Food and Chemical Toxicology 50 (2012) 1288–1294

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

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

# Anti-inflammatory activities of oleanolic acid on HMGB1 activated HUVECs

Eun-Ju Yang [a,1], Wonhwa Lee [b,c,1], Sae-Kwang Ku [d,1], Kyung-Sik Song [c], Jong-Sup Bae [c,*]

[a] BK21 Research Team for Developing Functional Health Food Materials, Kyungpook National University, Daegu 702-701, Republic of Korea
[b] Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Daegu 702-701, Republic of Korea
[c] College of Pharmacy, Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu 702-701, Republic of Korea
[d] Department of Anatomy and Histology, College of Oriental Medicine, Daegu Haany University, Gyeongsan 712-715, Republic of Korea

ARTICLE INFO

*Article history:*
Received 28 October 2011
Accepted 14 February 2012
Available online 22 February 2012

*Keywords:*
Oleanolic acid
Barrier integrity
LPS
HMGB1

ABSTRACT

As a late mediator of inflammation, high mobility group box 1 (HMGB1) protein up-regulates pro-inflammatory cytokines in several inflammatory diseases. Further, high plasma levels of HMGB1 correlate with poor prognosis and increased mortality in patients with severe inflammation. Oleanolic acid (OA), a triterpenoid known for its anti-inflammatory and anti-cancer properties, is commonly present in several medicinal plants but the effects of OA on HMGB1-mediated pro-inflammatory responses of human endothelial cells is not well-studied. In this study, we investigated this question by monitoring the effect of OA on lipopolysaccharide (LPS)-mediated release of HMGB1 and the HMGB1-mediated modulation of inflammatory responses in human umbilical vein endothelial cells (HUVECs). OA potently inhibited the release of HMGB1 by HUVECs as well as down-regulated HMGB1-dependent adhesion and migration of the monocytic cell line THP-1 to activated HUVECs. OA also down-regulated the cell surface expression of the receptor of HMGB1, thereby inhibiting HMGB1-dependent pro-inflammatory responses by inhibiting activation of nuclear factor-κB (NF-κB) and production of tumor necrosis factor-α (TNF-α) by HMGB1. Given these results, OA showed anti-inflammatory activities and could be a candidate as a therapeutic agent for various inflammatory diseases through the inhibition of the HMGB1 signaling pathway.


## 1. Introduction

The intracellular architectural protein that is termed high mobility group box protein 1 (HMGB1) was identified as a potent pro-inflammatory mediator when present extracellularly (Wang et al., 1999). HMGB1 is a highly conserved chromatin binding protein which is abundantly expressed in mammalian tissues (Muller et al., 2001). The presence of extracellular HMGB1 is not only a telltale that a cell or tissue has suffered from damage, but also provides danger signals to a variety of cells that constitute the innate immune system (DeMarco et al., 2005; Harris and Raucci, 2006). To act as a danger signal and inflammatory mediator, HMGB1 must be transported extracellularly (Wang et al., 1999). This occurs in two principally different manners; active secretion and passive release. HMGB1 is passively released by necrotic cells (Scaffidi et al., 2002) and could be actively secreted by stimulated macrophage or monocytes in a process requiring acetylation of the molecule which enables translocation from the nucleus to secretory lysosomes (Andersson et al., 2000; Wang et al., 1999). Subsequent transport out of the cells depends on a secretion signal mediated by either extracellular lysophophatidyl-choline or ATP (Andersson et al., 2000; Wang et al., 1999). Binding of HMGB1 to the receptor for advanced glycation end products (RAGE) on endothelial cells and monocytes increases the expression of adhesion molecules and stimulates the production of an array of pro-inflammatory cytokines (Andersson et al., 2000; Fiuza et al., 2003). The involvement of toll like receptor (TLR) 2 and 4 in cell activation by HMGB1 has also been demonstrated (Park et al., 2004).

The mistletoe (*Viscum album*) plant is a semi-parasite growing on many kinds of trees all over the world. The aqueous extracts of mistletoe had been used as anti-cancer agents for almost a century (Li, 2002). Korean mistletoe (KM) is also used as a traditional herb medicine for treatment of cancer, cardiovascular disease, and arthrosis (Kim et al., 2010). The studies on the biological effects of KM and its chemical compositions demonstrated that the lectins of KM induced apoptotic cell death on cancer cells (Yoon et al., 1999). The lectins of KM also showed immunomodulating activity to augment antigen-specific cellular and humoral immune responses (Yoon et al., 2001). In addition, we found that the lectins of KM inhibited experimental lung metastasis of tumor cells in mice and its antimetastatic activity was partly due to activation of

* Corresponding author. Present address: College of Pharmacy, Research Institute of Pharmaceutical Sciences, Kyungpook National University 1370 Sankyuk-dong, Buk-gu, Daegu 702-701, Republic of Korea. Tel.: +82 53 950 8570; fax: +82 53 950 8557.
*E-mail address:* baejs@knu.ac.kr (J.-S. Bae).
[1] First three authors contributed equally to this work.

0278-6915/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2012.02.026

macrophages and NK (natural killer) cells (Yoon et al., 2003). Previous studies have demonstrated that LPS stimulates HMGB1 release in murine macrophages and human endothelial cells (El Gazzar, 2007; Mullins et al., 2004). Following its release to intravascular spaces, HMGB1 is known to interact with specific cell surface receptors to amplify inflammatory responses by inducing the expression of pro-inflammatory cytokines (Fiuza et al., 2003). Recently, Kawahara et al. showed an inhibitory effect of OA on LPS-mediated HMGB1 release in RAW264.7 cell (Kawahara et al., 2009). Even though the lectins, alkaloids and viscotoxins isolated from KM were proven as the active compounds related to anti-cancer and immunomodulating activities, the effects of a triterpene, oleanolic acid (OA) on HMGB1-mediated pro-inflammatory responses are not well known. Although neutralizing anti-HMGB1 antibodies are shown to protect animals from the lethality of endotoxemia (Wang et al., 2001), the safety and efficacy of antibodies in a mouse model underestimates toxicity and overestimates efficacy in human, since in many case the antibodies do not cross-react with normal tissue of the mouse. Therefore, new compound from herbal medicine which has no cyto-toxicity but has anti-inflammatory activities against HMGB1-mediated proinflammatory responses could be a candidate as a therapeutic agent for various inflammatory diseases. Thus, the aim of this study was to investigate the effect of OA which was extracted from the mistletoe (*V. album*) plant on HMGB1-mediated pro-inflammatory responses in human endothelial cells.

## 2. Materials and methods

### 2.1. Reagents

Bacterial lipopolysaccharide (LPS, #4391), Evans blue, crystal violet, 2-mercaptoethanol and antibiotics (penicillin G and streptomycin) were purchased from Sigma (St. Louis, MO). Human recombinant HMGB1 was purchased from Abnova (Taipei City, Taiwan). Fetal bovine serum (FBS) and Vybrant DiD were purchased from Invitrogen (Carlsbad, CA, USA).

### 2.2. General

Organic solvents, such as ethanol (EtOH), dichloromethane ($CH_2Cl_2$), ethyl acetate (EtOAc), methanol (MeOH) and *n*-butanol (*n*-BuOH) were purchased from Duksan Chemical (Anseong, Korea). Nuclear magnetic resonance (NMR) spectra, $^1H$– and $^{13}C$–NMR, were recorded on a Bruker Avance Digital 400 NMR spectrometer (Karlsruche, Germany) with tetramethyl silane (TMS) as an internal standard. Chemical shifts ($\delta$) are expressed in ppm relative to TMS. All solvents were evaporated below 40 °C under reduced pressure. Thin layer chromatography (TLC) was performed on pre-coated plates (Kiesel gel 60 F254, Merck, NJ, USA). Silica gel for open column chromatography was Kiesel gel 60 (70–230 mesh, Merck).

### 2.3. Plant material, extraction, and isolation

The dried mistletoe (*V. album*) plant was purchased from Daeyu Oriental Pharm Co. (Daegu, Korea). The specimen is stored at the College of Pharmacy, Kyungpook National University, Deagu, Korea (voucher specimen number: KNUNPC-VAE-01). Dried mistletoe (2.7 kg) was refluxed with 95% EtOH for 5 h and then filtered through filter paper. The solution was evaporated to dryness to yield 466 g of ethanolic extract. The extract was successively partitioned with $CH_2Cl_2$, EtOAc and *n*-BuOH. The active $CH_2Cl_2$ soluble fraction (113 g) was chromatographed on the silica gel (12 × 75 cm, $CH_2Cl_2$:MeOH = 500:1→1:1) to give 14 fractions (Fr. 1–14). Compound 1 (3 g) was obtained from Fr. 10.

### 2.4. Compound 1 (OA)

$^1H$–NMR (400 MHz, $CDCl_3$) $\delta$: 0.74, 0.79, 0.90, 0.92, 0.93, 0.99 and 1.12 (each 3H, s), 3.22 (1H, dd, $J$ = 4.0 and 9.5 Hz, H-3), 5.28 (1H, m, H-12). $^{13}C$–NMR (100 MHz, $CDCl_3$) $\delta$: 16.0 (C-25), 16.5 (C-24), 18.0 (C-26), 19.0 (C-6), 23.7 (C-16), 23.8 (C-11 and C-30), 26.0 (C-27), 28.4 (C-2 and C-15), 28.7 (C-23), 31.1 (C-20), 33.2 (C-22 and C-29), 33.3 (C-7), 34.5 (C-21), 37.5 (C-10), 38.9 (C-1), 39.6 (C-4), 40.0 (C-8), 42.0 (C-14 and C-18), 46.5 (C-17 and C-19), 48.4 (C-9), 56.0 (C-5), 79.8 (C-3), 122.8 (C-12), 144.2 (C-13), 184.2 (C-28). $^1H$– and $^{13}C$–NMR data were consistent with previously published data (Cho et al., 2009). The chemical structure of compound 1 was identified as oleanolic acid and is presented in Fig. 1A.

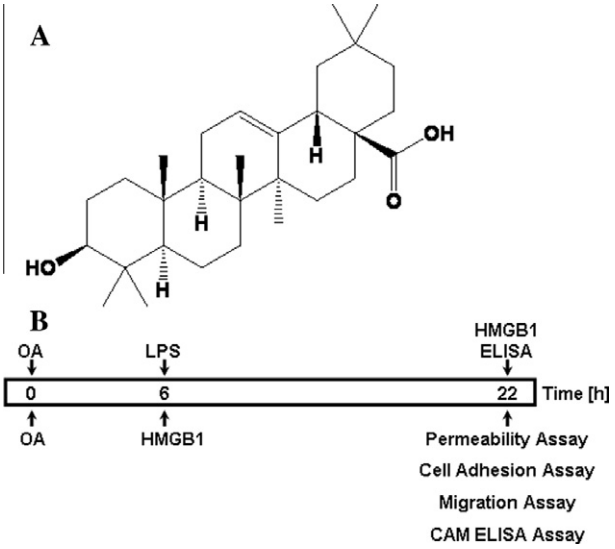


**Fig. 1.** Structure of oleanolic acid (OA), (A) and experimental timeline (B).

### 2.5. Cell culture

Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA) and maintained as described before (Bae and Rezaie, 2008). 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). THP-1 cells, a monocyte cell line, were maintained described before (Kim and Bae, 2010).

### 2.6. Permeability assay

Permeability was measured by the flux of Evans blue-bound albumin across functional HUVEC monolayers as previously described (Bae and Rezaie, 2008). Briefly, HUVECs ($7 \times 10^4$/well) were plated in transwells (3 μm, Corning, Lowell, MA, USA) for 3 days. The confluent monolayers were incubated with OA for 6 h followed by HMGB1 (1 μg/ml) for 16 h. Inserts were washed with PBS before adding 0.5 ml Evans blue (0.67 mg/mL) diluted in growth medium containing 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.

### 2.7. Cellular antioxidant activity assay

Intracellular ROS (reactive oxygen species) production was detected using the non-fluorescent cell permeating compound, 2'-7'-dichlorofluorescein diacetate (DCF-DA). DCF-DA is hydrolyzed by intracellular esterases and then oxidized by ROS to a fluorescent compound 2'-7'-DCF. Peroxyl radicals are generated by thermolysis of 2,2'-Azobis (amidinopropane) (ABAP) at physiological temperature. ABAP decomposes at approximately $1.36 \times 10^{-6} s^{-1}$ at 37 °C, producing at most $1 \times 10^{12}$ radicals/ml/s (Bowry and Stocker, 1993; Niki et al., 1986; Thomas et al., 1997). HUVECs were plated onto 24 wells plates ($3 \times 10^6$ cells/well) and were incubated for 1 h with OA. Then HUVECs were preloaded with DCF-DA for 0.5 h, washed twice with PBS, and ABAP (0.6 mM final concentration) was then added. The fluorescence, which indicates ROS levels, was measured in a plate reader with excitation at 485 nm and emission at 520 nm.

### 2.8. Cell-Cell adhesion assay

THP-1 cell adherence to HUVECs was evaluated by fluorescent labeling of THP-1 cells as described (Akeson and Woods, 1993). Briefly, THP-1 cells ($1.5 \times 10^6$/ml, 200 μl/well) were labeled with the Vybrant DiD dye followed by their addition to washed and stimulated HUVECs. HUVEC monolayers were treated for 6 h with indicated OA followed by HMGB1 (1 μg/ml for 16 h). THP-1 cells were allowed to adhere and the non-adherent THP-1 cells were washed off and the fluorescence of the adherent cells was measured. The percentage of adherent THP-1 cells was calculated by the formula: % adherence = (adherent signal/total signal) × 100 as described. The data were expressed as the means from at least three independent experiments.

### 2.9. Trans-endothelial migration assay (TEM)

Migration assays were performed in transwell plates of 6.5 mm diameter, with 8 μm pore size filters (Corning, Lowell, MA, USA). HUVECs ($6 \times 10^4$) were cultured for three days to obtain confluent endothelial monolayers. Cell monolayers were