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Acteoside Improves Survival in Cecal Ligation and Puncture-Induced Septic Mice *via* Blocking of High Mobility Group Box 1 Release

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Acteoside, an active phenylethanoid glycoside, has been used traditionally as an anti-inflammatory agent. The molecular mechanism by which acteoside reduces inflammation was investigated in lipopolysaccharide (LPS)-induced Raw264.7 cells and in a mouse model of cecal ligation and puncture (CLP)-induced sepsis. *In vitro*, acteoside inhibits high mobility group box 1 (HMGB1) release and iNOS/NO production and induces heme oxygenase-1 (HO-1) expression in a concentration-dependent manner, while HO-1 siRNA antagonizes the inhibition of HMGB1 and NO. The effect of acteoside is inhibited by the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 and Nfr2 siRNA, indicating that acteoside induces HO-1 *via* p38 MAPK and NF-E2-related factor 2 (Nrf2). *In vivo*, acteoside increases survival and decreases serum and lung HMGB1 levels in CLP-induced sepsis. Overall, these results that acteoside reduces HMGB1 release and may be beneficial for the treatment of sepsis.

INTRODUCTION

The pathogenesis of severe sepsis and shock caused by invasive infection is characterized by the excessive release of early and late pro-inflammatory cytokines (Aziz et al., 2012). Among pro-inflammatory cytokines, high mobility group box 1 (HMGB1) accumulates in circulation during sepsis, leading to multiple organ collapse and a lethal outcome (Yang et al., 2004). HMGB1 is actively released by immune cells (macrophages, monocytes, natural killer cells, and platelets) into the extracellular environment (Andersson et al., 2000). Anti-HMGB1 antibodies and HMGB1 inhibitors significantly improve survival in septic animals, suggesting that HMGB1 is a possible therapeutic target in sepsis (Barnay-Verdier et al., 2011; Sama et al., 2004). Heme oxygenase-1 (HO-1), a stress-responsive protein induced by stimulants (inflammatory cytokines, heat shock, heavy metals, and oxidants) (Jang et al., 2012; Tsoyi et al., 2009) decreases circulating HMGB1 levels in animal models of sepsis and improves patient survival, demonstrating its therapeutic potential in inflammatory disorders (Wu et al., 2011). In addition, it is well known that the inducers of HO-1 expression inhibits the expression of the inflammatory genes (cyclooxygenase-2 and inducible nitric oxide synthase), and subsequently decreases PGE_2 and NO production (Oh et al., 2006; Suh et al., 2006). It also was recently reported that the HO-1 system provides a therapeutic effect in many experimental pathological conditions (Bonelli et al., 2012; Farombi and Surh, 2006; Luz et al., 2012). In oxidative injury and inflammation conditions, an increase in the synthesis of the HO-1 gene is linked to the transcription factors NFkB, Nrf2, and AP-1 (Paine et al., 2010; Srisook et al., 2005; Surh et al., 2009). Among the transcriptional factors, Nrf2 nuclear translocation requires the activation of several signal transduction pathways, such as mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K) and0 Akt (Martin et al., 2004).

Despite little knowledge of their mechanisms of action, herbal medicines derived from plant extracts are increasingly utilized to treat a wide variety of diseases. Among them, acteoside (Fig. 1A), a well-studied phenylethanoid glycoside, has been used in Ayurvedic and Chinese medicine for hundreds of years and has a wide range of pharmacological and biochemical effects (He et al., 2011). A growing number of studies have revealed that acteoside has many biological effects, including anti-tumor (Inoue et al., 1998), anti-oxidant (Wong et al., 2001), anti-nephritic (Hayashi et al., 1994), anti-hepatotoxic (Lee et al., 2004) and antiseptic activity (Houghton, 1984; Lee et al., 2005). More recently, acteoside protected beta-amyloid-induced neurotoxicity via regulation of HO-1 expression by modulating the Nrf2 signaling pathways (Wang et al., 2012). Until now, the role of HMGB1 release and the related molecular mechanisms have not been completely clarified by acteoside. The present study demonstrates that acteoside induces HO-1 through activation of p38 and Nrf2 expression in LPS-activated Raw264.7 cells, which in turn reduces HMGB1 release both in macrophages

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and CLP-induced septic mice.

MATERIALS AND METHODS

Materials

Reagents used in this study were purchased from the following sources: Acteoside from Chromadex (USA); MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazol iumbromide] and LPS from Sigma-Aldrich (USA); SB203580, PD98059, and SP600125 were purchased from Calbiochem (USA); RPMI-1640, fetal bovine serum and Trizol were supplied by Gibco BRL (USA); antibodies for p38, phospho-p38 MAPK, β-actin and HRP-conjugated anti-rabbit IgG from Cell Signaling Technology (USA); antibodies for HMGB1, iNOS, Nrf2 and HO-1 from Calbiochem (USA). The HO-1-ARE-luciferase reporter gene was kindly provided by Dr. J. Alam (Tulane University School of Medicine, USA). All chemicals and reagents were of analytical grade.

Cell culture

Raw 264.7 cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovein serum (FBS), 100 μg/ml penicillin and 100 μg/ml streptomycin, in 5% CO_2 at 37°C.

Cell treatment and viability assay

Acteoside was dissolved in dimethylsulfoxide (DMSO) and the stock solutions were added directly to the culture media. The control cells were treated with culture medium only. The cells (5×10^3/well) in 10% FBS-DMEM were seeded into the 48-well plates. After incubation for 24 h, various concentrations of acteoside and LPS were added to the well, and the plates were incubated at 37°C for an additional 24 h. The cells were used for the MTT-based assay by measuring the according to the manufacture's instructions. Relative cytotoxicity was quantified by absorption measurements at 550 nm using a microtiter plate reader (Molecular Devices, USA). This wavelength was not found to interfere with acteoside.

Nitric oxide assay

The nitrite and nitrate concentration in the medium was measured, as an indicator of NO production. One hundred ml of each supernatant was mixed with the same volume of Griess reagent; the absorbance of the mixture at 545 nm was determined with an ELISA plate reader.

HMGB1 release assay

The levels of HMGB1 in the culture medium and blood were determined using commercially available enzyme-linked immunosorbent assay kits (R&D Systems, USA) and were performed according to the manufacturer's instructions.

RNA preparation and mRNA analysis by real-time quantitative PCR

The total RNA was isolated from cells using Trizol (GibcoBRL, USA). Accumulated PCR products were directly detected by monitoring the increase in reporter dye (SYBR®). The expression levels of HO-1 in the exposed cells were compared to those in the control cells at each time point using the comparative cycle threshold (*Ct*) method (Johnson et al., 2000). The following primer sequences were used: the HO-1 primers were sense 5′-CGCCTTCCTGCTCAACATT-3′ and antisense 5′-TGTGTTCCTCTGTCA GCATCAC-3′. Ribosomal protein S18 (S18) sense 5′-AAGTTTCAGCACATCCTGCGAGTA-3′; and S18 antisense 5′-TTGGTG AGGTCAATGTCTGCTTTC-3′. The quantity of each transcript was calculated as described in the instrument manual and was normalized to the amount of S18, a housekeeping gene.

Western immunoblot analysis

Cells were harvested and washed 3 times with cold phosphate-buffered saline (PBS). The cytoplasmic and nuclear protein fractions were extracted using NE-PER extraction reagents according to the manufacturer's protocol (Pierce Biotechnology, USA). Cytoplasmic/nuclear protein extracts or whole protein extracts were used for Western blot analysis. Western blotting was performed using anti-iNOS, anti-HO-1, anti-HMGB1, anti-p38, anti-phospho-p38, anti-nrf2 and anti- β-actin antibodies. Protein samples were heated at 95°C for 5 min and were analyzed using SDS-PAGE. Immunoblot signals were developed by enhanced chemiluminescence (Pierce Biotechnology, USA).

siRNA knockdown

siHO-1 and scrambled siRNA were purchased from Invitrogen. siNrf2, and scrambled siRNA were acquired from Santa Cruz Biotechnology. siRNA was transfected into macrophages according to the manufacturer's protocol and using the transfection reagent, Lipofectamine 2000© (Invitrogen, USA). The cells were incubated with 100 nM of target siRNA or scramble siRNA for 4 h in serum- and antibiotic-free media. The cells were then incubated for 18 h in media containing antibiotics and FBS, and cells were washed, pretreated with or without acteoside for 1 h, and treated with LPS.

Transient transfection and luciferase assay

Cells (3×10^5 cells/well) were seeded in 24-well plates, incubated overnight and transiently co-transfected with ARE-promoter-luciferase construct and pRL-SV40 plasmid (*Renilla* luciferase expression for normalization) (Promega, USA) using LipofectAMINE™ 2000 reagent (Invitrogen, USA). Relative luciferase activities were calculated by normalizing ARE-promoter-driven firefly luciferase activity to *Renilla* luciferase activity.

Animal model of sepsis

Specific pathogen-free BALB/C mice (male aged 8 weeks, 25 ± 3 g) were obtained from Central Laboratory Animal Inc. (Korea). Animals were housed under normal laboratory conditions, i.e. at 21-24°C and 40-0% relative humidity under a 12 h light/dark cycle with free access to standard rodent food and water. To induce sepsis, BALB/C mice were anesthetized with zoletil (80 mg/kg) and Xylazine (10 mg/kg) and then a 2 cm midline incision was performed to allow exposure of the cecum with adjoining intestine. The cecum was tightly ligated with a 3.0-silk suture at 5.0 mm from the cecal tip and punctured once with a 22-gauge needle (top and bottom). The cecum was then gently squeezed to extrude a small amount of feces from the perforation sites and returned to the peritoneal cavity. The laparotomy site was then stitched with 4.0-silk. In the sham control animals, the cecum was exposed but not ligated or punctured and then returned to the abdominal cavity. All animals were raised under specific pathogen-free conditions, and the protocol was reviewed and approved by the Animal Subjects Committee of Asan Medical Center (Korea).

Statistical analysis

All the experiments were repeated at least three times. The results are expressed as a mean ± SD, and the data were analyzed using one-way ANOVA followed by a Student's *t*-test for significant difference. The Kaplan-Meier method was used to

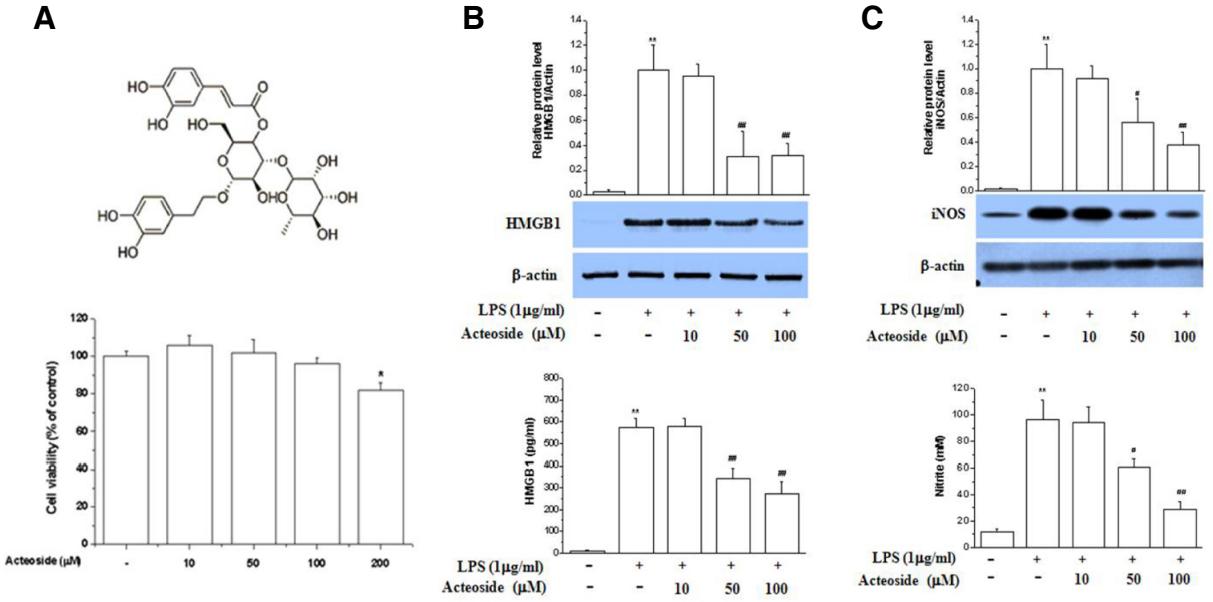

Fig. 1. Effect of acteoside on HMGB1 release and NO production in LPS-stimulated Raw264.7 cells. Cells were treated with acteoside for 24 h (10, 50, and 100 μM). Cell viability was measured by MTT assay (A). Cells were pretreated with acteoside for 1 h (10, 50, and 100 μM) and then stimulated with LPS (1 μg/ml) for 24 h. Cells were lysed, harvested and subjected to Western blotting for detection of HMGB1 (B, upper) and iNOS (C, upper). The culture medium was collected and subjected to HMGB1 release (B, lower) and NO production analysis (C, lower). Significance compared to control, $^{*}p < 0.05$; significance compared to LPS, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$.

compare the differences in mortality rates between groups. A p value < 0.05 were considered significant.

RESULTS

Acteoside inhibits HMGB1 release and iNOS expression/NO production

The optimal concentration range of acteoside was assessed in Raw264.7 cells (> 95% cell viability, Fig. 1A). To identify the potential anti-inflammatory mechanism of action of acteoside, cells were incubated with acteoside in the presence of LPS. As shown in Fig. 1, acteoside inhibited HMGB1 protein expression and release in a concentration-dependent manner (Fig. 1B) and reduced nitric oxide synthase (iNOS) protein expression and production of nitric oxide (NO) (Fig. 1C) in LPS-activated Raw264.7 cells. These results confirm a previous report according to which acteoside inhibits the expression of iNOS (Lee et al., 2005).

Anti-inflammatory effect of acteoside is mediated through HO-1

Since HO-1 decreases HMGB1 levels in septic conditions, acteoside was tested for potential induction of HO-1 in Raw264.7 cells (Wu et al., 2011). After cells were treated with acteoside for 24 h, the treatment dose-dependently increased both HO-1 mRNA and protein expression (Fig. 2A). RNA silencing was used to determine whether HO-1 induction reduced NO production and HMGB1 release. Results showed that HO-1 siRNA significantly reversed the anti-inflammatory effect of acteoside (Fig. 2B).

Acteoside induces HO-1 expression through p38 MAPK, but not ERK or JNK

As shown in Fig. 3A, the HO-1 increase by acteoside was sensitive to pretreatment with actinomycin D or cycloheximide, suggesting that acteoside increases the expression of the inducible heme oxygenase isoform. The sensitivity of the mRNA increase to cycloheximide suggests that induction of HO-1 transcription involves *de novo* protein synthesis, which is consistent with the results of a previous study (Hill-Kapturczak et al., 2000). Next, the signaling cascade of HO induction by acteoside was assessed. To identify which MAPK mediates the acteoside induction of HO-1, pharmacological inhibitors were used. As shown in Fig. 3B, the induction of HO-1 in Raw264.7 cells by acteoside was inhibited by the p38 inhibitor SB203580 but not by the MAPK inhibitor SP600125 or the JNK inhibitor PD98059. Moreover, acteoside significantly induced p38 activation in Raw264.7 cells in a concentration-dependent manner (Fig. 3C). These results show that p38 plays a key role in acteoside-mediated HO-1 induction.

Acteoside activates Nrf2 in LPS-activated Raw264.7 cells

Among the HO-1 transcription factors, Nrf2 regulates ARE-driven HO-1 gene expression (Alam and Cook, 2003; Srisook et al., 2005). To understand whether p38 MAPK has a key role for inducting HO-1 through Nrf2 activation, we checked the Nrf2 nuclear translocation ARE-luciferase activity by acteoside. Figure 4A shows that acteoside stimulated the nuclear translocation of Nrf2 in Raw264.7 cells in a concentration-dependent manner. In addition, acteoside increased ARE-luciferase activity, which was significantly reduced by SB203580, indicating that acteoside- induced Nrf2 binding DAN is dependent on p38 activity (Fig. 4B). To assess further whether acteoside-mediated HO-1 expression occurs *via* Nrf2 activation, Nrf2 was silenced. As shown in Fig. 4C, acteoside significantly reduced HO-1 expression, demonstrating the critical role of Nrf2 signal-

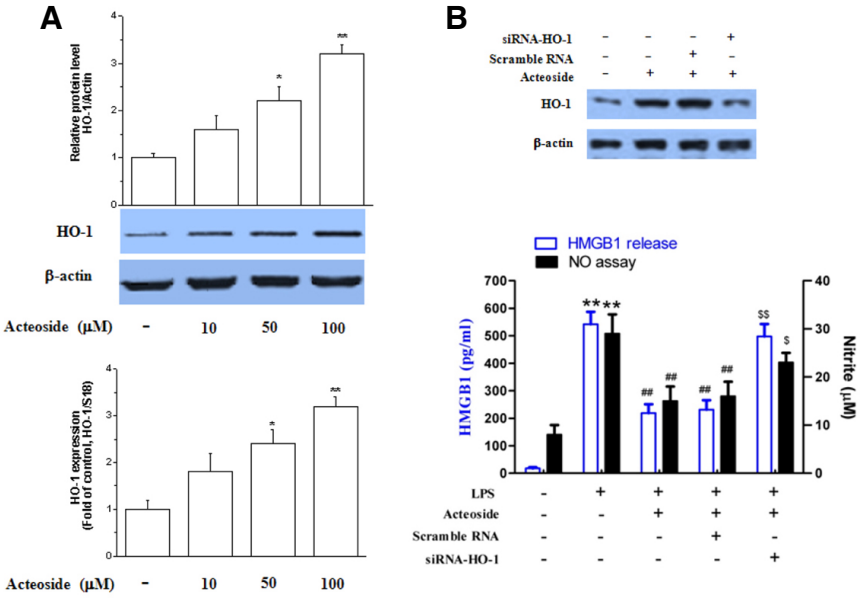

Fig. 2. Effect of acteoside on the expression of HO-1 and role in anti-inflammatory activity. Raw264.7 cells were exposed to acteoside for 24 h, after which HO-1 protein expression and mRNA were analyzed by Western blotting and RT-PCR (A). Significance compared to control, $^{*}p < 0.05$ and $^{**}p < 0.01$. Cells were transfected with scrambled siRNA or HO-1 siRNA and subjected to Western blotting to confirm the effect of HO-1 siRNA upon exposure to acteoside (100 μM) for 24 h (B, upper). Cells were stimulated with LPS (1 μg/ml) in the presence or absence of acteoside (100 μM) for 24 h. Supernatants were subjected to HMGB1 release and NO production analysis (B, lower). Significance compared to LPS, $^{*}p < 0.05$; significance compared to LPS + acteoside, $^{\#}p < 0.05$.

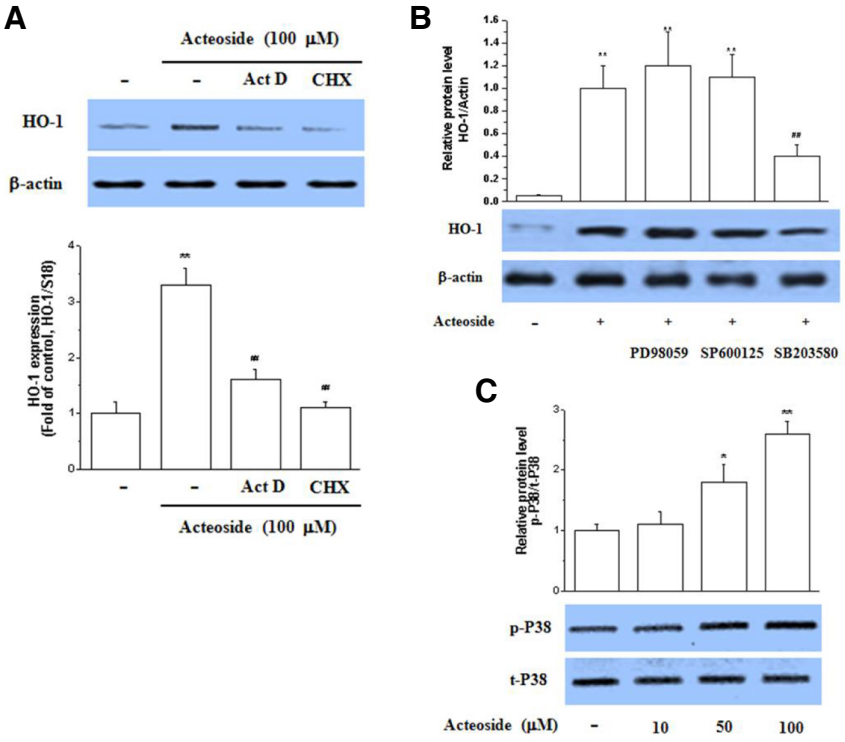

Fig. 3. Acteoside induces HO-1 through p38 MAPK. Raw264.7 cells were pretreated with cycloheximide (50 μM) or actinomycin D (10 μg/ml) for 2 h prior to the addition of acteoside (10 μM) for an additional period of 24 h. HO-1 protein expression and mRNA were analyzed by Western blotting and RT-PCR (A). Significance compared to acteoside, $^{**}p < 0.01$. Cells were pretreated with SB203580 (10 μM), SP600125 (10 μM), and PD98059 (10 μM) for 1 h, and then treated with acteoside (100 μM) for 24 h. After incubation, cells were harvested and subjected to Western blotting (B). Significance compared to control, $^{**}p < 0.01$; significance compared to LPS, $^{\#\#}p < 0.01$. Cells were exposed to acteoside for 3 h and protein was extracted. Parallel immunoblots were analyzed for total kinase levels with anti-p38 antibodies (C). Significance compared to control, $^{*}p < 0.05$, $^{**}p < 0.01$.

ing in HO-1 expression.

Anti-inflammatory effect of acteoside is mediated through p38 MARK / Nrf2 signal cascade pathways

The role of HO-1 induction in the anti-inflammatory activity of acteoside (Lee et al., 2004) was investigated Results showed that acteoside-induced HO-1 expression mediates the inhibition of HMGB1 release (Fig. 4A) and NO production (Fig. 4B), since these effects were antagonized by SB203580 and Nrf2 siRNA, thus demonstrating the importance of p38/Nrf2 signaling in acteoside-induced HO-1 expression and anti-inflammatory activity.

Acteoside inhibits HMGB1 accumulation *in vivo*

To assess the effect of acteoside on HMGB1 accumulation *in vivo*, the CLP preclinical mouse model of sepsis, which more closely resembles human sepsis than LPS-induced endotoxe-

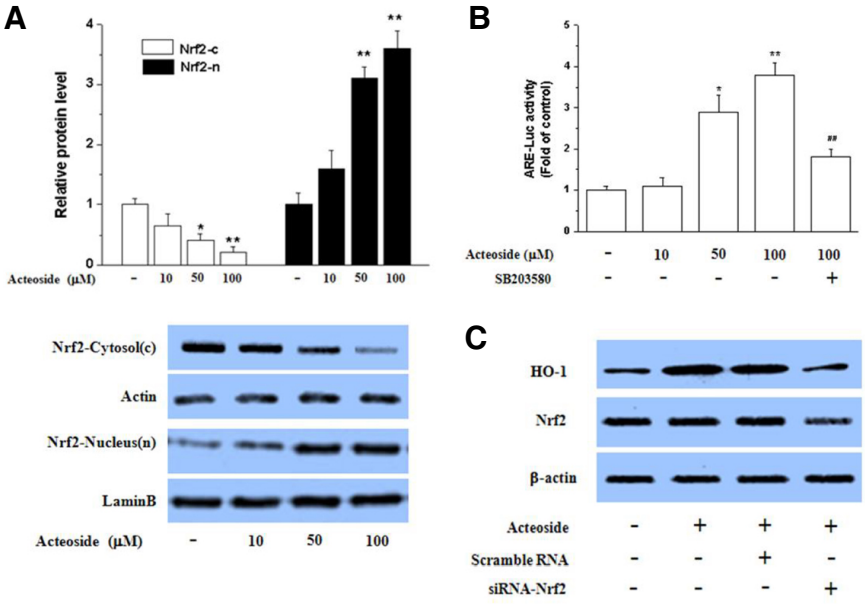

Fig. 4. Effect of acteoside on Nrf2 translocation into the nucleus and transcriptional activity. Raw264.7 cells were treated with acteoside for 3 h, then nuclear and cytosol extracts were prepared for Western blotting (A). Significance compared to cytosolic (c) control, $^{*}p < 0.05$, $^{**}p < 0.01$. Significance compared to nuclear (n) control, $^{\#\#}p < 0.01$. Cells were treated with acteoside (10 μM) for 24 h or pretreated with SB-203580 (10 μM) for 1 h before adding acteoside (100 μM) for an incubation period of 24 h. Cells were then subjected to luciferase assay (B). Significance compared to control, $^{*}p < 0.05$, $^{**}p < 0.01$. Significance compared to acteoside (100 μM), $^{\#}p < 0.05$. Cells were transfected with scrambled siRNA or Nrf2 siRNA. After a 24 h incubation period with acteoside (100 μM), cells were harvested and subjected to Western blotting for HO-1. Transfection efficiency was confirmed by measuring Nrf2 expression.

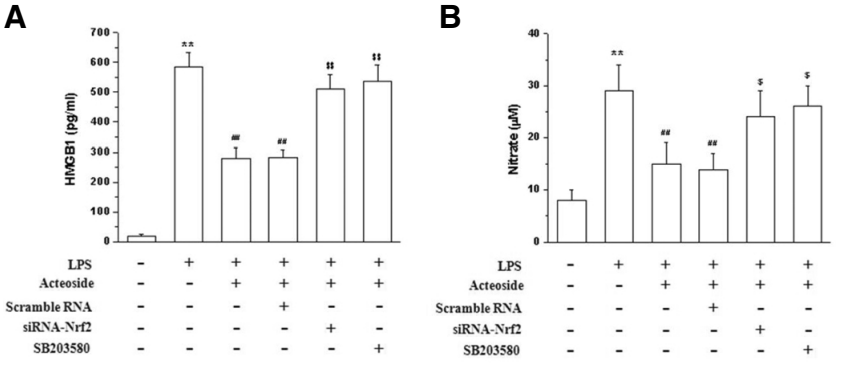

Fig. 5. Acteoside reduces inflammation by HO-1 down-regulation through p38 MARK and Nrf2 in Raw 264.7 cells. Cells were transfected with Nrf2 siRNA or scrambled siRNA and incubated with acteoside (100 μM) with or without SB203580 (10 μM) for 1 h and stimulated with LPS (1 μg/ml) for 24 h. Supernatants were subjected to HMGB1 release (A) and NO production assay (B). Significance compared to LPS, $^{**}p < 0.01$. Significance compared to acteoside + LPS, $^{\#\#}p < 0.01$.

mia, as reported previously (Tsoyi et al., 2011), was used. As shown in Fig. 6, in septic animals, the 6 day survivial rate of vehicle-treated control mice after CLP was 10%, while acteoside significantly increased survival to 60% (Fig. 6A). Moreover, HMGB1 levels, which were higher in the lung tissues and sera of CLP-induced septic mice than in those of non-CLP-induced (sham) mice, were significantly reduced by acteoside (100 mg/kg, i.p. 200 μl, twice) (Figs. 6B and 6C).

DISCUSSION

A previous study showed that acteoside up-regulates heme oxygenase-1 through phosphatidylinositol 3-kinase/Akt, ERK, and NF-E2-related factor-2 in neuronal cells (Wang et al., 2012). however, the precise anti-sepsis mechanism of action of acteoside in macrophages and septic animals remains unclear. The present study explored whether the anti-inflammatory action of acteoside was related to the induction of HO-1 in Raw264.7 cellsand CLP-induced septic mice. We first showed direct evidence that acteoside contributes to the reduction of HMGB1 and NO production by inducing HO-1 in macrophages. Other studies suggested that HMGB1 mediates cognitive impairment in sepsis survivors (Chavan et al., 2012; Huang et al., 2010) and that HO-1 inhibits the release of HMGB1 in Raw264.7 cells activated by LPS and in LPS- or CLP-induced septic mice (Tsoyi et al., 2009). Next, we assessed the signaling pathway through which acteoside induces HO-1. Since activation of MAPKs plays a central role in the induction of HO-1 gene expression [32], we investigated which MAPKs are responsible for acteoside-mediated HO-1 induction. p38 MAPK, but not JNK or ERK, mediated acteoside-induced HO-1 expression. Furthermore, the inhibition of HO-1 induction and that of the anti-inflammatory effect of acteoside by SB203580 suggests that p38 plays a key role in acteoside-mediated HO-1 induction.

The HO-1 gene promoter contains multiple regulatory transcription factor binding sites, including ARE, NF-κB, AP-1, and AP-2 responsive elements (Boberek et al., 2010; Lavrovsky et al., 1994; Surh et al., 2009). Among the transcriptional factors, Nrf2 is the transcription factor that can activate the transcription of anti-oxidant proteins including HO-1. Under the Nrf2 activation, it dissociates from Kelch-like ECH-associated protein 1 (Keap1) and translocates into the nucleus and positively regulates the ARE-mediated expression of phase II detoxification enzyme genes, including HO-1(Hwang and Jeong, 2010; Zhang

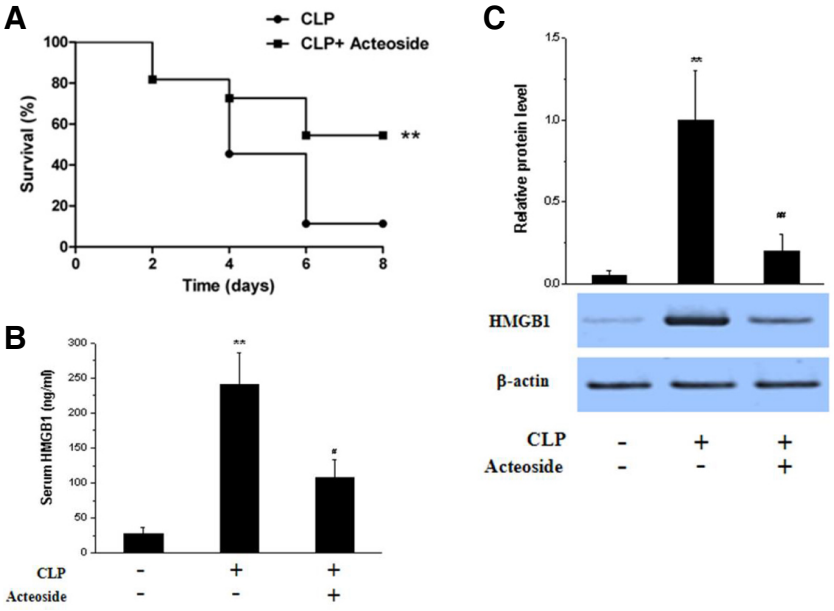

Fig. 6. Acteoside increases survival and inhibits HMGB1 levels in CLP-induced septic mice. BALB/c WT mice (n = 10) were subjected to CLP and received acteoside (n = 10, 100 mg/kg i.p.) or an equal volume of vehicle (n = 10, saline) at 0 and 24 h after the onset of sepsis. Survival was monitored daily for up to 8 days (A). To measure HMGB1 levels, WT mice were treated with acteoside (100 mg/kg i.p., n = 3) or an equal volume of vehicle (n = 3, saline) at 0 and 12 h after the onset of sepsis (CLP). After 24 h, lung tissues were harvested under anesthesia from the sham, CLP, and CLP + acteoside groups. Protein was extracted from lung tissues and expression of HMGB1 was analyzed by Western blotting (B). Blood was collected by cardiac puncture and HMGB1 release was analyzed by ELISA (C). Significance compared to sham, * $p < 0.05$. Significance compared to CLP, $^{\#}p < 0.05$ serum (C).

et al., 2004). Acteoside-induced HO-1 expression in Raw264.7 cells was significantly inhibited by Nrf2 siRNA, indicating the involvement of p38 MARK/Nrf2 signaling; however, others have produced compelling evidence for the lack of involvement of NFkB and p38 in HMGB1 release in LPS-treated macrophages, while emphasizing the role of Janus kinase 2/signal transducer and activator of transcription-1 and iNOS/ NO signaling in HMGB1 release (Jiang and Pisetsky, 2006; Oh et al., 2009). Thus, further study is needed to identify the pathways involved in the inhibition of HMGB1 downstream of HO-1. HMGB1 is released from stimulated macrophages and works as a late mediator of sepsis (Chavan et al., 2012; Yang et al., 2004) In addition, HMGB1 neutralizing antibodies potently improve survival in septic animals, suggesting a potential therapeutic benefit. Finally, the present study showed that the release of HMGB1 was decreased by acteoside in LPS-stimu-lated Raw264.7 cells as well as in CLP-induced septic mice. These results suggest that the traditional use of acteoside as an anti-inflammatory agent can be explained partly by the induction of HO-1.

In summary, acteoside is able to induce HO-1 in macrophages through p38MAPK/Nrf2 signaling and to decrease HMGB1 release in LPS-stimulated Raw264.7 cells and in CLP-induced septic mice. To the best of our knowledge, this is the first report showing that acteoside inhibits HMGB1 release in LPS-activated macrophages and CLP-induced septic animals. Thus, HO-1 induction accounts for the anti-inflammatory action of acteoside, which may have therapeutic value in systemic inflammatory conditions such as sepsis.

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