of factors, including inflammatory bowel disease (Caradonna et al., 2000), intestinal obstruction (Deitch, 1989), trauma (Peitzman, Udekwu, Ochoa, & Smith, 1991), ethanol ingestion (Purohit et al., 2008), chronic fatigue syndrome (Maes, Mihaylova, & Leunis, 2007), and consumption of a high-fat diet (Cani et al., 2007). NO, produced by iNOS, reacts with superoxide anion radicals and thus results in the generation of peroxynitrate anion radicals and hydrogen peroxide (Jorens, Matthys, & Bult, 1994). Consequently, overproduction of NO causes oxidative damage in many organs and results in fever, leucopenia, tachycardia, tachypnea and hypotension, which are symptoms of sepsis (Pinsky, 2007). In the liver, LPS-induced activation of Kupffer cells, which are resident macrophages, is a pivotal response during pathogenesis of LPS-associated hepatic tissue dysfunction (Laskin, Heck, Gardner, & Laskin, 2001). The inflammatory and immunomodulatory mediators (including interleukin-1, tumour necrosis factor-α, and NO), synthesised and released by Kupffer cells during endotoxemic episodes, mediate LPS-induced alterations such as fluctuations in metabolic pathways and pathological hepatic necrosis (Laskin et al., 2001).

The evergreen tree, *Eucalyptus globulus*, which is indigenous to Tasmania in Australia, is distributed worldwide. The leaves of this plant are infused and used as herbal tea or as an ingredient of functional foods (supplements). The essential oil, containing the major

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component 1,8-cineole, distilled from the leaves of this plant, is industrially produced and largely used as a flavour for foods, and also throat drops. In Japan, the aqueous ethanolic extract of the leaves (eucalyptus leaf extract; ELE) is included as one of the antioxidants in the List of Existing Food Additives (Notification No. 120 [16 April 1996]; Ministry of Health and Welfare, Japan); ELE is used as a natural food additive to prevent oxidative deterioration of the quality of fats and oils (Amakura et al., 2002; Amakura, Yoshimura, Sugimoto, Yamazaki, & Yoshida, 2009). The safety of ELE has been confirmed by the Japanese Ministry of Health, Labour and Welfare (Inoue, 2007). Some potent antioxidative compounds have been isolated from this plant, e.g. hydrolyzable tannins (Amakura et al., 2009; Sugimoto et al., 2009). In addition, the leaves have been used as a traditional folk medicine applied externally for the treatment of inflammation and incisura in China. It has been reported that ELE suppresses NO production and iNOS mRNA expression in the murine macrophage cell line J774A.1 when the cells are stimulated with LPS and interferon-γ (Vigo, Cepeda, Gualillo, & Perez-Fernandez, 2004).

In this study, we examined the protective effect of ELE on hepatic dysfunction induced by LPS in mice. Here, we report that the hydrolyzable tannins present in ELE, which are presumably hydrolysed to form gallic acid (GA) and ellagic acid (EA) in the digestive tract, suppress the LPS-induced iNOS expression in macrophages (Kupffer cells) and ameliorate hepatic disorders.

2. Materials and methods

2.1. Materials

One kilogramme of dried eucalyptus (*E. globulus*) leaves, which were harvested in Spain and purchased from K. Kobayashi & Co. Ltd. (Kobe, Japan), was extracted using 10 kg of ethanol–water (1:2, v/v) under reflux for 2 h. The extract obtained was filtered and evaporated to dryness *in vacuo*. The total polyphenol content of ELE was approximately 30%, as measured in terms of tannic acid equivalents by Folin–Ciocalteu's method (Julkunen-Tiitto, 1985).

1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (PGG) was obtained from ELE by performing repeated column chromatography according to a previously described method (Sugimoto et al., 2009).

2.2. Animal experiments

Male Balb/c mice (age, 7 weeks; weight, 20–22 g) were purchased from Kiwa Laboratory Animals (Wakayama, Japan), housed individually at controlled temperature (23 [2] °C), humidity (60 [10]%), and lighting (light period from 09:00 to 21:00 h), and fed a powdered standard diet and water *ad libitum*. One kilogramme of the standard diet contained 170 g of casein, 700 g of starch, 30 g of soybean oil, 35 g of mineral mixture (AIN-93G-MX; Clea Japan, Tokyo, Japan), 10 g of vitamin mixture (AIN-93VX; Clea Japan), 3 g of methionine, 2 g of choline chloride, and 50 g of cellulose. The test samples (ELE, GA and EA) were added to the respective diets by substituting a portion of cellulose with the corresponding test sample; the doses were as follows: (1) the ELE diet contained 1–10 g/kg of ELE and 40–49 g/kg of cellulose; (2) the GA diet contained 1 g/kg of GA and 49 g/kg of cellulose); and (3) the EA diet contained 1 g/kg of EA and 49 g/kg of cellulose.

When the mice were 8 weeks old, they were randomly divided into groups based on their diets (10 mice per group) and fed the ELE, GA, EA, or standard (control) diet for 10 d. During the feeding period, changes in food intake and body weight were noted. At the end of the feeding period, LPS (*Escherichia coli* 055:B5; Difco Laboratory, Detroit, MI) was dissolved in pyrogen-free saline to achieve a concentration of 250 μg/ml, and half of the mice in each group were intraperitoneally administered 50 μg of LPS. All of the LPS challenges were carried out at 10:00 h to exclude the effects of the circadian rhythm or hormone levels. Six hours after the LPS injection, all the mice (treated and untreated) were anesthetized with diethyl ether. Blood was sampled by cardiac puncture, and the liver was excised. The blood was allowed to clot at 4 °C for 2 h and then centrifuged to obtain the serum. After the liver was weighed and washed with phosphate-buffered saline (pH 6.8), it was immediately frozen in liquid nitrogen and macerated.

All experimental procedures involving laboratory animals were approved by the Animal Care and Use Committee of Osaka Prefecture University.

2.3. Blood and liver analyses

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the sera were measured using a commercially available kit (Transaminase C-II Test; Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. The hepatic thiobarbituric acid-reactive substance (TBARS) level was determined using the method described by Ohkawa, Ohishi, and Yagi (1979). Expression of iNOS protein in the liver was analysed using western blotting according to the method described by Matsuzaki, Kuwamura, Yamaji, Inui, and Nakano (2001). In brief, the livers were homogenised in 25 mM HEPES-KOH buffer (pH 7.4) at 4 °C. The homogenate was centrifuged (4500*g*, 4 °C) for 10 min, and the supernatant was used as a sample. The sample (75 μg of protein) was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (7% gel), and the proteins in the gel were electroblotted to a polyvinylidene difluoride membrane. The membrane was treated with rabbit anti-iNOS antibodies (1000-fold dilution) (Santa Cruz Biotechnology, Santa Cruz, CA), and thereafter with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (KPL, Gaithersburg, MD). Immunoreactive proteins were detected by a chemiluminescent method, using a commercial kit (3000-fold dilution) (Super Signals CL-HRP, Pierce, Rockford, IL), and the relative amounts of these proteins were estimated using scanning densitometry. The protein content was determined according to the method described by Bradford (1976) by using bovine serum albumin as a standard.

2.4. Cell culture and treatment

The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (high-glucose; Sigma Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin) at 37 °C in a humidified incubator containing 5% CO_2. The cells were subcultured every 3 days.

The test samples, that is ELE, PGG, GA, EA and quercetin (QC), were dissolved in dimethylsulphoxide and diluted to obtain a solution with a final dimethylsulphoxide concentration of 0.1%. The subcultured RAW 264.7 cells were allowed to reach confluence and then seeded at a density of 8×10^2 cells/mm^2 in a 60 mm dish and cultured for 2 d. ELE, PGG, GA, EA and QC solutions of several concentrations were added to the culture medium; 30 min later, these cells were stimulated with LPS (1 μg/ml) and incubated for an additional 12 h. The stimulated cells were washed twice with ice-cold phosphate-buffered saline and lysed with 100 mM HEPES-NaOH buffer (pH 7.5) containing 0.5% Nonidet P-40, 1 mM EDTA, 10 μM leupeptin, 10 μM aprotinin, and 100 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride for 10 min at 4 °C. After centrifugation (17,500*g*, 4 °C) for 20 min, expression of iNOS protein in the cell lysate was analysed using western blotting, and the relative amount of protein was estimated using scanning densitometry.