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Barrier protective effects of withaferin A in HMGB1-induced inflammatory responses in both cellular and animal models

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ABSTRACT

Withaferin A (WFA), an active compound from *Withania somnifera*, is widely researched for its anti-inflammatory, cardioactive and central nervous system effects. In this study, we first investigated the possible barrier protective effects of WFA against pro-inflammatory responses in human umbilical vein endothelial cells (HUVECs) and in mice induced by high mobility group box 1 protein (HMGB1) and the associated signaling pathways. The barrier protective activities of WFA were determined by measuring permeability, leukocytes adhesion and migration, and activation of pro-inflammatory proteins in HMGB1-activated HUVECs. We found that WFA inhibited lipopolysaccharide (LPS)-induced HMGB1 release and HMGB1-mediated barrier disruption, expression of cell adhesion molecules (CAMs) and adhesion/transendothelial migration of leukocytes to human endothelial cells. WFA also suppressed acetic acid-induced hyperpermeability and carboxymethylcellulose-induced leukocytes migration in vivo. Further studies revealed that WFA suppressed the production of interleukin 6, tumor necrosis factor-α (TNF-α) and activation of nuclear factor-κB (NF-κB) by HMGB1. Collectively, these results suggest that WFA protects vascular barrier integrity by inhibiting hyperpermeability, expression of CAMs, adhesion and migration of leukocytes, thereby endorsing its usefulness as a therapy for vascular inflammatory diseases.

Introduction

The intimal lining of all blood vessels is composed of a single continuous layer of simple squamous epithelial cells of mesenchymal origin which are called endothelial cells (ECs) (Mehta and Malik, 2006). Vascular endothelium was considered to be nothing more than a nucleated layer, functioning as a semipermeable barrier that separates blood from the surrounding tissues (Mehta and Malik, 2006). This endothelial barrier serves to separate the inner space of the blood vessel from the surrounding tissue and to control the exchange of cells and fluids between the two (Komarova et al., 2007; Mehta and Malik, 2006). This barrier is dynamic and highly susceptible to the regulation by various stimuli of physiological and pathological origin (Komarova et al., 2007; Mehta and Malik, 2006). It is well recognized that disruption of vascular barrier integrity results in marked increases in permeability to fluid and solute and is necessary to provide the access of leucocytes to the inflamed tissues and the central pathophysiologic mechanism of many vascular inflammatory disease processes such as sepsis and atherosclerosis (He, 2010; Komarova et al., 2007; Majno and Palade, 1961). Therefore, agents that enhance EC barrier function are desirable for a variety of inflammatory diseases (Dudek and Garcia, 2001; Komarova et al., 2007).

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 as a late acting mediator of LPS-induced (Wang et al., 1999) or sepsis-induced (Yang et al., 2004) lethality in mice. HMGB1 appears to have two distinct functions in cell systems. The intranuclear function of HMGB1 is to regulate transcription and affect chromatin structure

Abbreviations: WFA, withaferin A; HMGB1, high mobility group box 1 protein; HUVEC, human umbilical vein endothelial cell; CAM, cell adhesion molecule; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; EC, endothelial cell; LPS, lipopolysaccharide; VCAM, vascular cell adhesion molecule; ICAM, intercellular cell adhesion molecule; ELISA, Enzyme-linked immunosorbent assay; TEM, transendothelial migration.

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(Skoko et al., 2004). And extracellular HMGB1 can stimulate the secretion of proinflammatory cytokines from endothelial cells, monocytes, and macrophages, leading to inflammatory responses in target tissues (Andersson et al., 2000; Bae and Rezaie, 2011; Fiuza et al., 2003). Previous studies demonstrated that extracellular HMGB1 is a late cytokine mediator of delayed endotoxin lethality (Wang et al., 1999, 2004). HMGB1 was detected in the serum of patients with sepsis, and serum HMGB1 levels were significantly elevated in patients with poor prognoses (Chen et al., 2004; Sama et al., 2004).

The search for anticancer drugs and anti-inflammatory agents from natural products represents an area of great interest worldwide (Aggarwal et al., 2006). *Withania somnifera* has been used to treat burns, wounds and dermatological disorders to prevent infectious (Essawi and Srour, 2000). Withaferin A (WFA, Fig. 1) is a steroidal lactone derived from *W. somnifera*, a plant that has been used for centuries to treat several inflammatory disorders (Kaileh et al., 2007). However, the effect of WFA on vascular barrier integrity and the underlying mechanisms of its effect in both cellular system and animal model have not yet been elucidated. The objective of the present study was to test this naturally occurring anti-inflammatory agent for its vascular barrier protective effect on ECs and mice after HMGB1 exposure.

Materials and methods

Regents

WFA was purchased from Biomol (Plymouth Meeting, PA, USA). HMGB1 was purchased from Abnova (Taipei City, Taiwan). Bacterial lipopolysaccharide (LPS, #8274, ≥10,000 EU/mg, used at 100 ng/ml), Evans blue, crystal violet, carboxymethylcellulose sodium (CMC-Na) and MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma (St. Louis, MO, USA). Vybrant DiD (used at 5 μM) was obtained from Invitrogen (Carlsbad, CA, USA). Anti-(IκB-α) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against phospho-IκB-α (Ser32) was obtained from Cell Signaling Technology (Beverly, MA, USA).

Animals and husbandry

Female ICR mice (6 weeks old upon receipt, from Orient, South Korea) were used in this study after a 12-day acclimatization period. The animals were housed as 5 per polycarbonate cage under controlled temperature (20–25 °C) and humidity (40%–45%). The light/dark cycle was 12:12 h and normal rodent pellet diet and water were supplied during acclimatization ad libitum. All animals were treated in accordance with the Guidelines for Care and Use of Laboratory Animals of Daegu Haany University.

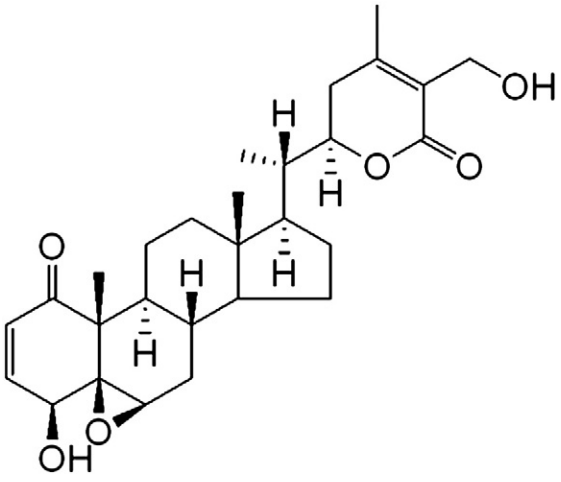

Fig. 1. Chemical structure of withaferin A (WFA).

Cell culture

Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA, USA) and maintained as described before (Bae and Rezaie, 2011). Briefly, the 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 with L-glutamine and 10% heat-inactivated FBS supplemented with 2-mercaptoethanol (55 μM) and antibiotics (penicillin G and streptomycin).

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) with 0.02% sodium azide, overnight at 4 °C. The plates were 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. Pre-incubated samples were transferred to pre-coated plate, and incubated for 30 min at room temperature. The plates were rinsed three times in PBS-T and incubated for 90 min at room temperature with the peroxidase-conjugated anti-rabbit IgG antibodies (diluted 1:2000 in PBS-T, Amersham Pharmacia Biotech). The plates were rinsed three times in PBS-T and incubated for 60 min at room temperature in dark space 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, the absorbance was read at 490 nm.

Permeability assay in vitro

Permeability was quantitated by spectrophotometric measurement of the flux of Evans blue-bound albumin across functional HUVECs monolayers using a modified 2-compartment chamber model as previously described (Bae and Rezaie, 2008). Briefly, HUVECs were plated (5×10^4/well) in 3-μm pore size and 12-mm diameter transwells for 3 days. The confluent monolayers were incubated with WFA for 6 h followed by HMGB1 (1 μg/ml) for 16 h or LPS (100 ng/ml) for 4 h. Inserts were washed with PBS, pH 7.4, before addition of 0.5 ml of Evans blue (0.67 mg/mL) diluted in growth medium containing 4% BSA. Fresh growth medium was added to the lower chamber, and the medium in the upper chamber was replaced with Evans blue/BSA. After 10 min, the optical density at 650 nm was measured in the lower chamber. Experiments were performed in triplicate and repeated multiple times.

Permeability assay in vivo

Mice were pretreated with WFA and after 6 h, 1% Evans blue dye solution in normal saline was injected intravenously in each mouse immediately followed by an intraperitoneal injection of 0.7% acetic acid (1.4 μg/200 μl per mouse). Thirty minutes later, the mice were sacrificed and the peritoneal exudates were collected after being washed with 5 ml of normal saline and centrifuged at 200×*g* for 10 min. The absorbance of the supernatant was read at 650 nm. The vascular permeability was expressed in terms of dye (μg/mouse), which leaked into peritoneal cavity according to a standard curve of Evans blue dye.