Food and Chemical Toxicology 50 (2012) 2188–2195

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Vascular barrier protective effects of phlorotannins on HMGB1-mediated proinflammatory responses *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 8 March 2012
Accepted 28 March 2012
Available online 13 April 2012

Keywords:
Phlorotannins
Vascular barrier integrity
Endothelium
Hydroxyl group

ABSTRACT

The phlorotannins (phloroglucinol, eckol, and dieckol) are active compounds found in *Eisenia bicyclis*, and have been widely investigated for their antioxidant, anti-tumor, and anti-cancer activities. In this study, we investigated the protective effects of these phlorotannins against pro-inflammatory responses in human umbilical vein endothelial cells (HUVECs) and in mice treated by high mobility group box 1 protein (HMGB1), and the signaling pathways involved. The protective activities of the phlorotannins were determined by measuring permeability, leukocyte adhesion and migration, and the activations of pro-inflammatory proteins in HMGB1-activated HUVECs. We found that the phlorotannins inhibited; lipopolysaccharide (LPS)-induced HMGB1 release, HMGB1-mediated barrier disruption, the expressions of cell adhesion molecules (CAMs), and the adhesion/transendothelial migration of leukocytes to human endothelial cells. The phlorotannins also suppressed acetic acid induced-hyperpermeability and carboxymethylcellulose-induced leukocytes migration *in vivo*. Further studies revealed that the hydroxyl groups on dieckol positively regulated these vascular barrier protective effects. Collectively, these results suggest that phloroglucinol, eckol, and dieckol protect vascular barrier integrity by inhibiting hyperpermeability, the expressions of CAMs, and the adhesion and migration of leukocytes, which confirms their potential usefulnesses for the treatment of vascular inflammatory diseases.

1. Introduction

High-mobility group proteins are small DNA-binding proteins that serve an important role in transcriptional regulation (Bustin et al., 1990). One of these proteins, HMGB1, has been identified to be a late acting mediator of LPS-induced (Wang et al., 1999) or sepsis-induced lethality in mice (Yang et al., 2004). Extracellular HMGB1 can stimulate the secretion of proinflammatory cytokines from endothelial cells, monocytes, and macrophages, which leads to inflammatory responses in target tissues (Andersson et al., 2000; Bae and Rezaie, 2011; Fiuza et al., 2003). Furthermore, it triggers the activations of endothelium and leukocytes by binding to at least three pathogen-associated cell surface pattern recognition receptors, such as, toll-like receptors (TLR) 2 and 4 and the receptor for advanced glycation end products (RAGE), and by so doing induces TNF-α expression and NF-κB activation in target cells (Fiuza et al., 2003; Hori et al., 1995; Park et al., 2004). A high plasma concentration of HMGB1 in patients with inflammatory diseases is known to be related to poor prognosis and high mortality, and the pharmacological inhibition of HMGB1 is known to improve survival in animal models of acute inflammation in response to endotoxin challenge (Sama et al., 2004). Therefore, the preventions of HMGB1 production and of HMGB1-mediated proinflammatory responses are considered promising therapeutic strategies for the treatment of vascular inflammatory diseases.

Previous studies have focused on disease preventing roles of dietary factors, such as, phenolic compounds and polyphenols, in the contexts of serious diseases, such as, cancer, coronary heart disease, and inflammatory diseases (Kohyama et al., 1997; Koshihara et al., 1984; Yang et al., 1999). Accordingly, the search for anticancer drugs and anti-inflammatory agents in natural products represents an area of great interest (Aggarwal et al., 2006). *Eisenia bicyclis* (Kijellman) Setchell is a common perennial brown alga of the family Laminariaceae that inhabits the middle Pacific coast around Korea and Japan (Okada et al., 2004), and the phlorotannins, which are oligomers and polymers of phloroglucinol, are found exclusively in brown algae (Koivikko et al., 2007). *E. bicyclis* have been reported to have several biological activities, which include antioxidant, anti-tumor, anti-cancer, and bactericidal activities, and to Alzheimer's disease (Jung et al., 2010; Nagayama

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http://dx.doi.org/10.1016/j.fct.2012.03.082

et al., 2002; Okada et al., 2004). However, anti-inflammatory effects of the phlorotannins of *E. bicyclis* on HMGB1-mediated proinflammatory responses in endothelial cells and in mice have not been previously studied. In the present study, we evaluated the effects of three phlorotannins from *E. bicyclis*, namely, phloroglucinol, eckol, and dieckol, on HMGB1-activated human endothelial cells and in mice.

2. Materials and methods

2.1. Reagents

Phloroglucinol (Fig. 1), bacterial lipopolysaccharide (LPS, #4391, used at 100 ng/ml), Evans blue, crystal violet, and MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma (St. Louis, MO, USA). Human recombinant HMGB1 was purchased from Abnova (Taipei City, Taiwan).

2.2. Plant materials, extraction, and the isolations of eckol and dieckol

Fresh *E. bicyclis* was washed three times with water to remove salt, lyophilized, and ground into a powder. Dried *E. bicyclis* powder (1.0 kg) was extracted with MeOH (10 L × 3) at room temperature, and the solvent was then evaporated *in vacuo*. The combined crude MeOH extract (164.3 g) was suspended in 10% MeOH (1.0 L), and then partitioned sequentially with *n*-hexane (1.0 L × 3), CH_2Cl_2 (1.0 L × 3), EtOAc (1.0 L × 3), and *n*-BuOH (1.0 L × 3) to yield dried *n*-hexane- (42.3 g), CH_2Cl_2 (2.5 g), EtOAc- (23.0 g), *n*-BuOH (26.5 g), and H_2O-soluble (69.1 g) residues, respectively. A portion (10.0 g) of the EtOAc extract was chromatographed on a Sephadex LH-20 column (4.0 cm i.d. × 50 cm) using MeOH as eluant and fractioned into seven subfractions (EB01–EB07). Subfractions EB02 and EB07 were subjected to column chromatography over a LiChroprep RP-18 column (1.1 cm i.d. × 37 cm) using aqueous MeOH to yield pure eckol (**1**) (*tr* 4.0 min, 25.2 mg) and dieckol (**2**) (*tr* 8.1 min, 17.2 mg) as shown in Fig. 1.

2.3. Spectroscopic data of Me-dieckol

For 7-[2,6-dimethoxy-4-(2,4,7,9-tetramethoxydibenzo [*b*,*e*] [1,4]dioxin-1-yloxy)phenoxy]-1-(3,5-dimethoxyphenoxy)-2,4,9-trimethoxydibenzo [*b*,*e*] [1,4]dioxine; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.67 (s, 3H), 3.70 (s, 6H), 3.72 (s, 6H), 3.74 (s, 6H), 3.75 (s, 3H), 3.85 (s, 3H), 3.86 (s, 3H), 3.94 (s, 3H), 5.94 (d, J = 3.0 Hz, 1H), 6.11–6.16 (m, 4H), 6.20–6.22 (m, 2H), 6.30 (s, 1H), 6.32 (s, 2H), 6.34 (d, J = 2.7 Hz, 1H); LC-MS (ESI) m/z 897 ($[M + 1]^+$).

2.4. Cell culture

Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA) and maintained as described previously (Bae and Rezaie, 2008). Briefly, cells were cultured to confluency at 37 °C and 5% CO_2 in EBM-2 basal media supplemented with growth supplements (Cambrex Bio Science). A human monocyte cell line, THP-1 cells, was maintained at a density of 2×10^5 to 1×10^6 cells/ml in RPMI 1640 containing L-glutamine and 10% heat-inactivated FBS supplemented with 2-mercaptoethanol (55 μM) and antibiotics (penicillin G and streptomycin).

2.5. Animals and husbandry

Female ICR mice (6 weeks old upon receipt, from Orient (South Korea)) were used after a 12-day acclimatization period. The animals were housed five per polycarbonate cage under controlled temperature (20–25 °C) and humidity (40–45%) under a 12:12 h light/dark cycle. A normal rodent pellet diet and water were supplied *ad libitum* during acclimatization. All animals were treated in accordance with the guidelines issued by Kyungpook National University regarding the Care and Use of Laboratory Animals.

2.6. Competitive ELISA (enzyme-linked immunosorbent assay) for HMGB1

Ninety-six well plastic flat microtiter plates (Corning, NY, USA) were coated with HMGB1 protein in 20 mM carbonate–bicarbonate buffer (pH 9.6) containing 0.02% sodium azide, overnight at 4 °C. The plates were then rinsed three times in PBS-0.05% Tween 20 (PBS–T) and kept at 4 °C. Lyophilized culture media were pre-incubated with anti-HMGB1 antibodies (Abnova, diluted 1:1000 in PBS-T) in 96-well plastic round microtiter plates for 90 min at 37 °C, and these pre-incubated samples were then transferred to pre-coated plates, and incubated for 30 min at room temperature. The plates were then rinsed three times in PBS-T, incubated for 90 min at room temperature with peroxidase-conjugated anti-rabbit IgG antibodies (diluted 1:2000 in PBS-T, Amersham Pharmacia Biotech), re-rinsed three times with PBS-T, and incubated for 60 min at room temperature in the dark with 200 μl substrate solution (100 μg/ml *o*-phenylenediamine and 0.003% H_2O_2). After stopping the reaction with 50 μl of 8 N H_2SO_4, absorbances were read at 490 nm.

2.7. ELISA for toll like receptor 4 (TLR4) expression

The expression of TLR4 on HUVECs was determined by whole-cell ELISA as described (Kim et al., 2011b). Briefly, confluent monolayers of HUVECs were treated with phloroglucinol, eckol, and dieckol for 6 h followed by LPS (100 ng/ml) for 3 h. The medium was removed, and cells were washed with PBS and fixed with 50 μL of 1% paraformaldehyde for 15 min at room temperature. After washing, 100 μL of TLR4 antibodies (H-80, Santa Cruz, CA, USA) were added. After 1 h (37 °C, 5% CO_2), the cells were washed three times and then 100 μL of 1:2000 peroxidase-conjugated anti-rabbit IgG antibodies (Sigma, Saint Louis, MO) were added

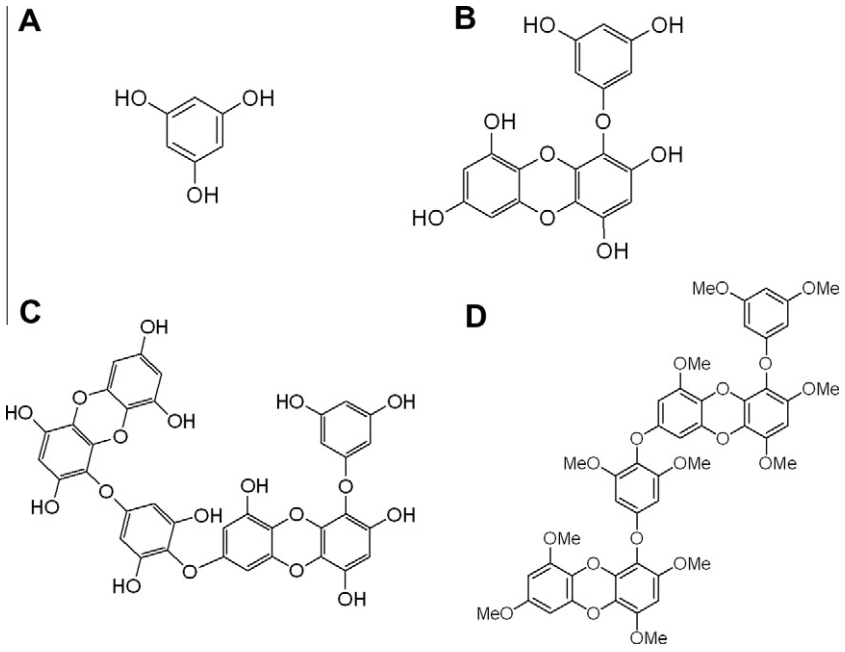

Fig. 1. Chemical structure of the three phlorotannins and of Me-dieckol. A, phloroglucinol. B, eckol. C, dieckol. D, Me-dieckol.