Original Research Article

Astragaloside IV exerts anti-inflammatory role in endometriosis by downregulating TLR4/NF-κB pathway

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Abstract

Purpose: *To investigate the effect of astragaloside IV administration on the inflammatory response in endometriosis and the underlying mechanism of action.*
Methods: *Mice were divided into two groups: endometriosis (EMs) mice and control mice (n = 12). EMs induction in mice was achieved by transplantation of mouse uterine tissue. The same procedure was performed in control mice except that a separate suture was inserted instead of endometrial tissue. After 5 weeks, EMs mice were treated with or without astragaloside IV (AIV). The tissue lesions in EMs and control mice were stained with hematoxylin and eosin staining. The activation of toll-like receptor 4 (TLR4) and nuclear factor-κB (NF-κB) p65 signaling was evaluated by western blot, while expression of inflammatory cytokines was evaluated by quantitative real-time-polymerase chain reaction (qRT-PCR), and enzyme-linked immunosorbent assay (ELISA).*
Results: *Astragaloside IV repressed the inflammation of murine Ems lesions, and also dampened the activation of TLR4/NF-κB signaling in vivo and vitro (p < 0.01 and p < 0.001, respectively). In addition, the expression levels of inflammatory cytokines (IL-1β, IL-6, Ccl-2, and TNF-α) decreased following AIV treatment in vivo.*
Conclusion: *The results indicate that TLR4/NF-κB signaling pathways are closely related to the inhibition of Ems inflammation by astragaloside IV. Thus, astragaloside IV may be a novel drug for the prevention and treatment of endometrioses.*

Keywords: *Murine model, Astragaloside IV, Endometriosis, Inflammation, TLR4/NF-κB*

INTRODUCTION

Endometriosis (EMs) is a common and highly-recurrent disorder [1] which causes about 10 to 15 % of cases of gynecological diseases such as dysmenorrhea, infertility, and chronic pelvic pain in women of childbearing age [2]. A well-known lipopolysaccharide (LPS) receptor, TLR4, transduces immune signals to the nucleus via activation of NF-κB transcription factors. Many factors leading to the inflammatory environment associated with endometriosis are upregulated by NF-κB [3,4]. It is speculated when various stimulating factors act on normal endometrial cells, they can specifically up-regulate NF-κB expression, initiate an inflammatory response, and promote various inflammations involved in

ectopic endometrial cell adhesion, substrate degradation, and angiogenesis. Recent studies have shown that inhibition of NF-κB activation by pyrrolidine dithiocarbamate or baicalein effectively inhibits EMs [5,6].

Astragaloside IV (AIV) is a high-purity drug extracted from *Astragalus membranaceus*, which is called "super Astragalus polysaccharide." Astragaloside enhances the body's immunity and improve the body's disease resistance [7]. Moreover, AIV has been reported to inhibit migration and invasion of lung cancer cells A549 by regulating PKC-α-ERK1/2-NF-κB [8]. In addition, the anti-inflammatory effect of AIV is mediated through its ability in inhibiting the activation of NF-κB signaling [9,10]. However, whether AIV can attenuate EMs remains unknown, and its underlying mechanism remains to be elucidated. Therefore, in present study, the effect of AIV on inflammation and the TLR4/NF-κB pathway was studied using an EMs mouse model as well as in *vitro* experiments.

EXPERIMENTAL

Drug preparation

Sigma (St. Louis, MO, USA) and Takeda Pharmaceutical Company Limited (Osaka, Japan) provided AIV (purity above 99.8 %, HPLC) and TAK-242, respectively. For *in vivo* studies, AIV was suspended in 1 % carboxymethyl cellulose (CMC) solution as a vehicle and administrated to mice through oral gavage. Astragaloside IV was administered once a day. For *in vitro* studies, AIV and TAK-242 were dissolved in dimethyl sulfoxide (DMSO) and diluted with the appropriate medium containing less than 0.1 % DMSO.

Mouse model

The Experimental Animal Center (Shanghai, China) provided female mice (6 weeks of age). Before starting the experiment, the female mice were adapted for 7 days in normal laboratory conditions. A mouse model of endometriosis was established by autologous transplantation. The upper end of the urethra was opened, the skin was cut longitudinally, the right side of the uterus was separated, and the ovary was ligated 0.5 cm away. The mouse uterus was longitudinally dissected and the endometrial tissue and the muscular layer of tissue were separated.

The isolated endometrial tissue was divided into small pieces of approximately 0.5 cm and attached to the inner side of the bilateral abdominal wall (the inner membrane layer was opposite to the planting site when attached). The endometrium was evaluated using a caliper to measure two vertical diameters (D_1, D_2) per lesion, which was accurate to 0.1 mm. Lesion volume (V) was calculated according to Eq 1 [11].

$$V = \frac{D_1 \times D_2 \times \pi}{4} \text{ .... (1)}$$

The same procedure was performed in mice except that the suture insertion alone was measured rather than endometrial tissue. The dose of AIV administered to the mice was 0, 5, 10, or 30 mg/kg/day for 5 weeks after surgical insertion of endometrial tissue; treatment lasted for 8 weeks. The animal experiments were approved by the Ethics Committee of Jiangxi Provincial People's Hospital (approval no. 2018-0112) and conducted according to international guidelines for animal [12].

Cell culture and drug treatment

Samples of endometriotic tissue were obtained from the EMs mice. Biopsy material was immediately transferred to phosphate buffered saline (PBS) after laparoscopy and digested for 6 h at room temperature. Collagenase A (0.25 %) and dispase (1.5 U/ml) (Roche, Germany) were added to the PBS. Red blood cells and debris were removed using a (45 %, v/v) Percoll pad (Amersham-Pharmacia, Germany) by centrifugation. Appropriate tissue culture vessels were provided for dissociated cells to seed. Antibiotics and 10 % fetal calf serum were added into Dulbecco's modified Eagle medium for storage of dissociated cells. The primary endometriotic cells were treated with AIV at 0, 5, 10, or 20 μM. The endometriotic cells were treated with the TLR4 inhibitor TAK-242 at 1 μM. Lipopolysaccharide (LPS)-induced inflammation is suppressed by small-molecule antisepsis agent TAK-242, which strongly inhibits the production of cytokines and nitric oxide (NO) of TLR4 by monocytes and macrophages, and does not inhibit LPS binding to cells [13].

HE staining

A 4 % PBS was added to the uterus tissue of EMs and sham mice for 30 min and 5 μm sections of the tissues were cut. Slides were then stained for 10 sec with 70 % ethanol (EtOH), 5 sec with diethylpyrocarbonate-treated water, 20 s with hematoxylin and RNAase inhibitor, 30 sec with 70 % EtOH, 20 s with eosin Y in 100 % EtOH, and 2 min with xylene (after 30 sec of dehydration with a series of EtOH). Finally, after being washed three times, the histological

structure of the uterus tissue was observed under a microscope (400 ×).

Western blotting

First, the tissues and cells were homogenized on ice for 30 min with RIPA buffer (Beyotime, China) and protease inhibitor. Then, the tissue homogenate was centrifuged for 15 min at 12000 rpm and the supernatant was collected and stored at -20 °C. A 12 % sodium dodecylsulphate polyacrylamide gel electrophoresis, SDS-PAGE (SDS-PAGE) with Tris-glycine running buffer was used for electrophoresis and the protein was electrically transferred onto polyvinylidene fluoride (PVDF) membrane. Non-specific binding was blocked in tris-buffered saline with 3 % bovine serum albumin and tris-buffered saline containing 0.1 % Tween-20 (TBS and 0.1 % Tween 20) under gentle shaking at room temperature for 1 h. The blots were incubated with anti-NF-κB p65 (No. #4764, CST, USA), anti-TLR4 (No. #76B357.1, Abcam, USA) and anti-β-actin (Abcam) after washing in TBS-T buffer three times. Goat-anti mouse IgG or goat-anti rabbit IgG secondary antibody was added and incubated at room temperature for 1 h after washing in TBS-T buffer three times. Strip intensity was quantified with ImageJ Software. Protein expressions of cells and tissues were standardized to β-actin levels.

RT-PCR

Trizol reagent (Invitrogen, CA) was used to isolate total RNA from tissues and cells according to the manufacture's specifications. Then, the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, USA) was used to reverse transcribe the total RNA to cDNA. ABI Prism7900 Sequence Detection System (Applied Biosystems, USA) with SYBR Green PCR Master Mix (Takara Bio Inc, China) was used in quantitative RT-PCR analysis of the major inflammatory cytokines including interleukin (IL)-1β, IL-6, monocyte chemotactic protein-2 (Ccl-2), and tumor necrosis factor-α (TNF-α) in the tissues and cells. A control containing no cDNA was evaluated in parallel for each assay. The $2^{-\triangle\triangle CT}$ method was used to calculate target gene expression as described previously [14]. All experiments were performed in duplicate or triplicate.

Determination of cytokine levels

Human ELISA Development Kit (R & D System, Inc., MN) was used to determine inflammatory cytokine levels in the culture medium according to the manufacturer's instructions. The culture medium was collected and centrifuged. Then, the supernatant was collected. The concentrations of IL-1β, IL-6, Ccl-2, and TNF-α in the culture medium was calculated from a standard curve.

Statistical analysis

Statistical Package for Social Sciences (SPSS) 22.0 software was used for data analysis. All protocols were repeated as independent experiments at least three times and expressed as mean ± standard deviation (SD). ANOVA was used to determine statistical differences with $p < 0.05$ considered statistically significant.

RESULTS

EMs induce inflammatory response in EMs mice

At 5 weeks post-surgery, the EMs mice showed inflammatory cell infiltration (Figure 1 A). The activities and expressions of TLR4 and NF-κB in endometriosis lesions were measured in EMs mice. A significant increase in TLR4 and NF-κB activities in endometriosis lesions from EMs mice was observed ($p < 0.01$; Figure 1 C). In addition, IL-1β, IL-6, Ccl-2, and TNF-α mRNA expressions increased in endometriosis lesions of EMs mice compared with control mice (Figure 1 B; $p < 0.01$). Similarly, when compared with control mice, IL-1β, IL-6, Ccl-2, and TNF-α protein levels in endometriosis lesions also significantly increased in EMs mice (Figure 1 D; $p < 0.01$). These results indicate that EMs induced an inflammatory response in EMs mice.
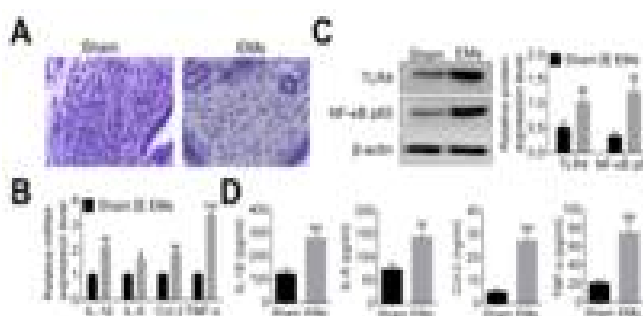

Figure 1: Effect of EMs on inflammatory response. (A) Hematoxylin and eosin (HE) staining of uterine tissue from control and EMs mice. (B) IL-1β, IL-6, Ccl-2, and TNF-α mRNA expression in control and EMs mice were evaluated by qRT-PCR. (C) TLR4 and NF-κB levels in control and EMs mice were evaluated by western blot. (D) ELISA evaluation of IL-1β, IL-6, Ccl-2, and TNF-α expression of control and EMs mice; *p < 0.05, **p < 0.01, ***p < 0.001, vs. control

Effect of AIV on the development of endometriosis lesions in EMs mice

Compared to untreated EMs mice, AIV treatment reduced the severity of endometriosis lesions in EMs mice, as shown by hematoxylin and eosin (HE) staining test (Figure 2 A). In western blot analysis, AIV inhibited TLR4 and NF-κB activity in a dose-dependent fashion in lesions from EMs mice. Moreover, protein expressions of TLR4 and NF-κB in EMs mice were also reduced in a dose-dependent manner by AIV treatment (Figure 2 B). These results indicates that the inhibitory effect of AIV in EMs mice is via the TLR4/NF-κB signaling pathway.

Compared with control mice, the mRNA levels of IL-1β, IL-6, Ccl-2, and TNF-α increased significantly in EMs mice ($p < 0.01$), whereas AIV treatment apparently downregulated the levels of IL-1β, IL-6, Ccl-2, and TNF-α mRNA in EMs mice. These effects were evident at a dose as low as 5 mg/kg/day and reached peak effect at 30 mg/kg/day of AIV in a dose-dependent manner (Figure 3 A). Similarly, treatment with AIV decreased L-1β, IL-6, Ccl-2, and TNF-α protein levels in EMs mice in a dose-dependent fashion (Figure 3 B). The results showed that AIV inhibited IL-1β, IL-6, Ccl-2, and TNF-α expression via its anti-inflammatory effect.

Effect of AIV on the development of inflammation *in vitro*

TLR4 and NF-κB p65 protein levels in epithelial cells of endometriosis lesions were significantly suppressed by AIV and TAK-242 treatment in a dose-dependent manner (Figure 4, $p < 0.001$). The data revealed that the mechanism of the anti-depressive effect of AIV was closely related to alterations in TLR4/NF-κB activity. To assess the inflammation of the endometriosis lesions in EMs mice, IL-1β, IL-6, Ccl-2, and TNF-α mRNA expression after AIV or TAK-242 treatment was measured by qRT-PCR. AIV administration attenuated the IL-1β, IL-6, Ccl-2, and TNF-α mRNA expression in a dose-dependent manner in epithelial cells of murine endometrium (Figure 5 A). Similarly, TAK-242 diminished the mRNA levels of inflammation-related cytokines in epithelial cells. In addition, the expression of IL-1β, IL-6, Ccl-2, and TNF-α were measured by ELISA. Astragaloside IV and TAK-242 treatment decreased the expression of inflammation-associated cytokines (Figure 5 B). Therefore, inhibition of both AIV and TLR4 repressed inflammatory and angiogenic activity in endometriosis.
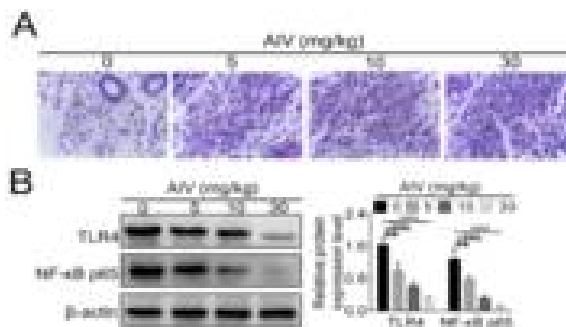

Figure 2: AIV treatment attenuated inflammatory responses in EMs mice. (A) Hematoxylin and eosin (HE) staining of uterine tissue from EMs mice. (B) TLR4 and NF-κB levels in EMs mice were evaluated by western blot. Five weeks after surgery, AIV treatment was started and lasted 8 weeks. AIV dosage-mediated protein expression in EMs mice at 0, 5, 10, or 30 mg/kg/day; ** $p < 0.01$, *** $p < 0.001$ vs. treatment with AIV at 0 mg/kg/day
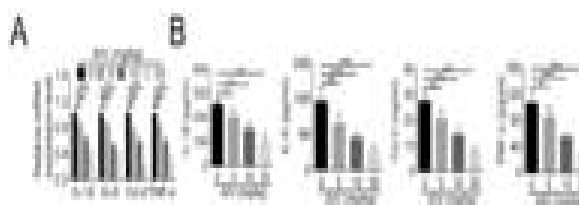

Figure 3: AIV reduced the level of inflammation-related factors in the EMs mice. (A) Effect of AIV on the IL-1β, IL-6, Ccl-2, and TNF-α mRNA expression in EMs mice by qRT-PCR. (B) Effect of AIV on the IL-1β, IL-6, Ccl-2, and TNF-α protein levels in EMs mice by ELISA; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs treatment with AIV (0 mg/kg/day)

Figure 4: Effects of AIV and TAK-242 on expression of TLR4 and NF-κB p65 in the epithelial cells of endometriosis lesions in EMs mice were evaluated by western blot. The expression of indicated proteins in epithelial cells of endometriosis lesions in EMs mice treated with AIV (0, 5, 10, or 20 μM) or TAK-242 (an inhibitor of TLR4, 1 μM) was determined by western blot. Representative blots (upper) and statistical analysis (lower) were shown. *** $p < 0.001$

Figure 5: Effects of AIV and TAK-242 on the expression of inflammatory proteins in the epithelial cells of endometriosis lesions in EMs mice. (A) The IL-1β, IL-6, Ccl-2, and TNF-α mRNA expression levels in murine epithelial cells of endometriosis lesions in EMs mice by qRT-PCR. (B) The IL-1β, IL-6, Ccl-2, and TNF-α protein levels in murine epithelial cells of endometriosis lesions in EMs mice by ELISA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. treatment with AIV (0 mg/kg/day)

DISCUSSION

In previous studies, an EMs mouse model provided an in-depth understanding of the pathogenesis of peritoneal endometriosis [15,16]. This EMs mouse model was used in the present study to examine the anti-inflammatory effects of AIV and further identify its underlying mechanism. In the EMs mouse model, the mice developed inflammatory responses. Remarkably, TLR4/NF-κB activation and overexpression were significantly inhibited by AIV treatment and TLR4/NF-κB was a major transcription factor that regulated chemokines and adhesion molecules, especially proinflammatory cytokines [17].

In addition, the anti-inflammatory effects of AIV were evidenced by a reduction in proinflammatory cytokine levels via regulation of IL-1β, IL-6, Ccl-2, and TNF-α protein expression. Moreover, TLR4/NF-κB expression levels and its related proinflammatory cytokines (IL-1β, IL-6, Ccl-2, and TNF-α) were further decreased by AIV treatment in the epithelial cells in endometriosis lesions. Therefore, these results indicate that AIV was associated with inhibition of inflammation by targeting of the TLR4/NF-κB signaling pathway.

Recent studies have reported that innate immunity is closely related to the NF-κB signaling pathway, and that peritoneal endometriotic lesions are activated by the NF-κB signaling pathway [18]. The activity of NF-κB p65 in red lesions was higher than in black lesions, and further evidence indicated that the inflammation and cell proliferation in the endometriotic cells were increased but apoptosis was inhibited by the activity of NF-κB [5,19,20]. In addition, TLR4 participated in the activation of the NF-κB signaling pathway, which was activated by lipopolysaccharide (LPS) derived from Gram-negative bacteria. In innate immunity, the survival of immune cells in the face of bacterial infections or irritation is protected through the NF-κB signaling pathway [21].

It is reported that AIV has strong anti-inflammatory activity, including inhibition of NF-κB activation and downregulation of adhesion molecule expression [22]. In this study, NF-κB p65 activation and overexpression were significantly inhibited by AIV treatment and were also dramatically decreased when treated with a TLR4 inhibitor TAK-242. NF-κB signaling helped modulate the activation of p50/p65 NF-κB dimers in the regulation of innate immunity, inflammatory response, and cell survival, which were closely related to the transcription of many genes. These results indicates that AIV inhibits the NF-κB signaling pathway by interacting with TPL4.

Cytokines play vital roles in immune responses and are, therefore, potential targets of immune regulation. NF-κB signaling plays an important role in acute inflammation and regulates a variety of promoter genes such as IL-1β, IL-6, Ccl-2, and TNF-α, which are critical to the inflammatory processes. IL-1β, IL-6, and TNF-α act as the proinflammatory cytokines and immediately cause an inflammatory response when the sterile spaces of animals are infected with bacteria. Ccl-2 is an anti-inflammatory mediator that could inhibit endotoxic shock caused by bacterial sepsis. Ccl-2 reduced the production of chemokines and chemokine receptors, thereby increasing the innate immune defense. In fact, a balance between pro- and anti-inflammatory mediators is critical during acute infections.

It is well-accepted that NF-κB signaling modulates the function of inflammatory cytokines and adhesion molecules and the regulation of inflammatory responses [23,24]. Recent studies reported that AIV had anti-inflammatory activity by inhibiting NF-κB activation and adhesion molecule expression in HUVECs stimulated by lipopolysaccharide (LPS) [10]. Here, AIV pretreatment suppressed activation of inflammatory markers as evidenced by a decrease in the IL-1β, IL-6, Ccl-2, and TNF-α activity and expression both in *vivo* and in *vitro*.

CONCLUSION

The findings of this study demonstrate that EMs activates TLR4/NF-κB signaling pathway and inflammation by increasing the expressions of IL-1β, IL-6, Ccl-2, and TNF-α genes. Astragaloside IV prevents inflammatory response by suppressing TLR4/NF-κB pathway both *in vivo* and *in vitro*. These findings reinforce the

therapeutic rationale for the use of AIV in the management of EMs.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article. All liabilities related to the content of this article will be borne by the authors. Yongping Zhang and Ouping Huang designed all the experiments and revised the paper. Wei Zhang and Luxin Liu carried out the experiments, while Caiwei Xu wrote the manuscript.

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