



## Aloin reduces inflammatory gene iNOS via inhibition activity and p-STAT-1 and NF- $\kappa$ B

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### ABSTRACT

Aloin is the major anthraquinone glycoside obtained from the *Aloe* species and exhibits anti-inflammatory and anti-oxidative activities. Here, we aimed to determine the effects of aloin on heme oxygenase-1 (HO-1) induction and on the expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) 2 in lipopolysaccharide (LPS)-activated human umbilical vein endothelial cells (HUVECs). To the end, aloin was tested whether aloin reduces iNOS protein expression and inflammatory markers (interleukin (IL)-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$ ) in LPS-treated mice lung tissue. The results indicated that aloin affected HO-1 induction and reduced LPS-activated NF- $\kappa$ B-luciferase activity showed to preferential inhibition of iNOS/NO and COX-2/PGE2 that was partly related to inhibition of STAT-1 phosphorylation. In particular, aloin induced translocation of Nrf2 from cytosol into the nucleus by an increased Nrf2-ARE binding activity, and reduced IL-1 $\beta$  production in LPS-activated HUVECs. The reduced expression of iNOS/NO by aloin was reversed by siHO-1RNA-transfection. In LPS-treated mice, aloin significantly reduced iNOS protein in lung tissues, and TNF- $\alpha$  levels in the BALF. We concluded that aloin may be beneficial for treatment of lung injury.

### 1. Introduction

Heme oxygenase-1 (HO-1) is considered to be the main protein in diseases such as pulmonary diseases, systemic autoimmune disease, and cancer as a result of oxidative and inflammatory insults (Waza et al., 2018). The expression of HO-1 suppressed the production of pro-inflammatory proteins such as interleukin (IL)-1 $\beta$ , IL-6 and tumor necrosis factor (TNF)- $\alpha$  (Waza et al., 2018). In addition, gene expression of HO-1 is modulated by transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2). Under the oxidative stress or other stressful conditions, Nrf2 binds to anti-oxidant response element (ARE) on the promoters of phase II antioxidant enzyme genes and controls their expression (Raghunath et al., 2018). Thus, the Nrf2-ARE pathway is thought to be an important target for therapy of inflammation-related

diseases (Raghunath et al., 2018). In particular, HO-1 increased survival in cecal ligation and puncture (CLP)-induced septic mice (Tsoyi et al., 2009; Waza et al., 2018) and displayed beneficial effects in various vascular inflammatory disorders (Waza et al., 2018).

Natural substances have been traditionally administered to treat or prevent various diseases, such as cancer and infectious diseases. Aloin is the major anthraquinone glycoside obtained from the *Aloe* species. Although aloin has anti-tumor (Chen et al., 2007; Esmat et al., 2005, 2006; Niciforovic et al., 2007; Tabolacci et al., 2010), anti-viral (Li et al., 2014; Lin et al., 2008), hepato-protective (Arosio et al., 2000; Woo et al., 2002), anti-oxidative (Beppu et al., 2003; Tao et al., 2014; Wan et al., 2017), and immunomodulatory and anti-inflammatory (Park et al., 2009; Seo et al., 2014; Silva et al., 2014; Yu et al., 2006) activities, few studies are available for the induction of HO-1, reduction

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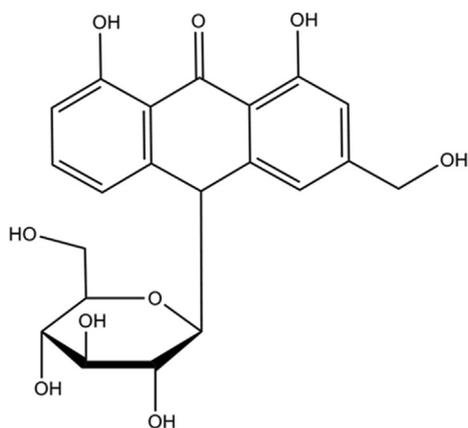


Fig. 1. Chemical structure of aloin.

of inflammatory mediators (NO, IL-1 $\beta$ , TNF- $\alpha$ ) in LPS-activated endothelial cells and in LPS-injected mice tissues. Therefore, in this study, we determined the effects of aloin on the induction of HO-1, reduction of inflammatory mediators and explored the inhibitory mechanism of aloin.

## 2. Materials and methods

### 2.1. Cell culture and reagents

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science (Charles City, IA) and maintained using a previously described method (Kim et al., 2019; Lee and Bae, 2019). HUVECs were used at cell culture passages 3–5. Aloin (Fig. 1. > 97%, dissolved in 0.5% DMSO), lipopolysaccharide (LPS, from *Escherichia coli*), antibiotics (penicillin G and streptomycin), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). Human HO-1 small interfering siRNA and control nonsense siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### 2.2. Animal care, LPS-induced lung injury model, and bronchoalveolar lavage fluid (BALF) analysis

Male C57BL/6 mice (6–7 weeks old, 27 g) were purchased from Orient Bio Co. (Sungnam, Republic of Korea) and were used in this study after an acclimatization period of 12 days. Five animals were housed per polycarbonate cage, which was kept under controlled temperature (20–25 °C), humidity (40–45% RH), and a 12:12 h light/dark cycle. The animals received a normal rodent pellet diet and were given *ad libitum* access to water during acclimatization.

For LPS lung injury model, LPS (15 mg/kg i.p., stock 1 mg/mL) with normal saline (vehicle control) was injected through a 28-gauge needle into the peritoneal space. Male C57BL/6 mice were administered with aloin (1.6–12.4 mg/kg) intravenously at 6 h after LPS injection. This protocol was approved by the Animal Care Committee at Kyungpook National University prior to conducting the study (IRB No. KNU 2017-102).

BALF was obtained by intratracheal injection of 0.7 mL cold PBS and gentle aspiration for 3 times. BALF was centrifuged at 3000 rpm for 10 min at 4 °C, and then the supernatants were preserved at –80 °C for further assay.

### 2.3. ELISA for iNOS, PGE2, p-STAT-1 (total or phosphor), HO-1, TNF- $\alpha$ , and IL-1 $\beta$

The activities of the total and phosphorylated forms STAT1 (Abcam, UK) in nuclear lysates were determined using ELISA kits (R&D Systems,

Minneapolis, MN). The concentrations of iNOS (Aviva Systems Biology, San Diego, CA), PGE2, HO-1, IL-1 $\beta$  and TNF- $\alpha$  in cell culture supernatants were determined using ELISA kits (R&D Systems, Minneapolis, MN). All measurements were performed in triplicate.

### 2.4. Nitrite determination

The production of NO was estimated from the accumulation of nitrite (NO $_2^-$ ) (a stable end product of NO metabolism) in the medium using the Griess reagent. Equal amounts of culture supernatant and Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1%  $\alpha$ -naphthylethylenediamine dihydrochloride in distilled water) were mixed and incubated for 15 min at room temperature. The reaction was measured at O.D.540 nm on a microplate reader, and the nitrate concentration was calculated using sodium nitrite as the standard.

### 2.5. Preparation of cytoplasmic and nuclear extracts and western blotting

The cells were harvested rapidly by sedimentation and nuclear and cytoplasmic extracts were prepared on ice, as previously described (Kim et al., 2019). For western blotting, anti-iNOS, anti-COX2, anti-Nrf2, anti-lamin B, or  $\beta$ -actin antibodies (Santa Cruz) were used.  $\beta$ -actin or lamin B was used as a loading control for cytoplasmic or nuclear extracts, respectively.

### 2.6. Quantitative real-time PCR

RNA was isolated using TRI-Reagent (Invitrogen), according to the manufacturer's suggested protocol. An aliquot (5  $\mu$ g) of extract RNA was reverse transcribed into first-strand cDNA with a PX2 Thermal Cycler (Thermo Scientific) using 200 U/ $\mu$ L M-MLV reverse-transcriptase (Invitrogen) and 0.5 mg/ $\mu$ l of oligo (dT)-adapter primer (Invitrogen) in a 20- $\mu$ L reaction mixture. The expression of iNOS and COX-2 was normalized to  $\beta$ -actin. The following primers were used for PCR analysis: iNOS forward 5'-GTT CTC AGC CCA ACA ATA CAA GA-3', iNOS reverse 5'-GTG GAC GGG TCG ATG TCA C-3'; COX-2 forward 5'-CCC CAT TAG CAG CCA GTT-3', COX-2 reverse 5'-CAT TCC CCA CGG TTT TGA-3';  $\beta$ -actin forward 5'-TCGTGCGTGACATCAAAGA-3',  $\beta$ -actin reverse 5'-CAT ACC CAA GAA GGA AGG CT-3'.

### 2.7. Transfection

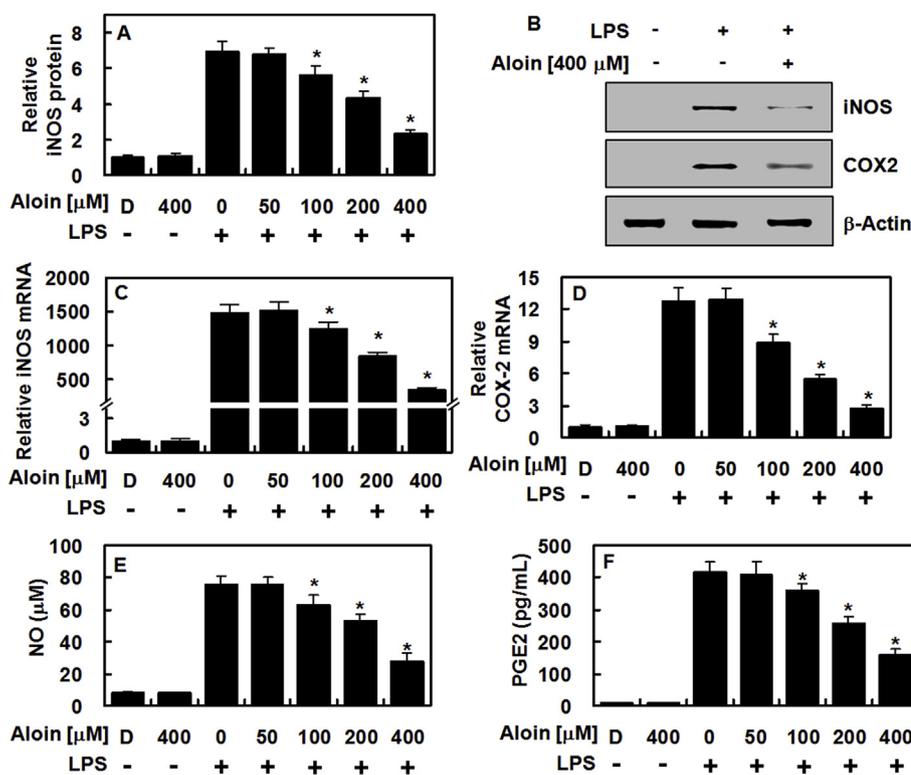
Cells were transfected with NF- $\kappa$ B luc vector, ARE luc vector, HO-1, and control nonsense siRNA using the SuperFect fragment (Qiagen, Valencia, CA). After incubation for 4 h, the medium was replaced with fresh one.

### 2.8. Luciferase activity

After experimental treatments, cells were washed twice with cold PBS, lysed in a lysis buffer provided in the dual luciferase kit (Promega, Madison, WI), and assayed for luciferase activity using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA), according to the manufacturer's protocol. All transfections were done in triplicate. The data are presented as a ratio between Firefly and renilla luciferase activities.

### 2.9. Hematoxylin & eosin (H&E) staining and histopathological examination

Male C57BL/6 mice were subjected to LPS and were administered aloin (12.4 mg/kg, i.v.) at 6 h after LPS (n = 5). And then, the mice were euthanized. To analyze the phenotypic change in the lung, H&E staining was applied as previously described (Lee et al., 2017).



**Fig. 2.** Aloin suppressed iNOS and COX-2 protein expression in LPS-activated HUVECs. After stimulation with LPS (1 μg/mL, 6 h), HUVECs were treated with the indicated concentrations of aloin for 6 h and the expressions of iNOS protein (A, B), COX-2 protein (B), iNOS mRNA (C), COX-2 mRNA (D), NO concentration (E), and PGE2 concentration (F) were measured, as described in Methods. The results shown are the mean ± SD from three separate experiments conducted in triplicate on different days. D = 0.2% DMSO is the vehicle control. \**p* < 0.05 versus LPS.

### 2.10. Statistical analysis

Data are expressed as mean ± standard deviation (SD) of 3 independent experiments. One-way analysis of variance (ANOVA) and Tukey's post-test were used for comparison among all different groups. When the ANOVA was significant, post-hoc testing of differences between groups was performed using Tukey's test. A *p* value < 0.05 was considered statistically significant.

## 3. Results and discussion

### 3.1. Effect of aloin on iNOS and COX-2 expression in LPS-activated HUVECs

To examine the effects of aloin (Fig. 1) on the expression of inflammatory gene, two representative pro-inflammatory proteins such as iNOS and COX-2 was investigated. After stimulation with LPS for 6 h, HUVECs were incubated for 6 h with different concentrations of aloin. The results showed that aloin concentration-dependently decreased the transcriptional and translational expressions of iNOS and COX-2 expression by quantitative ELISA, RT-PCR and immunoblot analyses (Fig. 2A–1D). To confirm these results, their corresponding products (NO, PGE2) were measured. As shown in Fig. 1E and F, aloin significantly and concentration-dependently reduced NO and PGE2, respectively. These results indicate that aloin suppressed LPS-induced NO production, primarily due to the aloin-mediated downregulation of iNOS.

### 3.2. Effect of aloin on NF-κB activity, phosphorylation of STAT-1 and expression of HO-1 protein

NF-κB is a crucial transcription factor for the generation of inflammatory gene expression. Therefore, we asked whether aloin inhibit NF-κB activity. Data showed that aloin concentration-dependently inhibited NF-κB luciferase activity (Fig. 3A). Noting that the activation of JAK/STAT signal plays an essential role for differential expression of

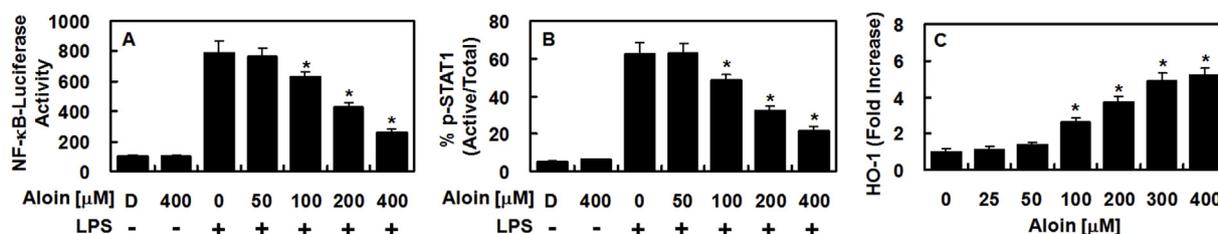
iNOS and COX-2 in LPS-activated condition (Tsoyi et al., 2008, 2010), we investigated whether aloin reduces phosphorylation of STAT-1 in LPS-activated HUVECs. As expected, aloin significantly reduced the p-STAT-1 expression (Fig. 3B). Next, we determined whether aloin up-regulate HO-1 expression. Fig. 3C clearly showed that aloin induced HO-1 expression.

### 3.3. Effect of aloin on Nrf2 translocation, ARE luciferase activity, and anti-inflammatory action

Because Nrf2 is an important transcription factor for inducing antioxidant proteins including HO-1, we measured whether aloin activates Nrf2. Indeed, aloin induced translocation of Nrf2 from cytosol into the nucleus (Fig. 4A), and increased Nrf2-ARE binding activity (Fig. 4B). To confirm further that inhibition of iNOS expression by aloin is due to induction of HO-1, small interference (siRNA) technique was applied. As deletion of HO-1 by siRNA-transfection, inhibition of iNOS protein expression and production of NO by aloin has been significantly reversed (Fig. 4C and D). These data indicated that aloin induce HO-1 expression which is, at least, responsible for inhibition of iNOS expression. Anti-inflammatory effect of aloin was further confirmed by inhibition of IL-1β production in LPS-treated HUVECs (Fig. 4E).

### 3.4. Effect of aloin on TNF-α and iNOS protein in LPS-injected mice lung tissues

Next, we asked whether administration of aloin shows anti-inflammatory effect *in vivo*. As shown in Fig. 5A, aloin significantly reduced TNF-α production in LPS-treated BALF. As the average circulating blood volume for mice is 72 mL/kg (Lee et al., 2018a, 2018b) and the average weight of mouse used was 27 g, the average blood volume was 2 mL. Hence, the amount of injected aloin (1.6, 3.1, 6.2 or 12.4 mg/kg) yielded a maximum concentration of 50, 100, 200 or 400 μM in the peripheral blood. In lung tissues, LPS significantly increased iNOS protein expression which was almost completely diminished by administration of aloin (Fig. 5B), indicating that aloin works *in vivo* as



**Fig. 3.** Aloin inhibited NF- $\kappa$ B luciferase activity and STAT-1 phosphorylation and enhanced HO-1 protein expression. After stimulation with LPS (1  $\mu$ g/mL, 6 h), HUVECs were treated with the indicated concentrations of aloin for 6 h. (A) NF- $\kappa$ B luciferase activity was measured in cells that were transiently transfected with NF- $\kappa$ B luciferase. (B) LPS-mediated activation of phosphorylated STAT1 (p-STAT1) in HUVECs was analyzed by ELISA. (C) The extracted proteins were subjected to ELISA for HO-1 expression. The results shown are the mean  $\pm$  SD from three separate experiments conducted in triplicate on different days. D = 0.2% DMSO is the vehicle control. \* $p$  < 0.05 versus LPS.

well. The histological analysis (H&E staining) also supports that aloin significantly ameliorated pulmonary injury incurred by LPS (Fig. 5C).

In this study, we demonstrated that aloin induced HO-1 expression in HUVECs in a time- and concentration-dependent manner. Interestingly, aloin inhibited LPS-induced iNOS/NO, COX2/PGE2, and NF- $\kappa$ B activity. Indeed, NF- $\kappa$ B has been implicated in the regulation of host inflammatory and immune responses, cell adhesion, developmental signals, cell proliferation, differentiation and in defending cells from apoptosis (Wullaert et al., 2011). In addition, NF- $\kappa$ B regulates the production of pro-inflammatory molecules, such as ROS, NO, COX2, IL-6 and TNF- $\alpha$  during inflammation. In particular, high level of NO also plays an important role in airway inflammatory response by regulating chemokine secretion. Indeed NF- $\kappa$ B activation is both required and sufficient for LPS-induced iNOS and COX-2 expression. Therefore, the inhibitory effects of aloin on the expressions of iNOS, NO, COX2, and ILs and induction of HO-1 by aloin were mediated via suppression of NF- $\kappa$ B activity.

We believe that inhibitory effect of aloin on TNF- $\alpha$  in LPS-administered BALF and on iNOS protein expression in LPS-treated mice lung tissue is possibly mediated through HO-1 induction. This conclusion was based on the results that the specific knockdown of the HO-1 gene using HO-1 siRNA significantly reversed the inhibitory effect of aloin on iNOS expression and production of NO. This finding suggests the importance of HO-1 for inhibition of inflammation, and TNF- $\alpha$  is a

potential target of HO-1.

Collectively, the present study showed that aloin was potent for induction of HO-1 and reduced inflammation (NO, iNOS, IL1- $\beta$ ) in LPS-activated HUVECs and (iNOS and TNF- $\alpha$ ) in LPS-injected mice tissues. Thus, aloin may be a potential drug candidate for inflammatory disorder, in particular, vascular pulmonary injury.

#### Conflicts of interest

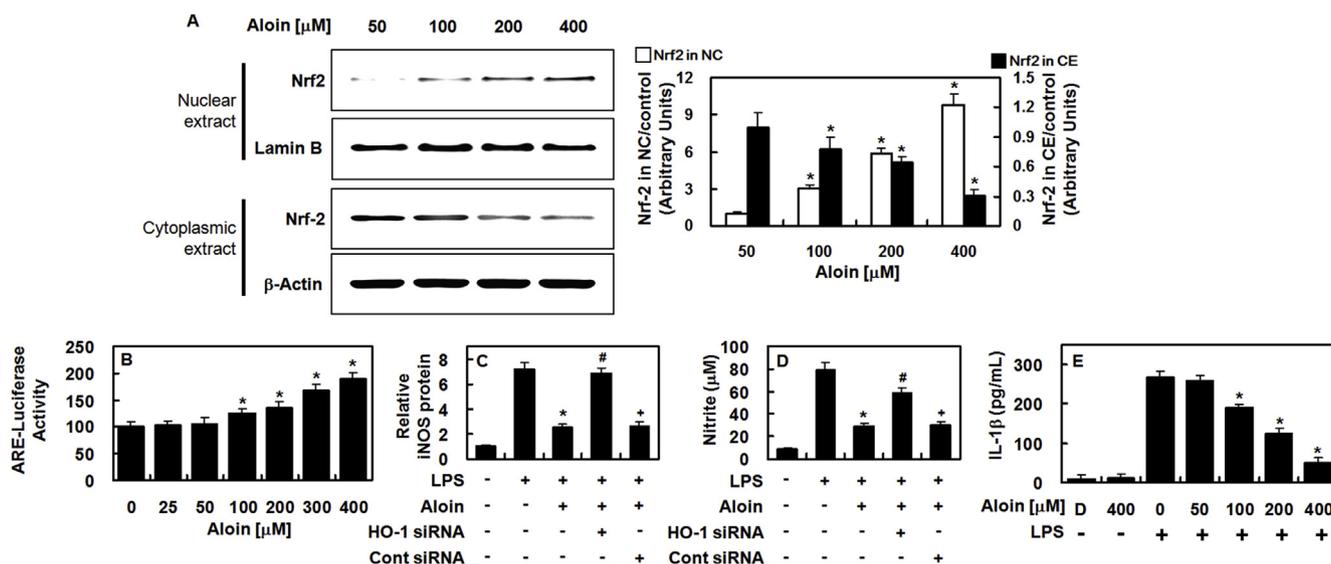
The authors have no conflicts of interest to declare.

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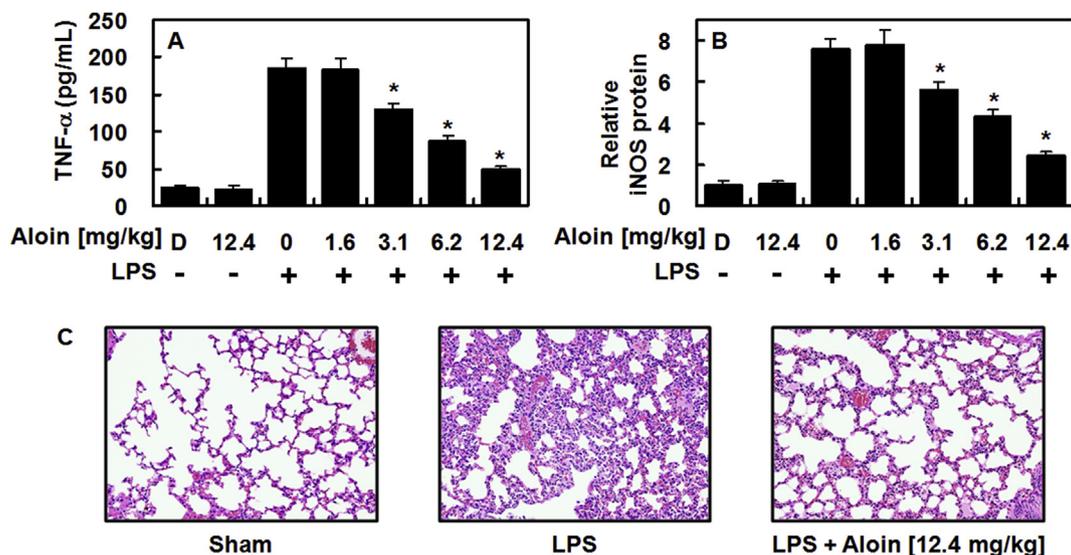
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**Fig. 4.** Aloin induced the translocation of Nrf2 and anti-inflammatory action in HUVECs. (A) HUVECs were harvested after treatment with aloin (5–400  $\mu$ M) for 6 h, and their cytosolic and nuclear fractions were extracted using a separation kit. The extracted proteins were subjected to western blotting for Nrf2. The graphs show the densitometric intensities of Nrf2 normalized to Lamin B or  $\beta$ -actin. (B) ARE luciferase activity was measured in cells transfected with ARE. (C, D) Silencing HO-1 was used to determine if iNOS (C) and NO (D) inhibitory action is involved in aloin-mediated HO-1 induction. (E) For measurement of IL-1 $\beta$  concentrations ELISA kit was used. The results shown are the mean  $\pm$  SD from three separate experiments conducted in triplicate on different days. D = 0.2% DMSO is the vehicle control. \* $p$  < 0.05 versus LPS, # $p$  < 0.05 versus LPS + Aloin, or + $p$  < 0.05 versus LPS + Aloin + HO-1 siRNA.



**Fig. 5.** Aloin inhibited the production of TNF- $\alpha$  and iNOS level and reduced lung tissue injury in LPS-injected mice. To induce lung injury model, mice were injected with bacterial endotoxin (LPS, 15 mg/kg, i.p. n = 5). Aloin [1.6–12.4 mg/kg (n = 5), i.v.] was administered for 6 h after LPS treatment. Control mice were not injected LPS (n = 5). 24 h after LPS challenge, the mice were anesthetized and sacrificed and the lung tissues and BALF were collected. Production of TNF- $\alpha$  (A) and levels of iNOS (B) were measured as described in the Methods. The results shown are the mean  $\pm$  SD from three separate experiments conducted in triplicate on different days. D = 0.2% DMSO is the vehicle control. \* $p < 0.05$  versus LPS. (C) H&E staining of each group of lung tissues was performed as described in the Method. Illustrations are representative images from three independent experiments conducted on different days with similar results.

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