

Arctigenin Protects against Lipopolysaccharide-Induced Pulmonary Oxidative Stress and Inflammation in a Mouse Model via Suppression of MAPK, HO-1, and iNOS Signaling

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Abstract—Arctigenin, a bioactive component of *Arctium lappa* (Nubang), has anti-inflammatory activity. Here, we investigated the effects of arctigenin on lipopolysaccharide (LPS)-induced acute lung injury. Mice were divided into four groups: control, LPS, LPS+DMSO, and LPS+Arctigenin. Mice in the LPS+Arctigenin group were injected intraperitoneally with 50 mg/kg of arctigenin 1 h before an intratracheal administration of LPS (5 mg/kg). Lung tissues and bronchoalveolar lavage fluids (BALFs) were collected. Histological changes of the lung were analyzed by hematoxylin and eosin staining. Arctigenin decreased LPS-induced acute lung inflammation, infiltration of inflammatory cells into BALF, and production of pro-inflammatory cytokines. Moreover, arctigenin pretreatment reduced the malondialdehyde level and increased superoxide dismutase and catalase activities and glutathione peroxidase/glutathione disulfide ratio in the lung. Mechanically, arctigenin significantly reduced the production of nitric oxide and inducible nitric oxide synthase (iNOS) expression, enhanced the expression of heme oxygenase-1, and decreased the phosphorylation of mitogen-activated protein kinases (MAPKs). Arctigenin has anti-inflammatory and antioxidative effects on LPS-induced acute lung injury, which are associated with modulation of MAPK, HO-1, and iNOS signaling.

KEY WORDS: arctigenin; lipopolysaccharide; acute lung injury; heme oxygenase; inflammation; oxidative stress.

INTRODUCTION

Acute lung injury and its severe form, acute respiratory distress syndrome (ARDS), are leading factors of morbidity and mortality in critically ill patients [1]. Histologically, acute lung injury/ARDS is characterized by hypoxemia, non-cardiogenic pulmonary edema, low lung compliance, and widespread capillary leakage [2]. Airway inflammation plays a critical role in the pathogenesis of ARDS. Lipopolysaccharide (LPS), a primary component of the outer membrane of gram-negative bacteria, can lead to various human disorders including acute lung injury by triggering severe inflammation [3]. In animal models, LPS challenge has been shown to promote rapid

infiltration of inflammatory cells, consequently resulting in the release of a variety of pro-inflammatory mediators, such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and macrophage inflammatory protein 2 (MIP2) [4, 5]. LPS can stimulate many intracellular signaling pathways, in particular mitogen-activated protein kinase (MAPK) signaling. Mammalian MAPKs include three subfamilies: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. It has been documented that MAPKs are involved in the inflammatory response of lung injury [6]

Severe inflammation is commonly linked to induction of oxidative stress [7]. Oxidative stress usually occurs when there is an imbalance between reactive oxygen species (ROS) production and elimination. Excessive ROS may exacerbate pathological inflammation and tissue damage [8]. Heme oxygenase-1 (HO-1) is an important antioxidant enzyme that can be induced by oxidative stress. It has been shown that activation of HO-1 confers protection against LPS-induced acute lung injury in rats [9]. Nitric oxide (NO) generated by inducible NO synthase (iNOS)

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also contributes to lung inflammatory injury due to oxidative stress [10]. Therefore, identification of therapeutic approaches to treat inflammation and oxidative stress is of importance for acute lung injury/ARDS.

Arctigenin, a bioactive component of *Arctium lappa* (Niubang), has various biological properties including anticancer activity [11], antioxidant activity [12], and anti-inflammatory activity [13]. A recent study has demonstrated that arctigenin attenuates LPS-induced acute lung injury in rats [14]. However, the underlying mechanism for arctigenin-mediated protective effects remains unclear. Arctigenin has been shown to inhibit LPS-induced iNOS expression and MAPK activation in RAW264.7 macrophages [15, 16]. In light of these findings, this study was designed to check whether the protective effects of arctigenin on LPS-induced acute lung injury are mediated through MAPK signaling.

MATERIALS AND METHODS

Animals

Male C57BL/6 wild-type mice (8–10 weeks old) were bought from the Experimental Animal Center of Zhengzhou University (Zhengzhou, China). All mice were kept on a 12-h light/dark cycle at a room temperature of 22 ± 2 °C with free access to food and water. The experiments were approved by the Animal Care and Use Committee of Affiliated Cancer Hospital of Zhengzhou University.

Mice were randomly divided into four groups ($n=16$ per group): control group receiving physical saline, LPS group injected with LPS alone, LPS+DMSO group injected with LPS and dimethyl sulfoxide (DMSO), and LPS+arctigenin group injected with arctigenin followed by LPS. Arctigenin was dissolved in 0.5 % DMSO at a concentration of 0.5 mg/ml. Mice were administrated intraperitoneally (i.p.) with 50 mg/kg/day arctigenin 1 h before exposure to LPS. To control for the toxic effects of the arctigenin solution, 0.5 % DMSO (a vehicle) was used. There was no evidence of lung injury after intraperitoneal injection of 0.5 % DMSO alone for 4 consecutive days (data not shown). Mice were anesthetized with i.p. ketamine and acetylpromazine (150 and 13.5 mg/kg, respectively) before exposure of the trachea. *Escherichia coli* LPS (*E. coli* serotype O111:B4; Sigma, St. Louis, MO, USA) was administrated intratracheally during inspiration at a dose of 10 mg/kg via a 20-gauge catheter. Physical saline intratracheally given to the mice did not cause evident damage to the lung and was thus used as a control for

LPS. At 4 days after instillation [17], mice were sacrificed and bronchoalveolar lavage fluids (BALFs) and lung tissues were collected. This time point was chosen because our prior experiments showed the onset of severe lung injury at 4 days after LPS challenge. The left lung was fixed and embedded in paraffin for morphological analysis. BALF samples were taken from the right lung. After the collection of BALFs, lung tissues were snap-frozen in liquid nitrogen and stored at -80 °C for Western blot analysis and NO production.

Histological Examination

Lung tissue samples were fixed for at least 4 h in 4 % paraformaldehyde. Tissues were dehydrated in an ascending series of ethanol to 100 %, embedded into paraffin, and cut into 4- μ m-thick sections. The sections were deparaffinized with xylene, rehydrated, and stained for 4–6 min with hematoxylin and eosin. Afterwards, the sections were dehydrated, cleared with xylene, and mounted with resinous mounting medium. Stained sections were examined under a light microscope. Degree of lung injury was scored in a blinded manner based on the following parameters, i.e., inflammatory cell infiltration, alveolar hemorrhage, alveolar wall thickening, and hyaline membrane formation [18]. The lung injury scoring system consists of 5 scales: 0, no damage; 1, mild damage; 2, moderate damage; 3, severe damage; and 4, very severe damage.

BALF Analysis

BAL was performed through a tracheal cannula with 5 ml saline solution. A 500- μ l aliquot of BALFs was used for the determination of total cell numbers and cell differentiation. Total cells were counted by a standard hemocytometer. Cell differentiation was done by counting at least 500 cells on a smear prepared by cytospin and Wright-Giemsa staining. The remaining BALFs were centrifuged at 800g for 5 min, and the supernatant was immediately stored at -80 °C. Total protein and albumin levels in BALFs were measured with the Bradford assay kit (Bio-Rad, Hercules, CA, USA) and Bromocresol green assay kit (Sigma), respectively.

Enzyme Linked Immunosorbent Assay (ELISA)

The levels of tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and macrophage inflammatory protein 2 (MIP-2) were examined by commercially available ELISA kits (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions.

Biochemical Assay

Commercially available kits for detection of lung superoxide dismutase (SOD), glutathione peroxidase (GSH), glutathione disulfide (GSSG), catalase (CAT), and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China). SOD, GSH/GSSG, CAT, and MDA were measured according to the manufacturer's instructions.

Measurement of Nitric Oxide (NO) Production

NO production in mouse lung tissues was examined by a nitric oxide quantification kit (Active Motif Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, lung tissues were thawed and homogenized, and the tissue supernatants were collected. The amount of total protein was quantified by the BAC Protein assay kit (Bio-Rad). Lung tissue samples were placed in a 96-well microtiter plate in triplets. Nitrate reductase and cofactors were added to convert nitrate to nitrite. Nitrite concentrations were determined by Griess reagent. Photometric measurements were performed by a spectrophotometer (Versa, Molecular Devices, Sunnyvale, CA, USA) operating at 540-nm absorbance and 620-nm reference wavelengths. Values are normalized to control group.

Semi-Quantitative Reverse Transcriptase Polymerase Chain Reaction Assay

Total RNA was extracted from mouse lung tissues with fresh TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed with the SuperScript[®] Double-Stranded cDNA Synthesis Kit (Invitrogen) according to the manufacturers' instructions. PCR amplification of HO-1 was performed with the following primers: forward 5'-GCCACCAAGGAGGTACACAT-3' and reverse 5'-CTTCCAGGGCCGTGTAGATA-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as an internal control was amplified in parallel with the following primers: forward 5'-AACGACCCCTTCATTGAC-3' and reverse 5'-TCCACGACATACTCAGCAC-3'. PCR products were electrophoresed on a 1.5 % agarose gel and detected by ethidium bromide staining. The relative HO-1 level was calculated after densitometric quantification of PCR products and normalization against GAPDH.

Western Blot Analysis

Lung tissue samples were collected and homogenized. Total protein and nuclear protein were extracted

separately by the ReadyPrep Protein Extraction Kit (Total Protein, Bio-Rad) and ReadyPrep Protein Extraction Kit (Cytoplasmic/Nuclear, Bio-Rad). The protein concentration was determined by BCA protein assay kit (Bio-Rad). Twenty micrograms of protein was loaded and separated by 10 % SDS-PAGE gel. After the protein was electrotransferred onto nitrocellulose membranes, the membranes were blocked with 5 % nonfat dry milk and incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma). The immunoblots were visualized with an enhanced chemiluminescence reagent (Amersham, Arlington Heights, IL, USA). The intensity of each band was quantified with NIH Image J software (Bethesda, MD, USA).

Statistical Analysis

All values are expressed as mean±standard deviation (SD). Differences among the groups were analyzed using a one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Arctigenin Inhibits LPS-Induced Acute Lung Injury

As shown in Fig. 1, control mice exhibited normal lung architecture. LPS instillation resulted in severe damage to the lung, including alveolar wall thickening and inflammatory cell infiltration. Arctigenin pretreatment significantly attenuated LPS-mediated lung injury, evidenced by decreased inflammatory cell influx.

Arctigenin Reduces LPS-Induced Lung Permeability

As shown in Table 1, the total protein and albumin levels in BALFs were significantly enhanced in LPS-treated mice (*P*<0.01, relative to control mice). The BALF total protein and albumin levels were significantly reduced by arctigenin. Moreover, LPS-induced elevation in the numbers of BALF total cells, neutrophils, macrophages, and lymphocytes was significantly decreased by arctigenin pretreatment (Table 1).

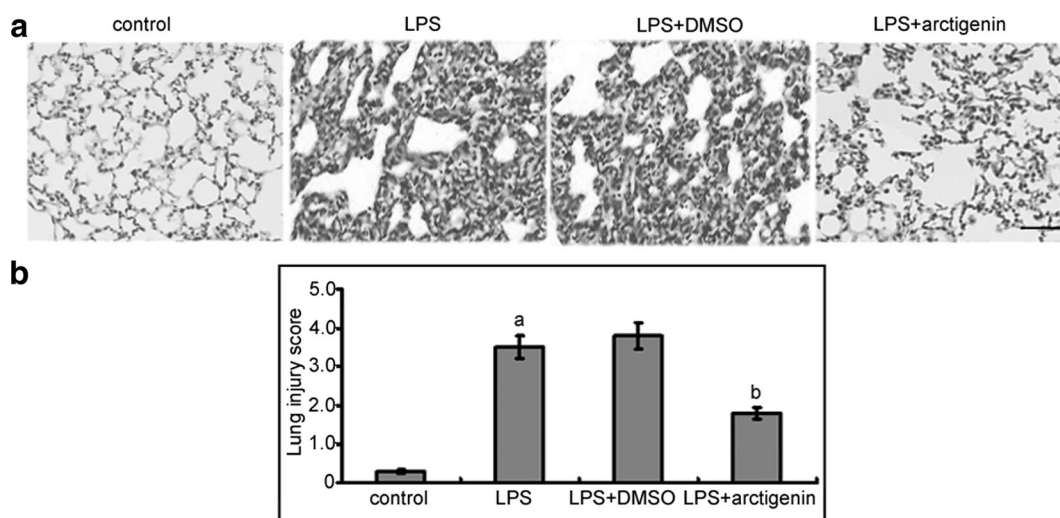


Fig. 1. Effects of arctigenin on histological changes in the lung of mice at 4 days after LPS administration. Representative sections stained with hematoxylin and eosin are shown. Lung tissues from LPS-treated mice showed alveolar wall thickening and inflammatory cell infiltration. The LPS-induced inflammatory changes were markedly prevented in the LPS+Arctigenin group. Scale bar=100 μ m.

Arctigenin Inhibits the Release of Pro-Inflammatory Molecules into BALFs

We also evaluated the effects of arctigenin on the levels of TNF- α , IL-6, and MIP-2 in BALFs. Compared to control mice, LPS enhanced the production of TNF- α , IL-6, and MIP-2 by 24-, 19-, and 23-folds, respectively (Table 2). Arctigenin significantly lowered the secretion of TNF- α , IL-6, and MIP-2 into BALFs (Table 2).

Arctigenin Reduces LPS-Induced Oxidative Lung Damage

The activities of SOD and MDA in the lung of LPS-treated mice were about 3.52- and 2.36- fold of those in the control mice (Table 3). However, the activity of CAT and the GSH/GSSG ratio was about 60.1 and 51.7 % of those in the control group (Table 3). The LPS-induced changes in

the oxidative stress parameters were significantly ($P<0.05$) reversed by arctigenin.

Arctigenin Lowers LPS-Induced NO Production and iNOS Expression

NO production in the lung of mice exposed to LPS was about threefold of that in the control mice ($P<0.01$, Fig. 2a). Arctigenin reduced NO levels by about 39 % (Fig. 2a). Western blot analysis revealed that LPS markedly enhanced iNOS expression in the lung, which was impaired by arctigenin pretreatment (Fig. 2b).

Arctigenin Increases the Expression of HO-1 in LPS-Treated Mice

The HO-1 mRNA level was low in the lung of control mice, but was significantly increased in LPS-treated mice

Table 1. Measurement of Total Protein and Albumin Levels and Cell Numbers in BALFs from each Group ($n=16$) at 4 days after LPS Challenge

Group	Control	LPS	LPS+DMSO	LPS+Arctigenin
Total protein (μ g/ml)	32.5 \pm 8.2	578.4 \pm 55.6 ^a	562.1 \pm 51.6	373.75 \pm 35.1 ^b
Albumin level (μ g/ml)	20.3 \pm 4.1	626.1 \pm 39.5 ^a	632.5 \pm 43.8	371.1 \pm 50.4 ^b
Total cells ($\times 10^6$ cells/ml)	0.11 \pm 0.02	2.4 \pm 0.31 ^a	1.7 \pm 0.09	1.1 \pm 0.07 ^b
Macrophages ($\times 10^6$ cells/ml)	0.1 \pm 0.004	2.2 \pm 0.12 ^a	1.7 \pm 0.01	1.0 \pm 0.05 ^b
Neutrophils ($\times 10^6$ cells/ml)	0.09 \pm 0.004	2.0 \pm 0.12 ^a	1.6 \pm 0.12	0.99 \pm 0.05 ^b
Lymphocytes ($\times 10^6$ cells/ml)	0.08 \pm 0.005	1.76 \pm 0.14 ^a	1.36 \pm 0.053	0.85 \pm 0.06 ^b

Data are expressed as mean \pm SD. ^a $P<0.01$ versus control group; ^b $P<0.01$ versus LPS+DMSO group

Table 2. Effects of Arctigenin on the Levels of TNF- α , IL-6, and MIP-2 in BALFs

Group	Control	LPS	LPS+DMSO	LPS+Arctigenin
TNF- α (pg/ml)	12.3 \pm 2.1	311.9 \pm 30.9 ^a	319.5 \pm 29.1	110.5 \pm 22.7 ^b
IL-6 (pg/ml)	31.1 \pm 9.1	588.5 \pm 59.3 ^a	452.25 \pm 47.1	272.5 \pm 36.1 ^b
MIP-2 (pg/ml)	14.8 \pm 2.8	328.4 \pm 31 ^a	322.6 \pm 24.2	120.2 \pm 21.6 ^b

TNF- α tumor necrosis factor-alpha, IL-6 interleukin-6, MIP-2 macrophage inflammatory protein 2

The data are expressed as mean \pm SD. ^a P <0.01 versus control group; ^b P <0.01 versus LPS+DMSO group (n =16)

(P <0.01, Fig. 3a). Arctigenin raised LPS-mediated upregulation of HO-1 mRNA by about threefold. Similar effects of arctigenin were observed on the expression of HO-1 protein (Fig. 3b).

Arctigenin Attenuates the Phosphorylation of MAPKs in LPS-Treated Mice

The baseline levels of phosphorylated ERK1/2, JNK, and p38 were very low in the lung (Fig. 4). LPS administration led to enhanced phosphorylation of ERK1/2, JNK, and p38 in the lung. LPS-induced MAPK phosphorylation was significantly blocked by arctigenin treatment.

DISCUSSION

Arctigenin has shown anti-inflammatory effects in animal models [13, 19]. It has been reported that arctigenin suppresses neuroinflammation in a focal cerebral ischemia-reperfusion rat model [19]. Our data revealed that arctigenin inhibited LPS-induced lung inflammatory injury, evidenced by decreased inflammatory cell infiltration and alveolar wall thickening. Massive inflammation causes vascular leakage in the lung of LPS-induced mouse [20]. We found that LPS elevated the levels of total protein and albumin in BALFs. Interestingly, arctigenin significantly inhibited LPS-induced vascular leakage. Inflammatory cell

migration and infiltration are an important factor leading to lung damage [21]. LPS enhanced the inflammatory cell influx into BALFs. Arctigenin pretreatment attenuated the numbers of leukocyte (macrophage, neutrophils, and lymphocytes) migrating into the alveoli. LPS challenge induces rapid infiltration of polymorphonuclear leukocyte, leading to the release of inflammatory mediators, such as cytokines and chemotactic factors [22, 23]. Subsequently, inflammation is induced in alveolar space and epithelial-endothelial barrier is disrupted, resulting in the lung injury. ELISA assay showed that arctigenin inhibited the production of TNF- α , IL-6, and MIP-2 in BALFs. Collectively, our results indicate that arctigenin inhibits LPS-induced acute lung inflammation through suppressing inflammatory cell influx and cytokine and chemokine secretion in BALFs.

The accumulation and activation of leukocytes in the lung increase the production and release of free radicals and lead to redox imbalance [24]. Antioxidants in the lung provide the protection against oxidative stress, which causes lung damage [25]. We investigated the activities of the antioxidant enzymes SOD and CAT, as well as the markers of oxidative stress, the GSH/GSSG ratio and MDA levels. We found that arctigenin enhanced the activities of SOD and CAT, raised the GSH/GSSG ratio, but decreased MDA amounts in the lung of LPS-exposed mice, indicating the antioxidative effect of arctigenin. The antioxidative activity of arctigenin has also been described

Table 3. Effects of Arctigenin on the Oxidative Stress Parameters in the Lung

Group	Control*	LPS	LPS+DMSO	LPS+Arctigenin
MDA	1 \pm 0.112	2.52 \pm 0.252 ^a	2.36 \pm 0.236	1.29 \pm 0.129 ^b
SOD	1 \pm 0.098	3.52 \pm 0.321 ^a	3.36 \pm 0.316	8.16 \pm 0.81 ^b
GSH/GSSG ratio	1 \pm 0.081	0.601 \pm 0.061 ^a	0.582 \pm 0.052	0.892 \pm 0.081 ^b
CAT	1 \pm 0.016	0.517 \pm 0.057 ^a	0.536 \pm 0.0526	0.791 \pm 0.072 ^b

MDA malondialdehyde, SOD superoxide dismutase, GSH/GSSG ratio glutathione peroxidase (GSH)/Glutathione disulfide (GSSG), CAT catalase

All data are expressed relative to control (arbitrarily assigned a value of 1) as mean \pm SD. ^a P <0.01 versus control group; ^b P <0.01 versus LPS+DMSO group (n =16)

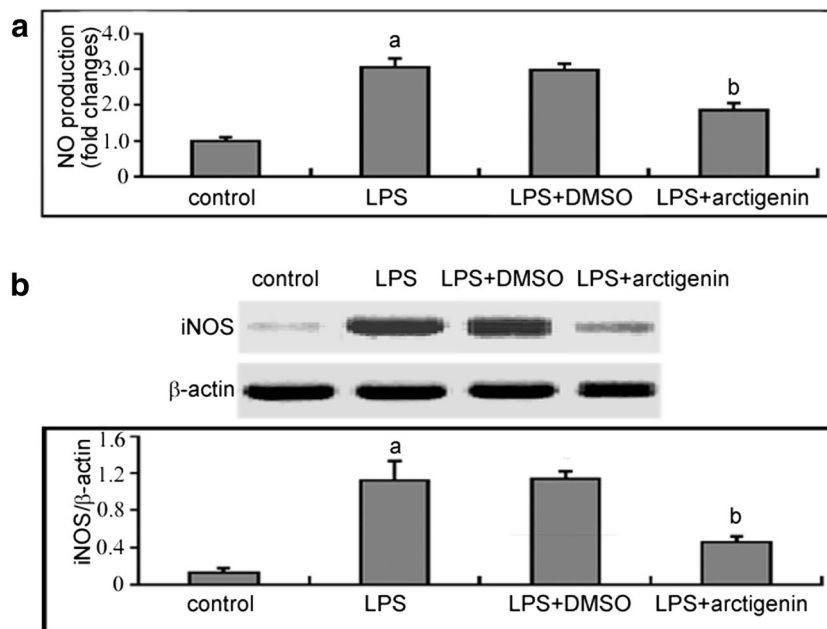


Fig. 2. Effects of arctigenin on NO production and iNOS expression in the lung at 4 days after LPS challenge. **a** Lung nitrate/nitrite (NOX) concentrations were measured. Fold changes of NO production are shown. **b** Western blot analysis of iNOS expression. Quantitative analysis of iNOS is shown in lower panels. The results are shown as mean±SD (*n*=16). ^a*P*<0.01 relative to control group; ^b*P*<0.01 relative to LPS+DMSO group.

in other different biological settings. For instance, Swarup *et al.* [26] reported that arctigenin attenuated the severity of

Japanese encephalitis induced by Japanese encephalitis virus via reduction of inflammation and oxidative stress.

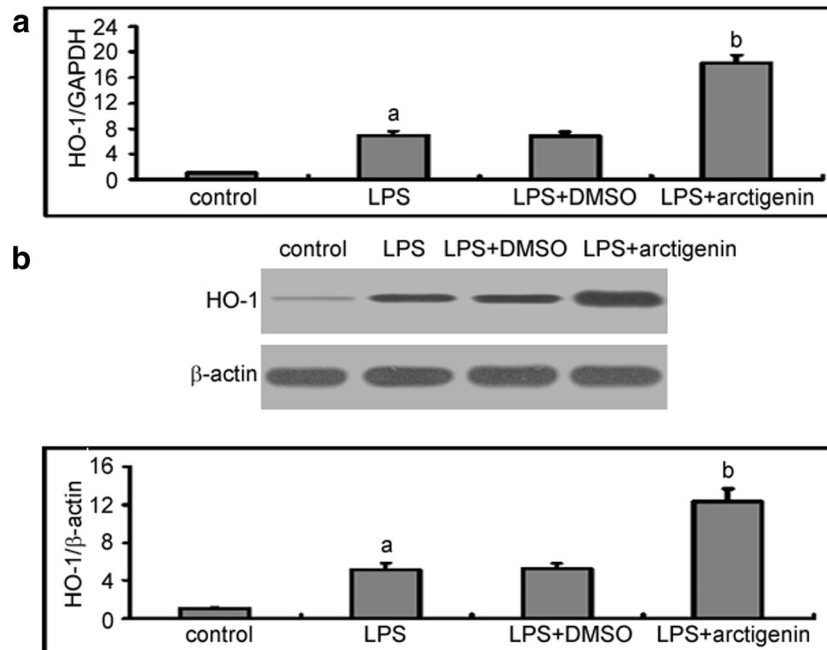


Fig. 3. Effects of arctigenin on HO-1 expression in the lung. At 4 days after LPS challenge, lung tissues were harvested. **a** The mRNA and **b** protein expression of HO-1 was measured by RT-PCR and Western blot analysis, respectively. Representative blots are shown in top panels in B. *Bar graphs* represent densitometric analysis of HO-1 mRNA and protein expression. ^a*P*<0.01 relative to control group; ^b*P*<0.01 relative to LPS+DMSO group.

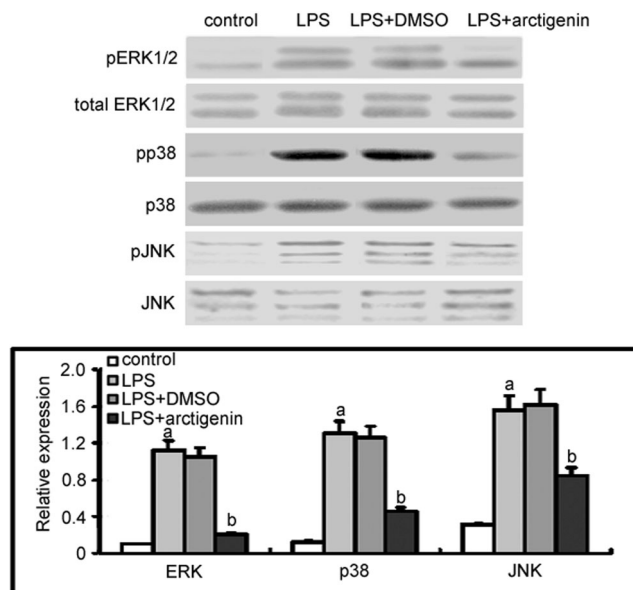


Fig. 4. Effects of arctigenin on the phosphorylation of MAPKs in the lung. Representative Western blots are shown in *top panels* and densitometric analysis of the blots is given in *lower panels*. Total ERK1/2, JNK, and p38 proteins were used as internal controls. ^a $P < 0.01$ relative to control group; ^b $P < 0.01$ relative to LPS+DMSO group.

Thus, arctigenin-mediated antioxidative activity provides an important mechanism for its protective effects against LPS-induced acute lung injury.

NO has been shown to be implicated in the pathogenesis of acute lung injury in animals [27]. iNOS is mainly responsible for the production of NO during inflammation [28]. Arctigenin has been shown to promote the ubiquitination and degradation of iNOS after LPS stimulation in murine macrophage-like RAW 264.7 cells [29]. In the lung, the major cells expressing iNOS are alveolar macrophages, alveolar epithelial cells, and inflammatory infiltrating cells [27]. To investigate the mechanism of how arctigenin inhibits LPS-induced lung inflammation, we analyzed the production of NO and the expression of iNOS. We found that arctigenin attenuated LPS-mediated NO production and iNOS expression. Our results demonstrated that the protective effects of arctigenin on LPS-induced acute lung inflammation may be mediated through the iNOS/NO signaling pathway.

HO-1, an inducible stress protein, has anti-inflammatory, anti-apoptotic, and anti-proliferative effects [30]. Ethyl pyruvate attenuates acute lung injury through modulating the expression of iNOS and HO-1 in endotoxemic rats [31]. Radix paeoniae rubra suppresses LPS-induced acute lung injury through p38 MAPK/iNOS/HO-1 signaling pathway [32]. Hyperbaric oxygen-mediated inhibition of LPS-induced acute lung injury involves the regulation of

HO-1 expression [33]. Here, we found that arctigenin enhanced the expression of both HO-1 mRNA and protein in LPS-treated acute lung injury. Thus, arctigenin induces protective effects against acute lung injury-induced by LPS might be through the induction of HO-1 and the subsequent inhibition of iNOS.

MAPKs control the synthesis and release of pro-inflammatory mediators in response to inflammation [34]. Activation of MAPKs is critical in the development of acute lung injury [35, 36]. Our results showed that LPS administration induced the phosphorylation of ERK, JNK, and p38 MAPK in mouse lungs. Arctigenin significantly inhibited LPS-induced phosphorylation of MAPKs. LPS-induced MAPK activation has been shown to regulate HO-1 expression [37]. Arctigenin-mediated upregulation of HO-1 might be through suppression of MAPK activation.

Some limitations of this study should be noted. First, acute lung injury can be evoked by multiple factors other than LPS stimulation. There is no information about the protective activity of arctigenin in other animal models of acute lung injury. Second, although we found that arctigenin modulated MAPKs, HO-1, and iNOS, the direct signaling pathways mediating the protective effects of arctigenin need to be further clarified.

In conclusion, our results demonstrate that arctigenin confers protection against LPS-induced lung inflammatory and oxidative damage, which is largely mediated through

inhibition of the activation of MAPK, HO-1, and iNOS. The clinical utilization of arctigenin warrants further exploration in the prevention and treatment of acute lung injury/ARDS.

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