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Research Article

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In this study we investigated the role of astragaloside IV (AS-IV), one of the major active constituents purified from the Chinese medicinal herb Astragalus membranaceus, in LPS-induced acute inflammatory responses in mice in vivo and examined possible underlying mechanisms. Mice were assigned to four groups: vehicle-treated control animals; AS-IV-treated animals (10 mg/kg b.w. AS-IV daily i.p. injection for 6 days); LPS-treated animals; and AS-IV plus LPS-treated animals. We found that AS-IV treatment significantly inhibited LPS-induced increases in serum levels of MCP-1 and TNF by 82% and 49%, respectively. AS-IV also inhibited LPS-induced upregulation of inflammatory gene expression in different organs. Lung mRNA levels of cellular adhesion molecules, MCP-1, TNFα, IL-6, and TLR4 were significantly attenuated, and lung neutrophil infiltration and activation were strongly inhibited, as reflected by decreased myeloperoxidase content, when the mice were pretreated with AS-IV. Similar results were observed in heart, aorta, kidney, and liver. Furthermore, AS-IV significantly suppressed LPS-induced NF-κB and AP-1 DNA-binding activities in lung and heart. In conclusion, our data provide new in vivo evidence that AS-IV effectively inhibits LPS-induced acute inflammatory responses by modulating NF-κB and AP-1 signaling pathways. Our results suggest that AS-IV may be useful for the prevention or treatment of inflammatory diseases.

1. Introduction

Endothelial cells are a primary target of inflammatory responses, and their injury can lead to vasculopathy and organ dysfunction [1]. The bacterial endotoxin LPS directly elicits several acute inflammatory responses in endothelial cells, including production of cellular adhesion molecules, such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1), and other proinflammatory mediators, such as TNFα, IL-6, and monocyte chemoattractant protein-1 (MCP-1). Together, these proinflammatory mediators elicit leukocyte adhesion to the vasculature and transmigration into the underlying tissue, causing endothelial injury and dysfunction associated with sepsis [1]. Although these proinflammatory mediators are required for an adequate host-defense response, their dysregulation can lead to refractory hypotension, cardiovascular hyporeactivity, intravascular coagulation, multiple organ failure, and death [2, 3].

The regulation of inflammatory gene transcription has been shown to be controlled by specific signaling pathways and transcription factors, such as NF-κB and AP-1 [4, 5]. In particular, the NF-κB pathway affects host defense against infectious agents by upregulating inflammatory genes that cause acute neutrophilic inflammation and the systemic inflammatory response syndrome [5]. Under normal condition, NF-κB is located in the cytoplasm in an inactive form in association with its inhibitor, IκB. In response to stimulation, for example, by LPS, IκB is phosphorylated by IκB kinase. Following phosphorylation, IκB is ubiquitinated and degraded, allowing NF-κB to translocate to the nucleus, bind to DNA promoter regions, and induce inflammatory gene transcription. Various agents that block NF-κB signaling have been shown to decrease expression of proinflammatory
mediators [6–9]. Thus, the inhibition of NF-κB activation is expected to be protective in pathological inflammatory states.

_Astragalus membranaceus_ is one of the most widely used Chinese medicinal herbs for the treatment of many diseases, including cardiovascular disease, nephritis, hepatitis, and diabetes [10]. It also has been available in Europe and the US for many years as a food supplement. Evidence from pharmacological research and clinical practice suggests that _Astragalus_ possesses a wide spectrum of activities, including immunomodulation [11, 12], cardiovascular protection [13–15], anti-inflammatory effects [16–18], hepatoprotection [19, 20], antidiabetes [21], anticancer [22], and neuroprotection [23].

The biologically active constituents of _Astragalus_ roots represent three classes of chemical compounds: saponins, polysaccharides, and flavonoids [24]. By chemical degradation and $^{13}$C nuclear magnetic resonance examination, the structure of astragaloside IV (AS-IV) was determined as 3-O-$\beta$-D-xylopyranosyl-6-O-$\beta$-D-glucopyranosyl-cycloastragenol (C$_{46}$H$_{68}$O$_{14}$; MW = 784.9) [25]. As one of the major active constituents of _Astragalus_, AS-IV is used as the characteristic marker for quality evaluation of _Astragalus_ in the Chinese Pharmacopeia and has been shown to exert potent cardioprotective and anti-inflammatory effects [6, 10–15]. We have previously shown that AS-IV inhibits LPS- and TNFα-induced adhesion molecule expression and NF-κB activation in cultured human endothelial cells [6]. However, _in vivo_ evidence supporting such activity is currently lacking. Therefore, in this study we investigated whether AS-IV can inhibit LPS-induced acute inflammatory responses in experimental mice.

2. Materials and Methods

2.1. Animals and Experimental Procedures. Female C57BL/6J mice, 12 weeks old and weighing 20–22 g, were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in specific pathogen-free conditions and a temperature- and humidity-controlled environment (12-h light/dark cycle) with unlimited access to tap water and Purina 5001 chow diet (Harlan Teklad, Madison, WI). The investigation conformed to the _Guide for the Care and Use of Laboratory Animals_ by NIH, and all animal procedures were reviewed and approved by the Oregon State University Institutional Animal Care and Use Committee.

AS-IV was purchased from Quality Phytochemicals LLC (Edison, NJ), and a stock solution was prepared with propylene glycol (Sigma Aldrich, St. Louis, MO) and further diluted with Hank's buffered saline solution (HBSS). LPS (serotype O55:B5 from _Escherichia coli_, Sigma Aldrich) stock solution was prepared in HBSS. Mice were randomly assigned to 4 groups as follows: (i) control animals received daily i.p. injection of the vehicle propylene glycol with HBSS for 6 days followed by a single i.p. HBSS injection; (ii) AS-IV-treated animals received 10 mg/kg b.w. AS-IV daily i.p. for 6 days followed by a single i.p. HBSS injection; (iii) LPS-treated animals received daily i.p. injection of propylene glycol with HBSS for 6 days followed by a single i.p. injection of 0.5 μg/g b.w. LPS; and (iv) AS-IV plus LPS-treated animals received AS-IV daily i.p. for 6 days followed by single i.p. injection of LPS. Animals were sacrificed 3 hours after HBSS or LPS injection. Based on our previous observations, the 3-h time point and LPS dose of 0.5 μg/g b.w. were chosen [31]. In some studies, mice were randomly assigned to receive i.p. injection of LPS and sacrificed after 1, 3, 8, or 24 h. Each group consisted of 4 to 5 animals. After sacrifice, blood and tissues were collected for further analysis.

2.2. Serum Inflammatory Mediators. Serum concentrations of MCP-1, TNFα, sVCAM-1, and sICAM-1 were measured by quantitative colorimetric sandwich ELISA (R&D Systems, Minneapolis, MN). The sensitivity of the assays is 2 pg/mL for MCP-1, 5 pg/mL for TNFα, and 30 pg/mL for sVCAM-1 and sICAM-1.

2.3. Tissue mRNA Levels of Inflammatory Mediators. Total RNA was isolated from different organs using TRIzol Reagent (Life Technologies, Foster City, CA). cDNA synthesis was performed using the high capacity cDNA archive kit (Life Technologies). mRNA levels of VCAM-1, ICAM-1, E-selectin, P-selectin, MCP-1, TNFα, IL-6, myeloperoxidase (MPO), Toll-like receptor-4 (TLR4), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantitated by real-time qPCR. All primers and probes were purchased as kits (Assays on Demand, Life Technologies). The assays are supplied as a 20x mixture of PCR primers and TaqMan minor groove binder 6-FAM dye-labeled probes with a nonfluorescent quencher at the 3’ end. TaqMan quantitative PCR (40 cycles at 95°C for 15 sec and 60°C for 1 min) was performed using TaqMan Universal PCR Master Mix (Life Technologies) in 96-well plates with an ABI Prism 7500 Sequence Detection System (Life Technologies). To obtain relative quantification, two standard curves were constructed in each plate with one target gene and the internal control GAPDH gene. Standard curves were generated by plotting the threshold cycle number values against the log of the amount of input cDNA and used to quantify the expression of the target genes and GAPDH gene in the same sample. After normalization to internal GAPDH in each sample, results were expressed as percentage of GAPDH or fold of control.

2.4. Lung Protein Concentration of Myeloperoxidase. A part of the right lung lobe was homogenized, and the cytosolic fraction of lung tissue homogenate was prepared using nuclear extract kits (Active Motif, Carlsbad, CA). Lung cytosolic MPO was quantitated using the MPO enzyme-linked immunosorbent assay kit (Hytest Biotechnology, Plymouth Meeting, PA) according to the manufacturer’s instructions. The lung MPO concentration of each sample was normalized with cytosolic protein concentration and expressed as ng MPO/mg tissue protein.

2.5. Nuclear Transcription Factors. Nuclear extracts were prepared from lung and heart using nuclear extract kits (Active Motif). For analysis of nuclear transcription factor activation, ELISA-based assays (Active Motif) were used to determine the DNA-binding activity of NF-κB (p65) and AP-1 (c-fos).
Mediators of Inflammation

Serum MCP-1 (ng/mL)

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(a)

Serum TNF-α (pg/mL)

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(b)

Figure 1: AS-IV inhibits LPS-induced increases in serum MCP-1 and TNF-α in mice. Mice were randomly assigned to 4 groups as follows: (i) control animals received daily i.p. injection of the vehicle HBSS for 6 days followed by a single i.p. HBSS injection; (ii) AS-IV-treated animals received 10 mg/kg b.w. AS-IV daily i.p. for 6 days followed by a single i.p. HBSS injection; (iii) LPS-treated animals received daily i.p. injection of vehicle HBSS for 6 days followed by a single i.p. injection of 0.5 μg/g b.w. LPS; and (iv) AS-IV plus LPS-treated animals received AS-IV daily i.p. for 6 days followed by single i.p. injection of LPS. Three hours after the HBSS or LPS injection, the animals were sacrificed and blood was collected. Serum MCP-1 (a) and TNF-α (b) were measured by ELISA. Data shown are mean values ± SEM of five animals per group. * P < 0.05 compared to animals treated with LPS only.

The specificity of binding was confirmed by competition with either wild-type or mutant oligonucleotides.

2.6. Statistical Analysis. The data were calculated as means ± SEM and analyzed by ANOVA with Fisher PLSD post hoc test. Statistical significance was set at P < 0.05.

3. Results and Discussion

The pathophysiology of acute inflammation triggered by the bacterial endotoxin LPS is characterized by the production of multiple proinflammatory cytokines and chemokines, expression of adhesion molecules, and infiltration of neutrophils and monocytes into inflamed tissues. Because of the complexity of the pathology of septic shock, major efforts have focused on identifying novel anti-inflammatory drugs that prevent the proinflammatory process at the early stage of gene expression of key inflammatory mediators [32].

We have previously shown that AS-IV inhibits LPS- and TNF-α-induced adhesion molecule expression and consequent adherence of monocytes by modulating NF-κB signaling pathway in cultured human endothelial cells [6]. We now provide new evidence showing that AS-IV exhibits strong anti-inflammatory activities in vivo by attenuating LPS-induced acute inflammatory responses through inhibition of NF-κB- and AP-1-mediated inflammatory signaling pathways in mice.

3.1. AS-IV Inhibits LPS-Induced Increases in Serum MCP-1 and TNF-α in Mice. Treatment of mice with AS-IV for 6 days had no effect on body weight changes compared to HBSS-treated control animals. The mean body weight was 20.8 ± 0.6 g before and 20.8 ± 0.5 g after 6-day AS-IV treatment. In control animals, the mean body weight was 20.2 ± 0.3 g before and 19.9 ± 0.4 g after 6-day HBSS treatment. We first investigated the effect of AS-IV on LPS-induced systemic inflammatory responses by determining serum levels of MCP-1, TNF-α, and the soluble cellular adhesion molecules, sVCAM-1 and sICAM-1. Treatment of mice with AS-IV alone for 6 days had no effect on serum levels of these inflammatory mediators compared to HBSS-treated control animals. As expected, 3 hours after LPS injection, significant increases in the serum levels of MCP-1 and TNF-α were observed (Figure 1). Interestingly, pretreatment of animals with AS-IV significantly inhibited LPS-induced increases in serum MCP-1 (Figure 1(a)) and TNF-α (Figure 1(b)) by 82% and 49%, respectively. Specifically, MCP-1 levels were 15.2 ± 2.0 ng/mL in mice treated with AS-IV plus LPS, compared to 81.8 ± 9.5 ng/mL in animals treated with LPS only; TNF-α levels were 142 ± 9 pg/mL and 279 ± 35 pg/mL, respectively (P < 0.05, n = 5). However, AS-IV did not inhibit the LPS-induced increases in serum sVCAM-1 and sICAM-1 concentrations (data not shown).

3.2. AS-IV Inhibits LPS-Induced Upregulation of Inflammatory Gene Expression in Mouse Lung and Other Tissues. To investigate whether AS-IV inhibits LPS-induced acute inflammatory responses in mouse organs, we assessed gene expression of inflammatory mediators in lung, heart, aorta, kidney, and liver of LPS-exposed mice, using real-time qPCR analysis. Treatment of mice with AS-IV alone did not affect gene expression of cellular adhesion molecules and proinflammatory mediators (Figures 2–6). As expected, treatment of mice with LPS for 3 hours strongly upregulated inflammatory gene expression in all tissues examined (Figures 2–6). Pretreatment of mice with AS-IV significantly inhibited
AS-IV inhibits LPS-induced inflammatory gene expression in mouse lung. Mice were randomly assigned to 4 groups as follows: (i) control animals received daily i.p. injection of the vehicle HBSS for 6 days followed by a single i.p. HBSS injection; (ii) AS-IV-treated animals received 10 mg/kg b.w. AS-IV daily i.p. for 6 days followed by a single i.p. HBSS injection; (iii) LPS-treated animals received daily i.p. injection of vehicle HBSS for 6 days followed by a single i.p. injection of 0.5 μg/g b.w. LPS; and (iv) AS-IV plus LPS-treated animals received AS-IV daily i.p. for 6 days followed by single i.p. injection of LPS. Three hours after the HBSS or LPS injection, the animals were sacrificed and tissues were collected. Total RNA was isolated from lung. Inflammatory gene expression was quantified using real-time quantitative PCR. After normalization to the internal control gene GAPDH, the results for each target gene were expressed as percentage of GAPDH. Data shown are means ± SEM of 5 animals per group. *P < 0.05 compared to animals treated with LPS only.

3.3. AS-IV Inhibits LPS-Induced Neutrophil Infiltration and Activation in Lung. As polymorphonuclear neutrophils (PMN) and other phagocytic cells, such as monocyte-macrophages, play critical roles in acute inflammation and tissue injury, we assessed lung myeloperoxidase (MPO), a well-documented PMN-specific biomarker [31, 33, 34], after LPS challenge. MPO is rapidly released when PMN are activated, which triggers transcriptional upregulation of mRNA and new protein synthesis of MPO. Therefore, upregulation of MPO mRNA levels also reflects PMN activation during acute lung inflammatory responses [31, 33]. As shown in Figure 3(a), lung MPO mRNA was at very low
levels in control animals; however, LPS treatment induced time-dependent upregulation of lung MPO gene expression, which peaked at 3 h and remained elevated for up to 8 h and then declined after 24 h. These data indicate infiltration and activation of interstitial PMN in the lungs of LPS-treated mice in a time-dependent manner, which is consistent with our previous results showing that TNFα treatment induced increases in MPO mRNA and protein levels, enzyme activity, and morphological accumulation of PMN in mouse lung tissues [33]. However, pretreatment of mice with AS-IV significantly inhibited the LPS-induced increase in MPO mRNA level by 27% (**P < 0.05, n = 5**) (Figure 3(b)). This was further confirmed by the lung MPO protein level, which increased from 6 ± 1 ng/mg tissue protein in control animals to 325 ± 67 ng/mg tissue protein in LPS-treated animals and significantly reduced by AS-IV treatment by 80% to 71 ± 9 ng/mg tissue protein (**P < 0.05, n = 5**) (Figure 3(c)). As the lung is the main target for activated PMN during acute inflammation [31, 33, 34], these data support the notion that AS-IV inhibits PMN infiltration/recruitment to the lung and subsequent tissue damage during LPS-induced acute inflammatory responses.

3.4. AS-IV Inhibits LPS-Induced NF-κB and AP-1 DNA-Binding Activity in Mouse Lung and Heart. It is well recognized that NF-κB plays a prominent role in LPS-induced transcriptional regulation of most inflammatory genes that contribute to the development of septic shock, multiple organ failure, and death [5, 32]. Of clinical relevance, NF-κB activation was increased in patients with acute inflammation and sepsis and correlated with clinical severity and mortality [35]. To investigate possible signaling pathways mediating the inhibitory effect of AS-IV on LPS-induced inflammatory gene transcription, we assessed the nuclear content of the NF-κB subunit, p65, and the AP-1 subunit, c-fos, as indicators of nuclear translocation and activation of these transcription factors. The DNA-binding activity of NF-κB (p65) and AP-1 (c-fos) was detectable at low levels in lung and heart tissues of control and AS-IV-only-treated animals. AS-IV alone did not cause NF-κB or AP-1 activation in either lung
Figure 4: AS-IV inhibits LPS-induced inflammatory gene expression in mouse heart. Mice were treated as described in the legend of Figure 2. Total RNA was isolated from heart, and inflammatory gene expression was quantified as described in the legend of Figure 2. Data shown are means ± SEM of 5 animals per group. * P < 0.05 compared to animals treated with LPS only.

or heart (Figure 7). LPS markedly increased NF-κB activity in lung and heart by 15.8- and 11.5-fold, respectively; AS-IV treatment significantly suppressed LPS-induced activation of NF-κB by 42% and 54%, respectively (P < 0.05, n = 5) (Figure 7(a)). LPS also strongly increased AP-1 activation by 9.6- and 25.3-fold, respectively, while AS-IV treatment significantly diminished LPS-induced AP-1 activity by 41% and 49%, respectively, in both lung and heart (P < 0.05, n = 5) (Figure 7(b)).

Our data provide new evidence that the inhibitory effect of AS-IV on LPS-induced inflammatory gene expression is mainly through modulating NF-κB and AP-1 DNA-binding activity. These data indicate that the ability of AS-IV to suppress the NF-κB and AP-1 pathways is the major underlying mechanism contributing to its anti-inflammatory potential in vivo. It is well documented that the “classic” TLR4 pathway plays a major role in LPS signaling during sepsis [36]. TLR4 recognizes LPS from Gram-negative bacteria and mediates the innate immune response by activating IκB kinase (IKK) and mitogen-activated protein kinase kinases (MKK), which in turn activate NF-κB and AP-1, respectively [5, 36]. Our results further show that AS-IV inhibits LPS-induced TLR4 gene expression in lung and heart by 30% and 65%, respectively (Figures 2 and 4), which is consistent with its inhibitory
Figure 5: AS-IV inhibits LPS-induced inflammatory gene expression in mouse aorta. Mice were treated as described in the legend of Figure 2. Total RNA was isolated from aorta, and inflammatory gene expression was quantified as described in the legend of Figure 2. Data shown are means ± SEM of 5 animals per group. * \( P < 0.05 \) compared to animals treated with LPS only.

A limitation of our study is that we had to apply AS-IV by i.p. injection because it is poorly bioavailable. Previous studies on dogs and rats have found that only 7.4% and 3.7%, respectively, of orally supplemented AS-IV were absorbed [41, 42]. This is mainly due to AS-IV’s low fat solubility and low transmittance in the small intestine [43]. In fact, in Chinese medicine AS-IV is clinically used as intravenous

species and subsequent activation of the redox-sensitive NF-kB and AP-1 pathways at different levels [40]. However, all these mechanisms observed in vitro need to be further investigated in vivo.

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(i.v.) therapy. Upon i.v. injection of AS-IV at 0.75 mg/kg in rats and 0.5 mg/kg in dogs, the maximum plasma concentrations reached were 3.79 µg/mL and 4.39 µg/mL, respectively, and the elimination half-life ($t_{1/2}$) was 98 min and 60 min, respectively [44, 45]. The highest concentration was found in lung and liver tissues (2.8–2.9 µg/g) after i.v. injection of 1.5 mg/kg AS-IV, whereas heart, muscle, skin, and kidney contained moderate amounts (0.16–1.0 µg/g) [45]. As Astragalus and AS-IV are now widely used in Chinese medicine and are also available in Europe and the US as dietary supplements, chemical modifications to improve the absolute bioavailability of AS-IV while maintaining its biological activity could be a focus of future research.

4. Conclusion

In conclusion, our data provide new evidence that AS-IV inhibits LPS-induced acute inflammatory responses in vivo by modulating the NF-κB and AP-1 signaling pathways. Our results might lead to the identification of AS-IV as a natural...
compound or chemically derived drug that may be useful for the prevention or treatment of inflammatory diseases. We believe that the molecular basis for its therapeutic efficacy is intriguing and warrants further investigation.

**Abbreviations**

- AP-1: Activator protein-1
- AS-IV: Astragaloside IV
- b.w.: Body weight
- GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
- ICAM-1: Intercellular adhesion molecule-1
- i.p.: Intraperitoneal
- i.v.: Intravenous
- IKK: IκB kinase
- IL: Interleukin
- LPS: Lipopolysaccharide
- MCP-1: Monocyte chemoattractant protein-1
- MPO: Myeloperoxidase
- NF-κB: Nuclear factor κB
- PMN: Polymorphonuclear neutrophils
- Redox: Reduction-oxidation
- ROS: Reactive oxygen species
- sICAM-1: Soluble intercellular adhesion molecule-1
- sVCAM-1: Soluble vascular cell adhesion molecule-1
- TL4R: Toll-like receptor-4
- TNFα: Tumor necrosis factor α
- VCAM-1: Vascular cell adhesion molecule-1.

**Disclaimer**

The content of this work is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

**Conflict of Interests**

The authors declare that they have no conflict of interests.

**Authors’ Contribution**

Wei-Jian Zhang designed and performed experiments; Wei-Jian Zhang and Balz Frei analyzed data and wrote and approved the final paper.

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**References**


