Withaferin A attenuates ovalbumin induced airway inflammation

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1. ABSTRACT

The molecular mechanism of how airway inflammation develops is not fully understood. Withaferin A (WA) is a natural phytochemical isolated from the plant Withania somnifera. It is a well-investigated bioactive compound that possesses a variety of health-promoting effects, including anti-inflammatory and anti-oxidative activities. In the present study, the effect of WA on ovalbumin (OVA)-induced airway inflammation in mice was investigated. The results indicated that pre-treatment with WA inhibited OVA-induced lung injury and fibrosis progression in mice. Furthermore, WA significantly downregulated inflammatory cell infiltration into the bronchoalveolar lavage fluid and significantly reduced pro-inflammatory cytokine expression in the lung tissue specimens. Additionally, WA significantly suppressed transforming growth factor-β1 expression in lung tissues. WA also caused the downregulation of collagen I, collagen III, α-smooth muscle actin and tissue inhibitor
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Withaferin A, as well as SMADs and extracellular signal related kinase 1/2 inactivation. Notably, WA significantly reduced the activation of the NLRP3 inflammasome. The results indicate that WA may be an effective novel candidate for the treatment of airway inflammation.

2. INTRODUCTION

A number of patients suffering from airway inflammation develop honeycomb lung and suffer mortality due to the irreversible loss of pulmonary function (1,2). At present, although various drugs have been explored for the treatment of airway inflammation, they are not particularly effective. Additionally, they are often accompanied by adverse side effects during long-term treatment (3,4). Therefore, the possible biological activities of compounds isolated from plants, including those used within Chinese medicine, have attracted the attention of researchers as a potential novel treatment for lung injury (5,6).

Withaferin A (WA) is a C₅, C₆-epoxy steroidal lactone derived from the Withania somnifera plant (7). WA has been previously demonstrated to possess pharmacological properties, including anti-inflammatory, hepatoprotective, anti-cancer and antioxidative activity (8,9). According to previous studies, WA could attenuate brain injury in animal models by suppressing inflammation (10). Additionally, WA has been suggested as a potential treatment to reduce the progression of fibrosis (11-13). Therefore, it may also be an effective candidate for the treatment of airway inflammation.

Inflammation of the airway epithelium has typically been considered as the first defensive barrier, which minimizes allergen access to the body (14). Ovalbumin (OVA) has been demonstrated to induce airway inflammation in animal models for experiments (15). Previous studies have indicated that transforming growth factor (TGF)-β1 is associated with pulmonary fibrosis, during which it initiates fibroblast differentiation into myofibroblasts, and subsequently causes excessive collagen accumulation and extracellular matrix (ECM) deposition (16,17). TGF-β1 overexpression has been identified in patients with lung fibrosis and animals with lung injuries (18). The expression of α-smooth muscle actin (α-SMA) indicates that fibroblasts have differentiated into myofibroblasts, which are sources of ECM, including collagen and fibronectin, which accelerate lung injury (19). Additionally, SMAD2/3 and extracellular signal related kinase (ERK)1/2 signaling pathways are essential for fibroblast to myofibroblast differentiation induced by TGF-β1 (20). Therefore, suppression of TGF-β1 is potentially beneficial for the treatment of pulmonary fibrosis.

Previous studies have indicated that pro-inflammatory cytokines, including interleukin (IL)-1β, IL-18, IL-6 and tumor necrosis factor (TNF)-α, are important in the pulmonary inflammatory response (21,22). The activation of caspase-1 is essential for the maturation of pro-inflammatory cytokines, including IL-1β and IL-18, from their inactive precursors (23). Caspase-1 activation requires the assembly of the inflammasome, which consists of NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and pro-caspase-1 (24). NLRP3 inflammasome assembly activates caspase-1, which leads to the maturation of pro-IL-1β and pro-IL-18 (25). The abnormal expression of NLRP3 has been reported to be associated with lung injury (26,27).

The present study was conducted to investigate whether WA ameliorates lung fibrosis and inflammation in a murine model of OVA-induced pulmonary fibrosis and to reveal the possible molecular mechanisms by which this occurs. The results of the present study suggest that WA protects mice against OVA-triggered pulmonary fibrosis and inflammation by suppressing the TGF-β1-induced SMAD2/3 and ERK1/2 signaling pathways, as well as by inactivating the NLRP3 inflammasome.

3. MATERIALS AND METHODS

3.1. Animals and treatments

A total of 60 male, 8-week-old, C57BL/6 mice (weight, 18-22 g), were purchased from the Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). All of the mice were maintained in a room with a 12-h light/dark cycle at 25±2°C and 50±5% humidity, with free access to food and water. All of the animal experiments were approved by the Animal Care and Use Committee of Nanjing Medical University (Huai’ian, China) and were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals, issued by the National Institutes of Health in 1996 (28).

The mice were randomly divided into five treatment groups (n=12/group): i) The control group (Con) without any treatments; ii) the OVA group; iii) the OVA+WA (20 mg/kg) group; iv) the OVA+WA (40 mg/kg) group; and v) the OVA+WA (80 mg/kg) group. OVA was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and was administered to mice to induce pulmonary fibrosis as previously described (29,30). WA (Sigma Aldrich; cat. no. W4394) was administered to mice daily via gavage from day 0 to day 37 until sacrifice. The mice in the four experimental groups were sensitized with an intraperitoneal injection of 0.2 ml saline containing 50 μg OVA and 2.25 mg aluminum hydroxide on days 0 and 15 (Figure 1A). At day 31, the mice in the OVA groups were challenged
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The food consumption of the mice was monitored daily and their water consumption was monitored twice a week. The body weights of the mice were measured throughout the experiment. Ocular blood samples were collected from mice immediately following the airway hyperresponsiveness (AHR) measurements on day 37 and stored at 4°C for 2 h prior to centrifugation at 5,000 x g for 15 min at 4°C. The serum was collected and stored at -80°C prior to further investigation. The mice were sacrificed and complete lung tissues were harvested on a 4°C glacial table and frozen in liquid nitrogen. The tissues were stored at -80°C prior to analysis.

3.2. ELISA analysis

TGF-β1 levels in the serum of mice were calculated using an ELISA kit (cat. no. 7754-BH; R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer’s protocol.
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3.3. Aspartate transaminase (AST) and alanine aminotransferase (ALT) measurements.

The levels of AST and ALT in the serum of the mice were calculated using an Aspartate Aminotransferase Assay kit and an Alanine Aminotransferase Assay kit, respectively, (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s protocol.

3.4. Cell culture and treatments

Human lung fibroblast MRC-5 cells and mouse lung epithelial MLE-12 cells were purchased from the Institute of Biomedical Sciences, Fudan University (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 kU/l penicillin and 100 mg/l streptomycin in an incubator at 37°C with a 5% CO2 atmosphere. TGF-β1 (R&D Systems, Inc.) was used to induce fibrosis in human MRC-5 cells. Prior to the experiments, the MRC-5 cells were treated with 5 ng/ml TGF-β1 in the absence or presence of WA at the indicated concentrations (0, 40, 80 and 160 μM) and for 24 h. Following treatment, all cells were harvested for further investigation.

3.5. Cell viability analysis

MRC-5 and MLE-12 cells were initially seeded in 96-well plates at a density of 2x10^4 and 3x10^4 cells/well, respectively, prior to incubation for 24 h at 37°C. DMEM was then replaced with complete medium containing the indicated concentrations of WA (0-160 μM), prior to incubation for the indicated time (0-72 h) at 37°C. Following incubation as indicated, 10 μl MTT (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) was administrated to the cells and they were incubated for 4 h at 37°C, according to the manufacturer’s protocol. Next, the supernatants were removed and 200 μl dimethyl sulfoxide was added into each well to dissolve the formazan crystals. The absorbance was read at 570 nm by a microplate reader. The cell viability was evaluated as the ratio of cells that survived.

3.6. Inflammatory cell infiltration in the bronchoalveolar lavage fluid (BALF)

Following treatment with OVA and WA, the mice in each group were sacrificed and their BALF was collected by washing three times with chilled PBS using a tracheal cannula. The BALF samples were then centrifuged at 1,000 g for 10 min at 4°C and the cell pellet was re-suspended in PBS. The total BALF cell number was determined using a hemocytometer. The cells were then cytopspun onto microscope slides and stained with Wright-Giemsa (50% in PBS, Sigma Aldrich; Merck KGaA) using a cytospin for total 6 min at room temperature for cell classification. The percentages of BALF neutrophils, macrophages and lymphocytes were obtained by counting the leukocytes under light microscopy at x400 magnification.

3.7. Hydroxyproline (OH-proline) analysis

The frozen lung tissues were weighed and homogenized in pre-chilled distilled H2O using a tissue homogenizer. The homogenate was subsequently mixed with trichloracetic acid and incubated on ice for 20 min. Following centrifugation at 3,000 x g for 20 min at 4°C, the samples were rinsed in cold ethanol and the pellet was dried. A total of 1 ml 6 M HCl was added to the pellet and it was incubated for 18 h at 95°C. The samples were cooled to room temperature, centrifuged at 3,000x g for 10 min at 20°C and the supernatant was collected. The supernatant (40 μl) and the hydroxyproline standard were mixed with chloramine T (460 μl) solution and incubated for 30 min at room temperature. A total of 500 μl Ehrlich’s reagent was added and the solution was incubated for 20 min at 65°C, the optical density value was evaluated at 550 nm.

3.8. Pulmonary fibrosis parameter analysis

Mice were intraperitoneally anesthetized with 6% chloral hydrate (400 mg/kg; Sigma-Aldrich; Merck KGaA) in saline and transferred to a plethysmographic chamber where their pulmonary function was calculated using an AniRes 2005 system (version3.0.; Biolabs Technology Co., Ltd., Beijing, China). The system automatically measures pulmonary parameters, including inspiratory resistance and expiratory resistance.

3.9. Immunohistochemical analysis

For histological analysis, the lungs, renal and liver tissue samples were harvested and fixed in 4% formalin for 48 h at room temperature and embedded in paraffin. The tissues were sectioned (3μm thickness) and stained using Mason’s trichrome for 15 min or hematoxylin and eosin for 10 min (H&E; Sigma Aldrich; Merck KGaA) to evaluate the morphological changes in the lungs. The H&E-stained sections were used to grade the extent of fibrosis according to the following scale: 0, no alteration in the lung structure, to 5, severe injury of the lung architecture. The slides were observed using a light microscope at x400 magnification. The paraffin-fixed lung sections were treated with xylene for 5 min; slides were then rehydrated in 2 changes of 100% ethanol for 3 min, followed by 95 and 80% ethanol for 1 min and rinsed in distilled water. Sections were boiled.
Table 1. Primer Sequences Used for Real-time PCR (5' to 3')

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<td>GCTGTCGATCTGCTGAGT</td>
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<tr>
<td>GAPDH</td>
<td>Mouse</td>
<td>GGGCAAGTGACGAGGAGC</td>
<td>GAGTGTGCGAGCTGCGAT</td>
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</table>

For immunofluorescence analysis the MRC-5 cells were washed three times with chilled PBS and fixed with 3.7% (v/v) formaldehyde in PBS for 15 min at room temperature. For 1 h, 0.1% Triton-X 100 in PBS was added to cells permeate the cells for blocking non-specific binding sites by using 2% BSA at room temperature. For NLRP3 fluorescence staining, the cells were incubated with 50 μg/ml mouse anti-NLRP3 antibody (ab4207; 1:200; Abcam), followed by incubation with Goat Anti-Mouse IgG H&L (Alexa Fluor® 594; ab150116; 1:200; Abcam) for 30 min. The cells were subsequently washed three times with PBS and the immunofluorescence was calculated using an LSM 710 confocal laser scanning microscope at x400magnification (Zeiss AG, Oberkochen, Germany) as previously described (31).

3.10. Biochemical analysis.

Aminotransferase (AST, C009-2) and alanine aminotransferase (ALT, C009-1) levels in serum of mice were measured using the standard diagnostic kits purchased from Jiancheng Biotechnology Co., Ltd. (Nanjing, China).

3.11. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from the cells and lung tissue samples using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol (32). The reaction was carried out at 42°C for 1 h and terminated by deactivation of the enzyme at 70°C for 10 min. cDNA was synthesized using oligo(dT) primers with the ReverTra First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.). PCR was performed on a CFX96 Touch™ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Amplification of each denatured product was conducted at 94°C for 60 sec followed by 45 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec and finally 95°C for 10 sec, 65°C for 45 sec, and 40°C for 60 sec. The primer sequences used were commercially synthesized and are listed in Table 1. GAPDH was included as internal reference. Fold changes in the mRNA level of the target gene were calculated as relative to the endogenous cyclophilin control. mRNA expression levels were quantified using the 2^ΔΔCq method (33).

3.12. Western blot analysis

Following treatments under different conditions, MRC-5 cells were harvested and the medium was removed. The cells were washed three times with chilled PBS and lysed in an ice-cold lysis buffer (150 mM NaCl, 100 mM NaF, 50 mM Tris-HCl (pH 7.6.), and 0.5% Nonidet P-40) in the presence of a fresh protease inhibitor cocktail (Roche, Mannheim, Germany). Frozen lung tissue samples were obtained from mice following treatments. A total of 100 μg tissue sample was lysed in 1 ml lysis buffer (25 mM...
Table 2. Primary antibodies for western blot analysis.

<table>
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<th>Primary antibodies</th>
<th>Dilution ratio</th>
<th>Corporation</th>
</tr>
</thead>
<tbody>
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<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>Rabbit anti-ERK1/2</td>
<td>1:1000</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>Rabbit anti-p-ERK1/2</td>
<td>1:1000</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>Rabbit anti-IL-18</td>
<td>1:1000</td>
<td>Abcam</td>
</tr>
<tr>
<td>Rabbit anti-FSP-1</td>
<td>1:500</td>
<td>Santa cruz</td>
</tr>
<tr>
<td>Mouse anti-Fibronectin</td>
<td>1:1000</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>Rabbit anti-Vimentin</td>
<td>1:1000</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>Rabbit anti-E-cadherin</td>
<td>1:1000</td>
<td>Abcam</td>
</tr>
<tr>
<td>Rabbit anti-Smad2</td>
<td>1:1000</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>Rabbit anti-IL-1β</td>
<td>1:1000</td>
<td>Abcam</td>
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<td>Rabbit anti-TNF-α</td>
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<td>Rabbit anti-NLRP3</td>
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<tr>
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<td>Santa cruz</td>
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Tris-HCl, pH 8.0., 1 mM EDTA, 5 μg/ml leupeptin, 1 mM Pefabloc SC, 50 μg/ml aprotinin, 5 μg/ml soybean trypsin inhibitor, 4 mM benzamidine). The lysates were then centrifuged at 15,000 x g for 15 min at 4˚C and the supernatant was collected. A BSA protein assay kit (Thermo Fisher Scientific, Inc., USA) was used to detect the protein concentrations, according to the manufacturer’s protocol. Protein extracts (40 μg) were separated by 10% SDS-PAGE and were then transferred to a polyvinylidene difluoride membrane (PVDF; EMD Millipore, Billerica, MA, USA). The PVDF membranes were blocked with 5% dry skimmed milk in 0.1% Tween-20 Tris-buffered saline for 2 h to block the non-specific sites on the blots at room temperature. The primary antibodies (Table 2) were dissolved in blocking buffer and incubated with the membranes overnight at 4˚C. The bands on the PVDF membranes were visualized by enhanced chemiluminescence using a Pierce ECL Western Blotting Substrate reagent (Thermo Fisher Scientific, Inc.), and then exposed to Kodak (Eastman Kodak Company, USA) X-ray film. Protein expression was quantified using ImageJ 1.3.8 software (National Institutes of Health, Bethesda, MA, USA) and standardized to GAPDH. All experiments were performed in triplicate.

3.13. Statistical analysis

Data were expressed as the mean ± standard error of the mean. Statistical analyses were performed using GraphPad PRISM (version 6.0.; Graph Pad Software, Inc., La Jolla, CA, USA) using one way analysis of variance with Dunnet’s least significant difference post-hoc tests. P<0.0.5 was considered to indicate a statistically significant difference.

4. RESULTS

4.1. WA administration ameliorates OVA-induced lung injury by suppressing pulmonary fibrosis in mice

WA has been previously reported to ameliorate the pathogenesis of selected diseases (7,12). However, whether it was an effective candidate for the treatment of OVA-induced lung injury of the airways was unknown. In the present study, mice were challenged with OVA to induce airway inflammation with or without WA treatment for 37 days and the severity of their lung fibrosis was calculated. The safety and hepatotoxicity of WA was evaluated at the highest concentration of 80 mg/kg (Figure 1B). No notable differences were identified between the H&E-stained sections from the Con and 80 mg/kg-treated groups, additionally no significant differences were identified between the AST and ALT assessments in the two groups. These results indicated that 80 mg/kg WA was not toxic to mice and was safe to use for further experiments in the study. OVA induction led to significant weight loss from day 12 in the OVA group and OVA-treated groups with WA administration at 20 and 40 mg/kg compared with the Con group (Figure 1C). The weight alterations were represented as a percentage of the weight from day 1 immediately prior to OVA treatment. These results indicate that OVA-induced lung injury caused a reduction in the body weight of mice. WA treatment (40 and 80 mg/kg) following the OVA challenge significantly improved the weight loss in comparison with the OVA only group, which may be associated with amelioration of lung injury.
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The amino acid OH-proline is primarily identified in collagen, where it is essential for its triple helix stabilization (34,35). The levels of OH-proline are often examined to determine the level of collagen present in tissue samples with fibrosis. In the present study, it was identified that OVA caused a significant increase of OH-proline in the lung tissue specimens compared with the Con group (Figure 1D). However, this level was significantly reduced following treatment with WA (40 and 80 mg/kg), which decreased the level of OH-proline in a dose-dependent manner.

In addition, H&E staining was performed to examine the histological conditions of the pulmonary tissue samples (Figure 1E). H&E staining demonstrated that significant pathologic reorganization and alveolar-capillary structural damage were induced by OVA, which was then reversed by the administration of WA. Additionally, Masson’s trichrome staining indicated a significant accumulation of collagen in the OVA-induced lung tissue sections, which was attenuated by WA treatment (Figure 1F). Notably, the pulmonary function of untreated mice was significantly worse in the OVA group compared with the Con group; however, this was significantly ameliorated by treatment with WA, as observed by the reduced inspiratory and expiratory resistance (Figure 1G and H).

Collagen type I consists of collagen α chains I and II, and collagen type III consists of collagen α chain 1 (36,37). α-SMA is a typical marker of myofibroblast differentiation, which indicates fibrosis progression (38). Tissue inhibitor of metalloproteinase-1 (TIMP)-1 is expressed on alveolar epithelial cells and macrophages and is increased during the process of pulmonary fibrosis (39). The downregulation of E-cadherin and the upregulation of vimentin are also associated with fibrosis progression (40). In the present study, it was revealed that OVA-induced a significant increase in collagen I (Figure 2A), collagen III (Figure 2B), α-SMA (Figure 2C), TIMP-1 (Figure 2D) and vimentin (Figure 2E) mRNA expression compared with the levels in the Con group. The administration of WA significantly reduced the mRNA expression levels of these genes in a dose-dependent manner. In contrast, E-cadherin mRNA expression was significantly reduced by OVA compared with the level in the Con group, and WA significantly increased the expression level compared with the OVA group (Figure 2F).

Immunohistochemical analysis further demonstrated that the elevated α-SMA expression due to OVA was reversed by the administration of WA (Figure 2G). These results suggest that WA may attenuate pulmonary fibrosis induced by OVA in mice.

Figure 2. WA reduces collagen accumulation in OVA-induced lung fibrosis. Reverse transcription-quantitative polymerase chain reaction analysis was performed to determine the mRNA expression levels of (A) collagen I, (B) collagen III, (C) α-SMA, (D) TIMP-1, (E) vimentin and (F) E-cadherin in lung tissue samples from mice. n=6 in each group. (G) Immunohistochemical analysis was performed to analyze α-SMA levels in lung tissue sections. n=6 in each group. Representative images and quantified levels were displayed. Scale bar =50 µm. ***P<0.0.01 vs. the Con group; **P<0.0.1 and +++P<0.0.01 vs. the OVA group. WA, withaferin A; OVA, ovalbumin; Con, control; α-SMA, α-smooth muscle actin; TIMP-1, tissue inhibitor of metalloproteinase-1.
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4.2. TGF-β1 expression is associated with the WA-attenuation of lung injury caused by OVA in mice

TGF-β1 is a key fibrogenic cytokine, which modulates various cellular functions (20). TGF-β1 is highly expressed in lung tissues under fibrosis (20,22). Suppression of TGF-β1 is believed to be an effective therapeutic target for pulmonary fibrosis (41). Western blot analysis was performed to measure the protein levels of TGF-β1 (Figure 3A). It was revealed that OVA significantly increased the expression of TGF-β1 compared with the Con group; however, treatment with WA significantly reduced the TGF-β1 levels in a dose-dependent manner. ELISA analysis indicated that TGF-β1 was significantly overexpressed in the serum of mice that underwent OVA treatment; however, this was significantly reduced by WA treatment (40 and 80 mg/kg; Figure 3B). TGF-β1 mRNA expression levels were also significantly increased following OVA treatment (Figure 3C). This was also significantly ameliorated by treatment with WA in a dose-dependent manner. Immunohistochemical staining revealed that TGF-β1 was highly expressed following OVA induction; however, this was restrained by WA treatment (Figure 3D).

Fibroblast activation has a close association with an increase in fibroblast specific protein-1 (FSP-1), fibronectin and vimentin, as well as a decrease in E-cadherin (42,43). In the present study OVA induction induced a significant increase in FSP-1, fibronectin and vimentin protein expression in mice, compared with the Con group; however, treatment with WA significantly reduced expression of these proteins (Figure 3E). Conversely, E-cadherin was significantly reduced in the OVA group compared with the Con group, while treatment with WA significantly increased its expression (40 and 80 mg/kg). These results indicate that WA-improved lung fibrosis was associated with the inactivation of TGF-β1, and the suppression of FSP-1, fibronectin and vimentin, whereas E-cadherin increased.
4.3. WA reduces OVA-induced lung fibrosis through downstream TGF-β1 signals in mice

The involvement of the TGF-β1 signaling pathway in pulmonary fibrosis has been previously reported (44). TGF-β1 activates SMADs, as well as SMAD-regulated target genes for transcription, which triggers EMT (20). In the present study, the role of WA in the SMAD-associated signaling pathway was investigated. Western blot analysis was performed and it was revealed that phosphorylated (p)-SMAD2 and 3 were significantly upregulated in the OVA group compared with the Con group; however, this upregulation was significantly inhibited by WA treatment (Figure 4). TGF-β1 also stimulates the mitogen-activated protein kinase (MAPK) signaling pathway (45). In the present study, the effect of WA on ERK1/2 phosphorylation in the lung tissue samples treated by OVA was investigated.

The results indicated that WA significantly downregulated the expression of p-SMAD2/3 and p-ERK1/2 induced by OVA in a dose-dependent manner, therefore suppressing pulmonary fibrosis progression.

4.4. Reduced inflammatory response caused by WA is associated with the activation of the NLRP3/ASC/caspase-1 signaling pathway in mice

A previous study indicated that OVA causes an inflammatory response, which contributes to lung injury (46). In the present study, the BALF cell number was calculated to investigate the role of WA in mediating the inflammatory response caused by OVA. OVA administration caused a significant increase in the numbers of inflammatory cells present in the BALF compared with the Con group, which was supported...
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by a significant increase in the total number of cells (Figure 5A). WA treatment (20-80 mg/kg) significantly reduced the number of macrophages, neutrophils and lymphocytes present in the BALF obtained from OVA-treated mice, as well as the total cell number.

Pro-inflammatory cytokines serve an essential role in pulmonary fibrosis pathogenesis induced by OVA in mice (47). Western blot analysis indicated that the protein expression levels of IL-1β, IL-18, IL-6 and TNF-α were significantly increased in OVA-treated mice compared with the level in the Con group (Figure 5B). The expression of these proteins was significantly downregulated by WA treatment (20-80 mg/kg). The mRNA expression levels of IL-1β, IL-18, IL-6 and TNF-α were also investigated, and similar results were observed (Figure 5C). OVA caused a significant increase in the mRNA expression levels of these cytokines and WA significantly suppressed the effects compared with the OVA group. These results suggest that in OVA-treated mice, WA inhibits lung inflammation by suppressing the expression of IL-1β, IL-18, IL-6 and TNF-α.

A previous report indicated that caspase-1 activation is required for IL-1β and IL-18 secretion (48). In the present study, the role of WA in caspase-1 regulation was investigated. Caspase-1 cleavage is tightly linked to the NLRP3 inflammasome (49). Treatment with WA caused a significant downregulation in NLRP3 and ASC protein expression compared with the OVA group (Figure 6A and B). Active and pro-caspase-1 protein levels were significantly elevated in the lung tissue specimens treated with OVA alone; however, WA administration significantly suppressed these levels (Figure 6A and B). The ratio of active caspase-1 to pro-caspase-1 was significantly increased following OVA treatment compared with the ratio in the Con group; however, this was significantly reversed by treatment with WA (Figure 6C). In addition, immunohistochemical analysis indicated that WA treatment at 80 mg/kg significantly reduced the number

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**Figure 5.** WA downregulates the inflammatory response in OVA-triggered lung fibrosis. (A) BALF was extracted from mice and the number of infiltrated macrophages, neutrophils and lymphocytes were investigated. In addition, the total cell number in the BALF was also calculated. n=5 in each group. (B) Western blot analysis was performed to measure the protein expression levels of IL-1β, IL-18, IL-6 and TNF-α, and the results were quantified. n=6 in each group. (C) Reverse transcription-quantitative polymerase chain reaction analysis was performed to measure the mRNA expression levels of the pro-inflammatory cytokines IL-1β, IL-18, IL-6 and TNF-α. n=6 in each group. **P<0.001 vs. the Con group; +P<0.05, ++P<0.01 and +++P<0.001 vs. the OVA group. WA, withaferin A; OVA, ovalbumin; Con, control; IL, interleukin; TNF, tumor necrosis factor; BALF, bronchoalveolar lavage fluid.
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**Figure 6.** WA-reduced inflammatory response is associated with the activation of the NLRP3/ASC/caspase-1 signaling pathway. (A) Western blot analysis detected the protein expression levels of NLRP3, ASC, and active- and pro-caspase-1. (B) The western blotting results were quantified. n=6 in each group. (C) The ratio of active caspase-1 to pro-caspase-1 was quantified based on the western blot analysis. n=6 in each group. (D) NLRP3-positive cells were examined by immunohistochemical analysis. n=6 in each group. (E) Reverse transcription-quantitative polymerase chain reaction was used to investigate NLRP3, ASC and caspase-1 mRNA expression levels in lung tissue samples from mice. n=6 in each group. Scale bar =50 µm. *P<0.05, **P<0.01 vs. the Con group; +P<0.05, ++P<0.01 vs. the OVA group. WA, withaferin A; OVA, ovalbumin; Con, control; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain.

**Figure 7.** WA demonstrates no cytotoxicity to lung epithelial cells in vitro. (A) MRC-5 human lung epithelial cells were treated with various concentrations (5-160 μM) of WA for 24 h and MTT analysis was performed to calculate the cell viability. n=6 in each group. (B) MRC-5 cells were treated with 160 μM WA for different times (0-72 h) and the cell viability was analyzed. n=6 in each group. (C) MLE-12 mouse lung epithelial cells were treated with various concentrations (5-160 μM) of WA for 24 h and MTT analysis was performed to calculate the cell viability. n=6 in each group. (D) MLE-12 cells were treated with 160 μM WA for different times (0-72 h) and the cell viability was analyzed. n=6 in each group. WA, withaferin A.
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of NLRP3-positive cells compared with the number in the OVA only group (Figure 6D). Mice that underwent WA-pre-treatment exhibited significantly lower levels of NLRP3, ASC and caspase-1 mRNA compared with the mice treated with OVA only (Figure 6E). These results suggest that WA may improve OVA-induced lung inflammation through modulation of the NLRP3 inflammasome and regulation of pro-inflammatory cytokine expression.

4.5. WA demonstrates no cytotoxicity to lung epithelial cells in cells in vitro

To confirm the role of WA in lung fibrosis in vitro, human and mouse epithelial cells, MRC-5 and MLE-12, respectively, were studied. MRC-5 cells were treated with various concentrations of WA (0-160 µM) for 24 h. MTT analysis was performed to calculate the cell viability. No significant differences were observed among the different groups at any concentration (Figure 7A). MRC-5 cells were treated with 160 µM WA for increasing time periods (6-72 h), followed by MTT analysis. No significant differences were observed among the groups, except for 96 h (data not shown). The MLE-12 cells were also treated with WA at different doses (0-160 µM) for 24 h. The WA doses tested induced no significant changes to cell viability (Figure 7C). MLE-12 cells were treated with 160 µM WA for increasing time periods (6-72 h) and a similar result was observed; there was no significant difference in cell viability. These results indicate that WA has no cytotoxicity to human and mouse epithelial cells, even at the highest concentrations for 72 h. WA at concentrations of 40, 80 and 160 µM were used to investigate the effect of WA in fibrosis induced by TGF-β1 (5 ng/ml) in human cells.

4.6. WA reduces fibrosis accumulation through the TGF-β1 signaling pathway in cells in vitro

MRC-5 cells were exposed to 5 ng/ml TGF-β1 for 24 h in the absence or presence of varying concentrations of WA (40-160 µM). Treatment of cells with TGF-β1 caused a significant increase in the protein expression level of TGF-β1 compared with the level in the Con group (Figure 8A). Treatment with WA significantly reduced this increase in a dose-dependent manner. The expression levels of fibrosis-associated proteins, which contribute to EMT, including FSP-1, fibronectin and Vimentin, were also significantly upregulated in MRC-5 cells following treatment with TGF-β1 compared with the level in the Con group (Figure 8B). Treatment with WA significantly reduced the protein expression levels of these cytokines in a dose-dependent manner. Conversely, E-cadherin expression was significantly downregulated by TGF-1 compared with the level in the Con group, and significantly upregulated by treatment with WA. These results were in line with the in vivo results. RT-qPCR analysis was performed to investigate the effect of WA on collagen accumulation in TGF-β1-treated MRC-5 cells. The results indicated that collagen I, collagen III, α-SMA and TIMP-1 mRNA levels were significantly upregulated following TGF-β1 exposure in vitro compared with the levels in the Con group; however, these levels were significantly reduced following WA treatment (Figure 8C).

The expression levels of p-SMAD1/2 and p-ERK1/2 proteins associated with the SMAD and MAPK signaling pathways were significantly upregulated following TGF-β1 exposure in vitro compared with the levels in the Con group (Figure 9A and B). Treatment of the cells with WA significantly reduced the protein expression levels of p-SMAD2, p-SMAD3 and p-ERK1/2, which may contribute to the EMT inhibition observed in in vitro studies. These results suggest that WA may serve a suppressive role in fibrosis progression in human lung epithelial cells in vitro.

4.7. WA attenuates the TGF-β1-induced inflammation response through suppression of the NLRP3 signaling pathway in cells in vitro

The NLRP3 signaling pathway was investigated in association with the inflammatory response. Western blot analysis demonstrated that NLRP3, ASC and caspase-1 protein levels in active and pro forms were significantly upregulated in MRC-5 cells following TGF-β1 administration compared with the levels in the Con group (Figure 10A and B). Treatment with WA was observed to significantly reduce the protein expression levels of NLRP3, ASC and caspase-1 in vitro. The ratio of active caspase-1 to pro-caspase-1 was significantly elevated following TGF-β1 exposure compared with the level in the Con group; however, this was significantly reduced following WA treatment to a level comparable with that in the Con group (Figure 10C).

Immunofluorescence analysis indicated that NLRP3-positive cells were significantly increased by TGF-β1 compared with the number in the Con group, and significantly reduced in a dose-dependent manner following WA treatment (Figure 10D). The mRNA expression levels of the pro-inflammatory cytokines IL-1β, IL-18, IL-6 and TNF-α were significantly upregulated following TGF-β1 induction compared with the level in the Con group; however, this was significantly reduced following WA treatment (Figure 10E). These results further indicate that WA has a potential role in suppressing the release of pro-inflammatory cytokines through the inactivation of NLRP3 inflammasome induced by TGF-β1 in vitro.
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5. DISCUSSION

In previous studies, OVA has often been used to induce airway inflammation in mice (15,16). At present, OVA treatment is considered to induce histological changes in animal lungs, which simulates inflammation (50,51). In the present study, the effect of WA treatment on airway inflammation through fibrosis and inflammatory responses induced by OVA or TGF-β1 were investigated in vivo or in vitro, respectively. During the in vivo studies, no significant toxicity was observed when the highest concentration of 80 mg/kg WA was administered to mice. To the best of our knowledge, no previous studies have been conducted to explore the effect of WA on lung epithelial cells in vitro. Therefore, the effect of WA at varying doses on cell viability was investigated. No significant cytotoxic effects were observed and 40-160 μM WA was selected as the dose range used to investigate the effect of WA on human cells in vitro. The results of the present study revealed that WA significantly reduced cell damage in a dose-dependent manner. The highest dose of 160 μM WA was the most effective at attenuating TGF-β1-induced cell injury.

A reduced body weight was observed in OVA-induced mice, which was reversed by WA administration. OVA induced lung injury in mice by enhancing inflammation and accelerating fibrosis development (52,53). Lung injuries affect metabolism and immune responses in animals, which are associated with body alterations, including weight loss (54,55). Fibrosis formation is associated with an inflammatory response (56,57). Inflammatory responses have been reported to possess a close association with the immune system (58,59). This was also observed in the present study as evidenced by the infiltration of inflammatory cells, including macrophages, neutrophils and lymphocytes, in the BALF. As previously reported, the number of inflammatory cells was increased in lung injury caused by various conditions (60-62). In response to an alteration in the immune system, the body may develop an abnormal metabolism, which influences food intake and energy metabolism, potentially causing a loss of body weight (63,64). However, the specific effects of WA on metabolism were not investigated in the present study and they require further analysis. The findings of the present study indicated that WA suppressed collagen deposition and inflammation, as well as ameliorated the loss of lung function. WA inhibited fibrosis-associated signals contributing to collagen deposition and reduced TGF-β1 expression, as well as inhibiting the TGF-β1-regulated SMAD and ERK1/2 signaling pathways. Furthermore, the NLRP3 inflammasome was inactivated by WA, resulting in

Figure 8. WA reduces fibrosis accumulation through TGF-β1-associated signaling pathways in vitro. MRC-5 human epithelial cells were treated with TGF-β1 to induce fibrosis in the presence or absence of various concentrations of WA (40-160 μM) for 24 h. (A) Western blot analysis was performed to assess TGF-β1 protein expression levels, these results were quantified. n=6 in each group. (B) Western blot analysis was performed to measure the protein expression levels of FSP-1, fibronectin, vimentin and E-cadherin. n=6 in each group. These results were quantified. (C) Reverse transcription-quantitative polymerase chain reaction analysis was performed to investigate the mRNA expression levels of collagen I, collagen III, α-SMA and TIMP-1 under different conditions. n=6 in each group. **P<0.0.1 and ***P<0.0.01 vs. the Con group; *P<0.0.5, **P<0.0.1 and ***P<0.0.01 vs. the TGF-β1 group. WA, withaferin A; TGF-β1, transforming growth factor-β1; Con, control; TIMP-1, tissue inhibitor of metalloproteinase-1; FSP-1 fibroblast specific protein-1; α-SMA, α-smooth muscle actin.
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Figure 9. WA reduces ovalbumin-induced lung fibrosis through downstream TGF-β1 signals in vitro. (A) The protein expression levels of SMAD2, SMAD3, ERK1/2, p-SMAD2, p-SMAD3 and p-ERK1/2 were detected by western blot analysis. (B) The results of western blotting were quantified. n=6 in each group. ***P<0.01 vs. the Con group; **P<0.05, *P<0.1 and +++P<0.001 vs. the TGF-β1 group. WA, withaferin A; TGF-β1, transforming growth factor-β1; Con, control; p, phosphorylated; ERK, extracellular signal related kinase.

Figure 10. WA attenuates TGF-β1-induced inflammation by suppressing the NLRP3 signaling pathway in vitro. (A) A western blot analysis was performed to detect the protein expression levels of NLRP3, ASC, active caspase-1 and pro-caspase-1 in MRC-5 cells treated with TGF-β1, with or without WA. n=6 in each group. (B) The western blot analysis results were quantified. (C) The ratio of active caspase-1 to pro-caspase-1 was calculated based on the western blotting results. n=6 in each group. (D) Immunofluorescent analysis was performed to measure the NLRP3 expression levels in MRC-5 cells. n=5 in each group. (E) Reverse transcription-quantitative polymerase chain reaction analysis was performed to measure the mRNA expression levels of IL-1β, IL-18, IL-6 and TNF-α. n=6 in each group. Scale bar =25 µm. ***P<0.01 and ****P<0.001 vs. the Con group; **P<0.05, *P<0.01 and +++P<0.001 vs. the TGF-β1 group. WA, withaferin A; TGF-β1, transforming growth factor-β1; Con, control; IL, interleukin, ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; TNF-α, tumor necrosis factor-α.
reduced secretion of pro-inflammatory cytokines. The results of the present study suggest that WA may be used as an effective anti-fibrotic and anti-inflammatory therapy for airway inflammation.

Typically, airway inflammation is caused by the inhalation of allergens, including pollens, air pollutants, house dust and inhalants. It is characterized by airway hyper-responsiveness, eosinophilic airway inflammation and mucus hypersecretion (65,66). Inflammatory cells secrete chemical regulators that are closely associated with the development of airway inflammation (1,2). In the present study, it was revealed that the expression of α-SMA, collagen I, collagen III, FSP-1, fibronectin and vimentin were significantly suppressed following WA treatment in OVA- and TGF-β1-induced fibrosis \textit{in vivo} and \textit{in vitro}. Previous research has demonstrated that α-SMA, collagen I, collagen III, FSP-1, fibronectin and vimentin inhibition attenuates fibrosis in lung tissue samples from mice (42). TIMP-1 is a prototypic and original ancestral member of the TIMP family, and is increased during airway inflammation and may activate fibrosis progression (41,67). The present study indicated that WA significantly reduced OVA-induced TIMP-1 expression, which may be associated with the attenuation of pulmonary fibrosis. Conversely, the upregulation of E-cadherin may also alleviate the course of fibrosis (42). WA reversed the OVA-induced downregulation of E-cadherin expression in the lung tissue samples from mice in the present study, and therefore improved their pulmonary fibrosis. Additionally, TGF-β1 is a critical pro-fibrotic cytokine, which is recognized as the "master switch" to induce fibrosis by promoting the differentiation of fibroblasts into myofibroblasts (18-20,43). Previous studies have described how interference with TGF-β1 signaling may attenuate fibroblast accumulation and subsequently reverse the established fibrosis (68,69). TGF-β1 is a prosclerotic cytokine that promotes EMT during fibrotic diseases (70). EMT is the conversion from an epithelial phenotype to a fibroblastic phenotype, and it serves a prominent role in experimental and clinical pulmonary fibrosis (71,72). EMT induced by TGF-β1 is a key issue in the pathogenesis of tissue or organ fibrosis; it has been reported that the inhibition of TGF-β1 may reverse EMT in hepatic fibrogenesis (73). The lung phenotype in pulmonary fibrosis is regulated by aberrant recapitulation of the TGF-β1/SMAD signaling pathway (74). Inhibition of SMAD activation infers a resistance to TGF-β1-induced EMT and fibrosis (75,76). Therefore, drugs or targets of TGF-β1 may be a novel approach for pulmonary fibrosis therapy. In the present study, WA significantly suppressed TGF-β1 expression \textit{in vivo} and \textit{in vitro}, in OVA-induced lung tissues of mice and TGF-β1-stimulated MRC-5 cells. This was in keeping with previous studies investigating the role of WA against fibrosis (9). TGF-β1 induces EMT by activating SMAD and ERK1/2, which are members of the MAPK signaling pathway (77,78). The administration of OVA to mice causes the phosphorylation of SMAD and ERK1/2. Pre-treatment of mice with WA significantly reduced the phosphorylation of SMAD and ERK1/2. WA may serve an effective therapeutic role in pulmonary fibrosis by impeding the TGF-β1 signaling pathway.

Cytokines, including IL-1β, IL-18, IL-6 and TNF-α, are reported to enhance the progression of airway inflammation (21,22). The findings of the present study indicated that IL-1β, IL-18, IL-6 and TNF-α levels in lung tissue samples were highly increased in mice following the administration of OVA. WA significantly inhibited the production of IL-1β, IL-18, IL-6 and TNF-α in the lung tissues, possibly exhibiting its anti-inflammatory effects. The observation that WA blocked OVA-induced airway inflammation may occur due to the decrease in IL-1β, IL-18, IL-6 and TNF-α. It has been suggested that OVA-induced IL-1β and IL-18 production and lung inflammation are closely associated with the inflammmasome-adaptor protein, ASC (79). Fibrosis development was reported to be associated with NLRP3 and its ability to form a caspase-1-activating inflammasome (80). NLRP3 expression increases in pulmonary fibrosis, as well as in response to TGF-β1 stimulation (81). These previous findings illustrate the involvement of the NLRP3 inflammasome in OVA-triggered lung inflammation and fibrosis. The present study demonstrated that pre-treatment with WA significantly reduced caspase-1 activation, and NLRP3 and ASC expression in OVA-treated lung tissue samples in mice. According to previous studies, WA exhibited an anti-inflammatory role by inactivating the nuclear factor (NF)-κB signaling pathway (82,83). NF-κB is a transcription factor that regulates hundreds of genes involved in innate immunity, cell survival, cell death and inflammation, and it is involved in the production of various cytokines (84,85). In the present study, WA was revealed to reduce the levels of pro-inflammatory cytokines, which may be related to NF-κB regulation (86). Additionally, NF-κB is a key activator of inflammation, it primes the NLRP3-inflammasome for activation by inducing pro-IL-1β and NLRP3 expression (87,88). Therefore, NF-κB activation is also likely to be involved in WA-ameliorated lung injury; however, this was not investigated in the present study. Further study is required to comprehensively investigate the effects of WA on airway inflammation and the underlying mechanisms by which they occur.

In summary, the present study indicated that WA efficiently attenuated OVA-induced lung inflammation and fibrosis by suppressing the infiltration of inflammatory cells. The protective effect of WA is associated with the downregulation of TGF-β1, p-SMADs, p-ERK1/2, collagen I/III, FSP-1, fibronectin, vimentin and α-SMA. It was also demonstrated that WA reduced the levels of pro-inflammatory...
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cytokines, including IL-1β, IL-6, IL-18 and TNF-α, as well as suppressing the activation of the NLRP3 inflammasome. These results indicate that WA may be a potential novel treatment for airway inflammation.

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7. REFERENCES


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Abbreviations: interleukin(IL), transforming growth factor-β(TGF-β), apoptosis-associated speck-like protein containing a caspase recruitment domain(ASC), fibroblast specific protein-1(FSP-1), extracellular signal related kinase(ERK), phosphorylated(p).

Key Words: airway inflammation, withaferin A, ovalbumin, fibrosis, inflammation

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