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Anti-Inflammatory Function of Withangulatin A by Targeted Inhibiting COX-2 Expression via MAPK and NF-κB Pathways

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ABSTRACT

Withangulatin A (WA), an active component isolated from *Physalis angulata* L., has been reported to possess anti-tumor and trypanocidal activities in model systems via multiple biochemical mechanisms. The aim of this study is to investigate its anti-inflammatory potential and the possible underlying mechanisms. In the current study, WA significantly suppressed mice T lymphocytes proliferation stimulated with LPS in a dose- and time-dependent manner and inhibited pro-inflammation cytokines (IL-2, IFN-γ, and IL-6) dramatically. Moreover, WA targeted inhibited COX-2 expression mediated by MAPKs and NF-κB nuclear translocation pathways in mice T lymphocytes, and this result was further confirmed by the COX-1/2 luciferase reporter assay. Intriguingly, administration of WA inhibited the extent of mice ear swelling and decreased pro-inflammatory cytokines production in mice blood serum. Based on these evidences, WA influences the mice T lymphocytes function through targeted inhibiting COX-2 expression via MAPKs and NF-κB nuclear translocation signaling pathways, and this would make WA a strong candidate for further study as an anti-inflammatory agent. J. Cell. Biochem. 109: 532–541, 2010. © 2009 Wiley-Liss, Inc.

KEY WORDS: WITHANGULATIN A; ANTI-INFLAMMATORY; COX-2; MAPK; NF-κB

Cyclooxygenase (COX) catalyzes the synthesis of prostaglandins from arachidonic acid. So far, two isoforms of COX, which are namely COX-1 and COX-2, have been characterized. COX-1 is constitutively present in most cells and plays a role in the maintenance of normal physiological function, while COX-2 is a rate-limiting enzyme in prostaglandin biosynthesis and is responsible for increased PGE2 levels in inflamed tissues, and is not expressed in most cells under normal physiological conditions [Tilley et al., 2001; Mancini et al., 2007]. As far as we know, inappropriate up-regulation of COX-2 has been frequently observed in various premalignant and malignant tissues [Mohan and Epstein, 2003], and accumulating evidences have confirmed COX-2 as a major therapeutic target in the treatment of inflammatory disorders [Korotkova et al., 2005]. At the same time, the COX-2 expression is regulated by MAPKs in various cell types [Bradbury et al., 2004; Cheng et al., 2004], and NF-κB also plays a key role in the regulation of COX-2 expression in the different disease status [Ghosh and Karin, 2002]. So far, the role and the regulation of COX-2 expression have received considerable attentions.

Non-steroidal anti-inflammatory drugs (NSAIDs) have been widely used in the treatment of acute and chronic inflammatory diseases, which play their therapeutic effects via inhibiting COX to prevent the production of pro-inflammatory prostaglandins [Berndt et al., 2005]. However, their long-term use shows the major

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side-effects of producing gastrointestinal irritation and ulceration thought to be due to topical irritation of the epithelium, as well suppression of COX-mediated endogenous prostaglandin synthesis [Schoen and Vender, 1989; Soll et al., 1991]. Thus developing drugs which targeted inhibited COX-2 with markedly lower toxicity and higher anti-inflammatory activity is of great interest in the therapeutic application.

Physalis angulata L., a traditional Chinese herb medicine, is used worldwide for its fruits. This herb medicine is widely used in the treatment of various inflammatory disorders, especially for rheumatoid arthritis and dermatitis [Caceres et al., 1995; Ankrah et al., 2003]. Previous pharmacological studies show that the crude extract of *Physalis angulata* L. has been showed cytotoxic activity against cancer cell lines [Hsieh et al., 2006; He et al., 2007; Lee et al., 2009a] and anti-inflammatory effects [Choi and Hwang, 2003; Bastos et al., 2008]. Interesting, its fruits are edible as health wine by many in China. In our previous study, a large number of components were isolated out from *Physalis angulata* L. under anti-inflammatory activity-guided screening assay. In those compounds, Withangulatin A (WA) has been reported to show potent pharmacological activities in changing the morphology [Lee et al., 1993] and suppressing general protein synthesis in 9L rat brain tumor cells [Lee et al., 1991]. However, anti-inflammatory activity of WA has not been reported before, so this research tries to investigate its anti-inflammatory activity and probe its underlying mechanisms.

In the current study, our aim is to investigate the anti-inflammatory effect of WA on mice T lymphocytes activation through MAPKs and NF-κB nuclear translocation signaling pathways. This anti-inflammatory effect is evidenced in significantly inhibiting LPS-induced T lymphocytes proliferation, decreasing pro-inflammation cytokines (IL-2, IFN-γ, IL-6) secretion, and targeted inhibiting COX-2 expression and PGE2 production. Further mechanistic studies demonstrate that the anti-inflammatory effect of WA is closely associated with p42/p44/MAPK and p38/MAPK pathways, as well as NF-κB nuclear translocation. Ear tumefaction of xylene-induced BALB/c mice is also inhibited by WA in vivo.

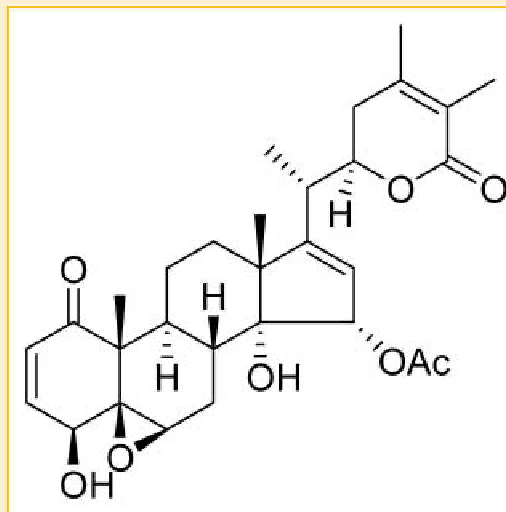

Fig. 1. Chemical structure of Withangulatin A (WA, (4β,5β,6β,14α,22R)-5,6:22,26-diepoxy-4,14 dihydroxy-1,26-dioxoergosta-2,16,24-trien-15-yl acetate).

MATERIALS AND METHODS

MATERIALS

3-[4,5-Dimethylthylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), cyclosporine A (CsA), and lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (Carlsbad, CA). [^{3}H]-thymidine (1 mCi/ml) was obtained from the Shanghai Institute of Atomic Energy (Shanghai, China). TRIzolTM reagent was product of Invitrogen (Carlsbad, CA). All cytokine ELISA kits, polyclonal antibody anti-β-actin, monoclonal antibodies anti-COX-1, COX-2 and NF-κB p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primers were synthesized by Shenggong Co., Led (Shanghai, China).

PREPARATION OF WA

WA (Fig. 1) was isolated from the root of *Physalis angulata* L., and identified on the basis of chemical and spectroscopic determination (IR, MS, and NMR). The purity of WA was determined to be 99.5% by HPLC on a waters 600E HPLC instrument with a Symmetry® C18 column (250 × 4.6 mm i.d.; 5 μm particle size), a waters 2996 PDA detector, eluting with MeOH/H_2O 40:60 ($t_R = 12.2$ min). WA was dissolved in DMSO, and the final concentration of DMSO was less than 0.1% in culture medium. The endotoxin level of solution was less than 0.5 endotoxin units (EU)/ml using a gel-clot Limulus amebocyte lysate assay.

ANIMAL AND CELL CULTURES

Male BALB/c mice (8 weeks old) were purchased from the Shanghai Experimental Animals Center (License number: SCXK 2007-0002). All mice were accustomed in our SPF standards animal laboratory for 1 week before experiments started. Animal experiments were conformed to the Guide for the Care and the Use of Laboratory Animals (1996).

Mice lymphocytes were isolated from BALB/c mice spleens [Feng et al., 2002]. Purified T lymphocytes were prepared by using immunomagnetic negative selection to deplete B cells and I-A+ antigen presenting cell [Zhu et al., 2006]. The purity of T lymphocytes was determined by flow cytometry and the purity was consistently >90%. LPS (10 μg/ml) was used to stimulate T lymphocyte activation. Jurkat cells were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and grown in Rosewell Park Memorial Institute 1640 medium containing 10% heat-inactivated bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml). Both cell types were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

MICE T LYMPHOCYTES ASSAY

Mice T lymphocytes (5×10^5 cells/ml) were cultured in triplicate separately with 10 μg/ml LPS plus either WA or CsA for 40 h. 0.5 μCi/well of [^{3}H] thymidine were added, and cells were incubated for another 8 h and then harvested with glass fiber filters. The

incorporated radioactivity was detected using a Beta Scintillation Counter (MicroBeta Trilux; PerkinElmer Life and Analytical Sciences, Boston, MA).

Cytotoxicity was assessed by the MTT colorimetric assay as previously described [Wu et al., 2005]. Briefly, mice T lymphocytes (1×10^6 cells/ml) were cultured in a 96-well flat-bottomed plate in triplicates separately with WA or CsA at concentrations for 44 h. MTT (500 μg/ml) was added and cells were then incubated for another 4 h. Cell viability was measured with a microplate reader (Bio-Rad, CA) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

ELISA ASSAY

Levels of IL-2, IFN-γ, and IL-6 cytokines secretion were measured in LPS-induced mice T lymphocytes incubated with WA (1.4, 2.8, and 5.6 μM) or CsA (3.2 μM). PGE2 production was measured in LPS-induced mice T lymphocytes incubated with WA at different concentrations or time. Cell supernatants were detected by commercially available ELISA kits according to the manufacturer's instructions.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION ANALYSIS

Total RNA (5 μg), which was extracted from mice T lymphocytes treated with WA at various concentrations for 48 h was applied to the access Reverse Transcriptase Polymerase Chain Reaction. Oligo-nucleotide primer sequences were as follows: mice COX-1 sense, 5′-TGG ATG GAG TGA TGC TTC-3′ and anti-sense, 5′-CTG CTT TGG GGG TAT CTC-3′ (corresponding to AF535139), mice COX-2 sense, 5′-ACAGGA GAG AAG GAA ATG GC-3′ and anti-sense, 5′-GGATTG AGG CAG TGT TGATG-3′ (corresponding to AI044905); mice GADPH sense, 5′-TCC CTC AAG ATT GTC AGC AA-3′ and anti-sense 5′-AGA TCC ACA ACG GAT ACA TT-3′ [Yoshida et al., 2002]. GAPDH was used as the load control. PCR reactions were carried out in the presence of 1.5 mM $MgCl_2$ for 30 cycles at the following temperatures and time: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 40 s; these cycles were followed by a final extension at 72°C for 10 min. The amplified PCR products were detected by electrophoresis in 1.2% agarose gel containing 0.2% ethidium bromide and then analyzed with Image Analysis software (Image Gauze 3.0.1, Fuji Photo Film, Tokyo, Japan).

WESTERN BLOT ANALYSIS

Whole cell extracts, cytosolic, and nuclear proteins were extracted [Rsiaf et al., 2007] respectively from mice T lymphocytes co-treated at various concentrations of WA for 48 h. Protein (50 μg) was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, protein was transferred to nitrocellulose membranes, and then the membranes were incubated with COX-1, COX-2, and NF-κB p65 antibody. β-actin was used as the loading control. Immunoreactive bands were detected by incubating with IgG antibodies conjugated with alkaline phosphatase visualized with BCIP/NBT substrate reagent (Promega, Madison, WI).

LUCIFERASE REPORTER GENE ASSAY

A fragment of 1,334 bp (−1747 to −413, from ATG) and 545 bp (−611 to −66, from ATG) from the human COX-1 5′-flanking region, the incorporation of *Kpn*I and *Hin*dIII restriction sites, as well as from the human COX-2 5′-flanking region, the incorporation of *Bgl*II and *Hin*dIII restriction sites were amplified from genomic DNA isolated from HeLa cells. The amplified products were then ligated and joined into a dual luciferase reporter vector pGL-3-Basic (BD Biosciences Clontech, Palo Alto, CA).

Jurkat cells were transiently transfected with 4 μg hCOX-1 and hCOX-2 reporter plasmid and 1 μg Renilla luciferase control vector pRL-TK (Promega) respectively in LipofectAmine 2000™ (Invitrogen Life Technologies). Sixteen hours later, the medium was removed and the cells were treated with WA for another 24 h. The promoter activities of the hCOX-1 and hCOX-2 were represented by firefly luciferase light units, which normalized with the light units of Renilla luciferase. The two luciferases were measured with Dual-Luciferase Assay Kit (KenReal, China) using a FB12 Luminometer (Berthold Detection System).

EVALUATION OF XYLENE-INDUCED MICE TUMESCENCE MODEL

Ear-swelling response in mice is a classic inflammatory experimental animal model. Briefly, WA was intraperitoneal injected into mice at three different doses (5, 10, and 20 mg/kg) separately for 5 days; meanwhile, mice treated with CsA (20 mg/kg) served as the positive group. On the 5th day, the left ear was wiped with xylene after 1 h administration; 40 min later, mice blood specimens of all groups were collected. Level of IL-2 and IL-6 was measured by ELISA kits. Inhibition of ear tumescence extent was calculated as follows: Degree of ear swelling $= (M_{left\ ear} - M_{right\ ear})/M_{mice}$.

STATISTICAL ANALYSIS

The results are expressed as the means ± standard deviation (SD). One-way ANOVA was used to determine significance between groups where appropriate $P < 0.01$ was considered significant.

RESULTS

WA INHIBITS REDUCES LPS-INDUCED MICE T LYMPHOCYTES PROLIFERATION

Cell proliferation is one of the most outstanding hallmarks of T lymphocyte activation. To verify the anti-inflammatory of WA on the inhibitory effect of mice T lymphocytes proliferation, we firstly examined the effect of WA on mice T lymphocytes proliferation stimulated by LPS using the [^{3}H] thymidine incorporation method. Mice T lymphocytes showed the high proliferative responses (42×10^3 cpm) to LPS stimulation (Fig. 2), and the response was impaired after WA (17×10^3 cpm) and CsA (18×10^3 cpm) treatment in LPS-induced T lymphocytes, as half inhibitive concentration (EC_{50}) for WA and CsA was 2.89 and 3.22 μM respectively (Fig. 2A). Moreover, the results illustrated the time-dependent inhibitory effect of WA on the proliferation of LPS -induced T lymphocytes (Fig. 2B). In addition, to determine whether this anti-proliferative effect of WA on mice T lymphocytes results from the cytotoxic effect, the cytotoxicity experiments were performed using the MTT method. The results clearly showed no marked cytotoxicity of WA at

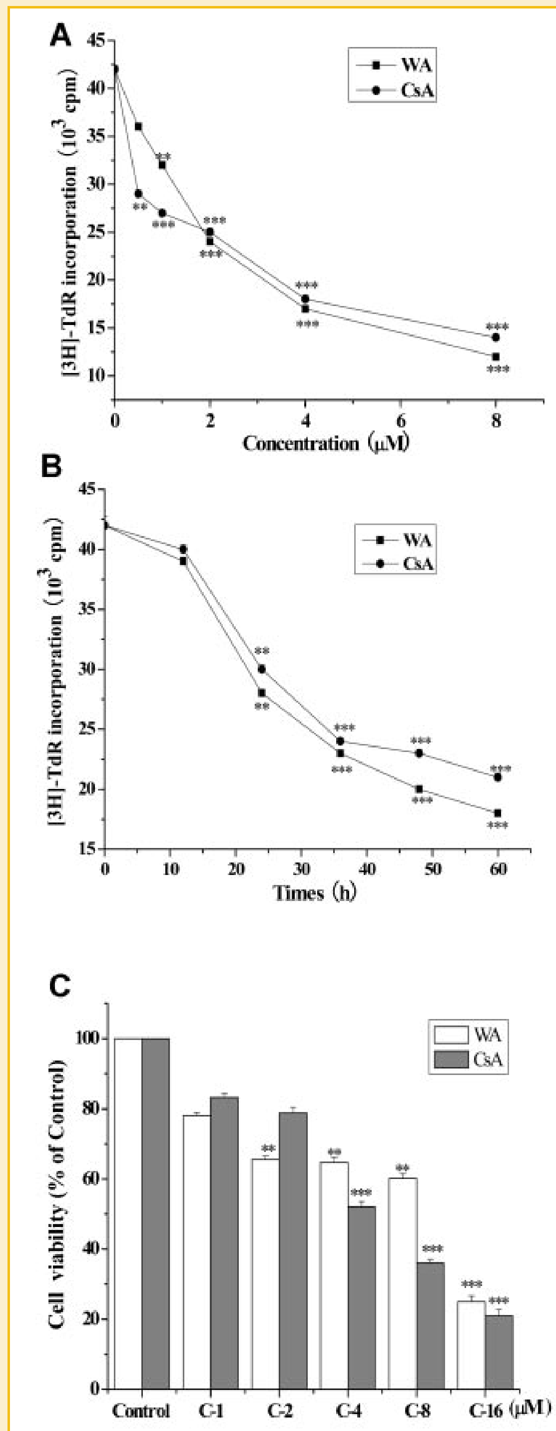

Fig. 2. Inhibition of Withangulatin A (WA) in mice T lymphocytes proliferation. A: LPS-induced mice T lymphocytes were cultured with various concentrations of WA or CsA for 48 h; cultures were pulsed with 0.5 μCi/well [^{3}H] thymidine for the final 8 h to access [^{3}H] thymidine incorporation. B: Time-dependent inhibitive effect curve of WA in LPS-induced mice T lymphocytes. Cells were cultured with LPS in the presence of WA (2.8 μM) or CsA (3.2 μM) for different hours. C; Cytotoxicity evaluation of WA and CsA in mice T lymphocytes. Cells were cultured with various concentrations of WA and CsA for 48 h. Results were representative of three independent experiments. Significant differences from LPS group were indicated by $^{**}P < 0.01$, $^{***}P < 0.001$.

the indicated concentrations, which covered the range of effective concentrations of WA used in the anti-proliferation experiments, and even the data also indicated that WA was less cytotoxic than CsA with IC_{50} as 10.28 and 4.62 μM (Fig. 2C).

WA REVERSES THE TH1 POLARIZATION IN VITRO

IL-2, IFN-γ, and IL-6 are released upon T lymphocytes activation. Mechanistic studies indicate that suppression of pro-inflammation cytokines (IL-2, IFN-γ, and IL-6) secretion is involved in the anti-inflammatory effect of WA on mice T lymphocytes. WA performed dose-dependent inhibitory effect on IL-2, IFN-γ, and IL-6 in LPS-activated T lymphocytes (Fig. 3), even though the extent of reduction in the production of IL-2 and IFN-γ was greater significant than IL-6. For IL-2, the production in LPS-induced group was 610 pg/ml, it fell sharply to 65 pg/ml in WA high group. For IFN-γ, the production was from 760 to 80 pg/ml in WA high group. Moreover, the production of IL-6 was reduced from 480 to 150 pg/ml in WA high group. Thus WA inhibited pro-inflammation cytokines such as IL-2, IFN-γ, and IL-6 to reverse the Th1 polarization.

WA INHIBITS PGE2 PRODUCTION AND COX-2 EXPRESSION THROUGH p42/p44/MAPK AND p38/MAPK PATHWAYS IN MICE T LYMPHOCYTES

Incubation of mice T lymphocytes with WA (1.4, 2.8, 5.6 μM) produced concentration-dependent reduction in PGE2 production. When the cells were incubated with WA (2.8 μM), the concentration of PGE2 in the culture medium was almost maintained at a background level similar to that in the unstimulated cells (Fig. 4A). Moreover, the results illustrated the time-dependent inhibitory effect of PGE2 production in LPS-induced mice T lymphocytes by WA, 48 h later, the inhibitory effect of PGE2 production reached a steady state (Fig. 4B).

In order to clearly elucidate the mechanisms of the inhibitory effect of WA on PGE2 production, we further determined the effects of WA on COX-1 and COX-2 expression in LPS-induced mice T lymphocytes. The effect of WA on COX-2 mRNA expression shown in Figure 4C was in line with that on COX-2 protein expression, which showed an evident effect on reducing COX-2 expression and the inhibition was more significant in WA high group, while the expression level of COX-1 did not change markedly. Furthermore, WA reduced COX-2 protein level in a time-dependent manner at 2.8 μM, it decreased sharply after 36 h and reached the lowest level after 48 h (Fig. 4D). Interesting, WA also inhibited COX-2 protein levels in Jurkat cell in a dose-dependent manner (Fig. 4E). Meanwhile, both PD98059 and SB203580 similarly reversed COX-2 expression at protein levels in the LPS-induced mice T lymphocytes treated with WA, which showed that suppression of p42/p44 and p38/MAPK activities, but not of JNK/MAPK activity, abolished the WA-reduced COX-2 expression (Fig. 4F). These data suggested p42/p44/MAPK and p38/MAPK pathways participated in WA-reduced COX-2 expression in mice T lymphocytes.

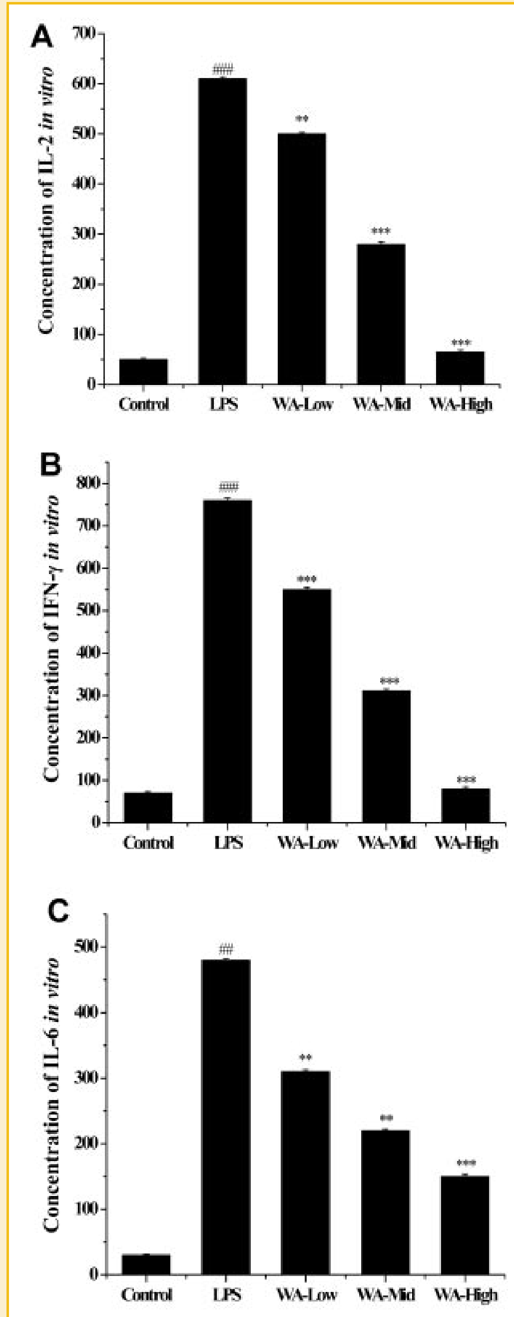

Fig. 3. The mice T lymphocytes were pretreated with LPS for 4 h and cultured in 6-well culture plates coated with different concentrations of Withangulatin A (1.4, 2.8, and 5.6 μM) for 48 h to determine IL-2, IFN-γ, and IL-6 (A–C) by ELISA. Data were representative of three independent experiments. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared with control group and $^{**}P<0.01$, $^{***}P<0.001$ compared with LPS group.

WA INHIBITS NUCLEAR FACTOR κB (NF-κB) p65 NUCLEAR TRANSLOCATION

Whether WA-inhibited COX-2 expression was related to NF-κB p65 nuclear translocation is studied in this research. WA inhibited nuclear translocation of NF-κB p65, as increasing amounts of NF-κB p65 in the cytosol and correspondingly decreasing amounts in the nuclear extract in a dose-dependent manner (Fig. 5A). In other words, there appeared to be NF-κB-dependent pathways which regulated COX-2 expression and subsequent PGE2 release. Furthermore, WA-inhibited NF-κB nuclear translocation was not abrogated by the inhibition of p38/MAPK, p42/p44 MAPK, and JNK/MAPK in LPS-stimulated mice T lymphocytes (Fig. 5B).

WA REGULATES TRANSCRIPTIONAL ACTIVATION OF COX-1 AND COX-2 GENE

Jurkat cells were transfected with hCOX-1 and hCOX-2 reporter construct, and luciferase activity was subsequently measured to reveal the transcriptional activation of COX-1 and COX-2 gene. WA exhibited adverse effect on the transcriptional activation COX-2 gene in a dose-dependent manner, but obvious effect on the COX-1 gene was not observed (Fig. 6A). WA at 5.6 μM significantly reduced COX-2 luciferase activity by 37% compared to an empty vector control. The time-dependent activity was also addressed, and the results in Figure 6B illustrated the time-dependent inhibitory effect of WA on COX-2 luciferase activity.

WA INHIBITS XYLENE-INDUCED MICE EAR TUMEFACTION

According to the results (Fig. 7), WA evidently inhibited xylene-induced ear tumefaction in BALB/c mice, and it was more effective than CsA at middle and high dose, fortunately, the weight of mice in any groups was not significantly varied. Meanwhile, WA inhibited IL-2 and IL-6 secretion compared with the control group, which the middle and high group were more effective than the positive group, so the above results showed that WA showed significant inhibition of mice ear tumefaction, and decreased the production of pro-inflammation (IL-2 and IL-6) secretion in the experimental blood specimens. In other words, WA possessed potential anti-inflammatory activity in vivo.

DISCUSSION

In this report, we demonstrate that WA restricts the overactive T lymphocytes and pro-inflammation cytokines to reverse Th1 polarization in vitro; In addition, WA significantly inhibits COX-2 expression and PGE2 production through p42/p44/MAPK and p38/MAPK pathways, as well as NF-κB nuclear translocation. The selective inhibition of COX-2 is further confirmed by the COX-2 luciferase reporter assay. Xylene-induced BALB/c mice ear tumefaction is inhibited by WA in vivo. Taken together, these data argue that WA might be a potential drug against inflammatory diseases, and is possible responsible for the anti-inflammatory properties of *Physalis angulata* L.

T lymphocytes-mediated inflammatory diseases are associated with T lymphocytes overactivation or unbalanced Th1-/Th2-type immune responses [Schulze-Koops and Kalden, 2001]. WA inhibited LPS induced-T lymphocytes proliferation in dose- and time-dependent manner; the results suggested WA could restrict the overactive T lymphocytes. Moreover, WA decreased the concentration of pro-inflammation cytokines (IL-2, IFN-γ, and IL-6) to reverse the Th1 polarization in vitro and the production of IL-2 and IL-6 in mice blood in vivo. So the anti-inflammatory function of WA is

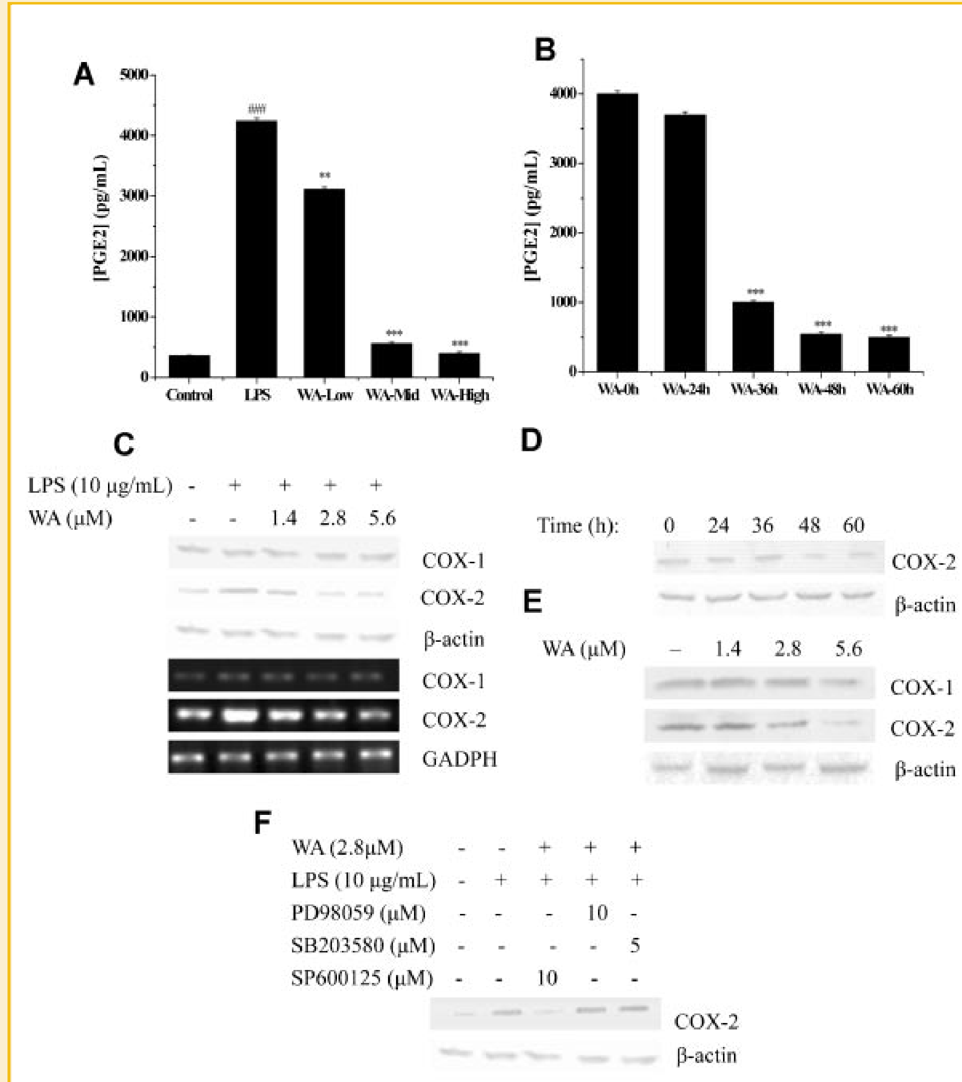

Fig. 4. Withangulatin A (WA) inhibited PGE2 production in LPS-induced mice T lymphocytes. A: Mice T lymphocytes were pretreated with LPS for 4 h and cultured in 6-well culture plates coated with different concentrations of WA (1.4, 2.8, and 5.6 μM) for 48 h to determine PGE2 production. B; Time-dependent effect of PGE2 production by WA. C; The levels of COX-1 and COX-2 protein and mRNA in cells were analyzed by western blot and RT-PCR; β-actin and GADPH were shown as the loading control. D: Time-course experiment on the effects of COX-2 protein levels by WA. E: COX-2 protein expression in Jurkat cells treated with various concentrations of WA. F: Effect of MAPK inhibition on WA-reduced COX-2 protein expression in LPS-induced mice T lymphocytes. Cells were pretreated with the indicated concentrations of PD98059, SB203580 or SP600125 for 1 h and then treated with 2.8 μM WA for 48 h. The levels of COX-2 protein were detected by Western blot. The data were representative of three independent experiments. $^{###}P < 0.001$ compared with control group and $^{**}P < 0.01$, $^{***}P < 0.001$ compared with LPS group.

closely associated with its ability to influence the pro-inflammation cytokines secretion. Whether WA possesses effect on other cytokines-mediated inflammatory diseases needs further investigation.

Based on recent findings, PGE2 is implicated as a major contributor to the inflammatory function [Funk, 2001], and COX-2 is responsible for the catalysis of PGE2 production [Lee et al., 2009b]. Although there are well known that COX-1, COX-2, and PGE2 biosynthesis are induced in activated monocytes, the high expression of COX-1, COX-2, and PGE2 biosynthesis are also produced in T lymphocytes [Íñiguez et al., 1999; de Gregorio et al., 2001; Tilley et al., 2001]. So one of the major contributions of this study is to clarify that PGE2 production and COX-2 expression could be selective inhibited by WA in LPS-induced T lymphocytes. In our study, PGE2 production and COX-2 expression were significantly induced after LPS stimulus in mice T lymphocytes, and they were inhibited by WA in a dose-dependent fashion, while COX-1 expression is not influenced. As shown in Figure 4, in the presence of WA at 2.8 μM, the PGE2 levels and COX-2 expression returned to the basal non-stimulated levels.

This is confirmed by the second important finding that WA affects COX-1 and COX-2 promoter activity. However, because mice T lymphocytes could be transfected poorly, we select Jurkat cell as a model to be transfected by hCOX-1 and hCOX-2 promoter. The results showed that WA inhibited transcriptional activation of COX-2 promoter in dose- and time-dependent manner, however,

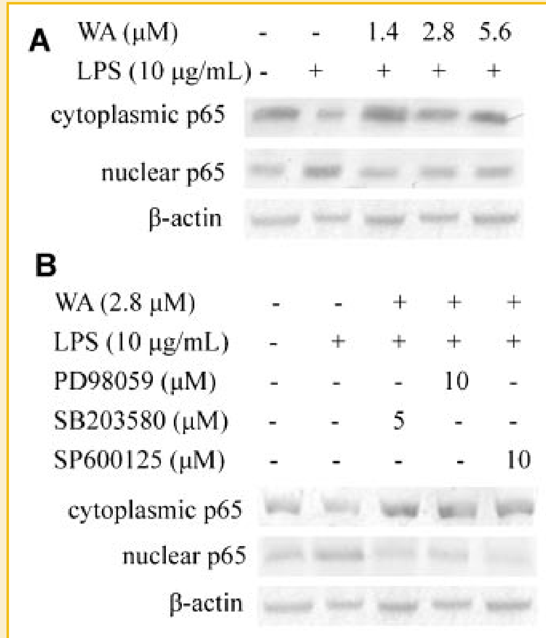

Fig. 5. Inhibitory NF-κB nuclear translocation effect in mice T lymphocytes by Withangulatin A (WA). Mice T lymphocytes were pretreated with LPS for 4 h, and then cultured with different concentrations of WA (1.4, 2.8, and 5.6 μM) for 48 h. A: Cytoplasmic and nuclear extracts were extracted and then analyzed by Western blot with anti-p65 antibody. B: Effect of MAPK on WA-inhibited NF-κB nuclear translocation. Cells were pretreated with the indicated concentrations of PD98059, SB203580, or SP600125 for 1 h and then treated with 2.8 μM WA for 48 h. The levels of p65 antibody protein were detected. The data were representative of three independent experiments.

no function on COX-1 was noticed. According to COX-1 and COX-2 reporter construct model and the former results, WA is expected to be a promising anti-inflammatory drug with lower side-effect than NSAIDs, at the same time, the dual-luciferase report gene of COX-1/2 model also could be used to analysis the molecular mechanism of target inhibiting COX-2 expression.

MAPKs pathway integrates multiple signals from various second messengers, resulting in cellular survival, proliferation, or target gene expression, as well as in autoimmune and inflammatory diseases [Schett et al., 2000]. In addition, recent paper indicates that p42/p44 MAPK plays a part in various stimuli such as LPS and ATP-induced expression of COX-2 [Luo et al., 2003]. The involvement of MAPKs in macrophage activation by LPS has been demonstrated [Hambleton et al., 1996]. PD98059 specifically inhibits the upstream kinase MEK of p42/p44 and does not inhibit other MAPKs, while SB203580 and SP600125 specifically inhibit p38 and JNK separately [Finn et al., 2005]. These properties make them useful tools to study the roles of MAPKs in COX-2 expression in LPS-stimulated mice T lymphocytes. To further clarify the mechanism of selectively inhibiting COX-2 expression, which is regulated through a complex signaling network [Chun and Surh, 2004], and the COX-2 gene promoter bears multiple regulatory elements that interact with NF-κB, activator protein-1 (AP-1), and cyclic AMP-response element binding protein (CREB) [Tanabe and Tohnai, 2002; Nie et al., 2003], our third important finding is that MAPKs pathway and

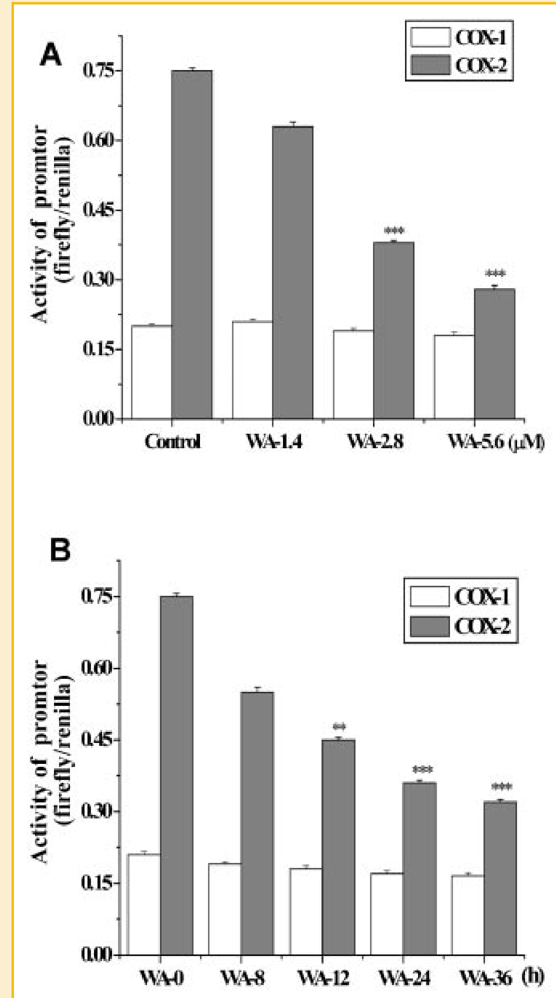

Fig. 6. Inhibitory COX-1 and COX-2 promoter activity effect in Jurkat cell by Withangulatin A (WA) using Dual-Luciferase Assay Kit. A; Jurkat cells were transfected with hCOX-1 of hCOX-2 reporter plasmid and Renilla luciferase control vector pRL-TK Vector in LipofectAmine 2000^{TM}, and then treated with various concentrations of WA (1.4, 2.8, and 5.6 μM). B: Time-dependent inhibition of COX-1 and COX-2 promoter activity by WA in Jurkat cells. Results were representative of three independent experiments. Significant differences were indicated by $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group.

NF-κB nuclear translocation could be impeded by WA and then subsequently decreased COX-2 expression and PGE2 production. As we expected, chemical inhibition of p42/p44 MAPK and p38 MAPK reversed COX-2 expression in LPS-induced mice T lymphocytes treated with WA, as both kinases are considered as important regulators of pro-inflammatory signaling pathways. This result is in line with previous observations, which demonstrated a pivotal role of the p42/p44 MAPK and p38/MAPK pathway involved in the regulation of COX-2 expression [Nagano et al., 2002]. NF-κB activation could regulate production of a series of pro-inflammatory cytokines and then potentate the upset and progression of inflammatory and autoimmune conditions [Hayden et al., 2006]. Regulation of NF-κB activation is a promising area in inflammatory diseases that could potentially give rise to targets for therapeutic intervention [Gilmore, 2006]. Based on our findings, WA prevented

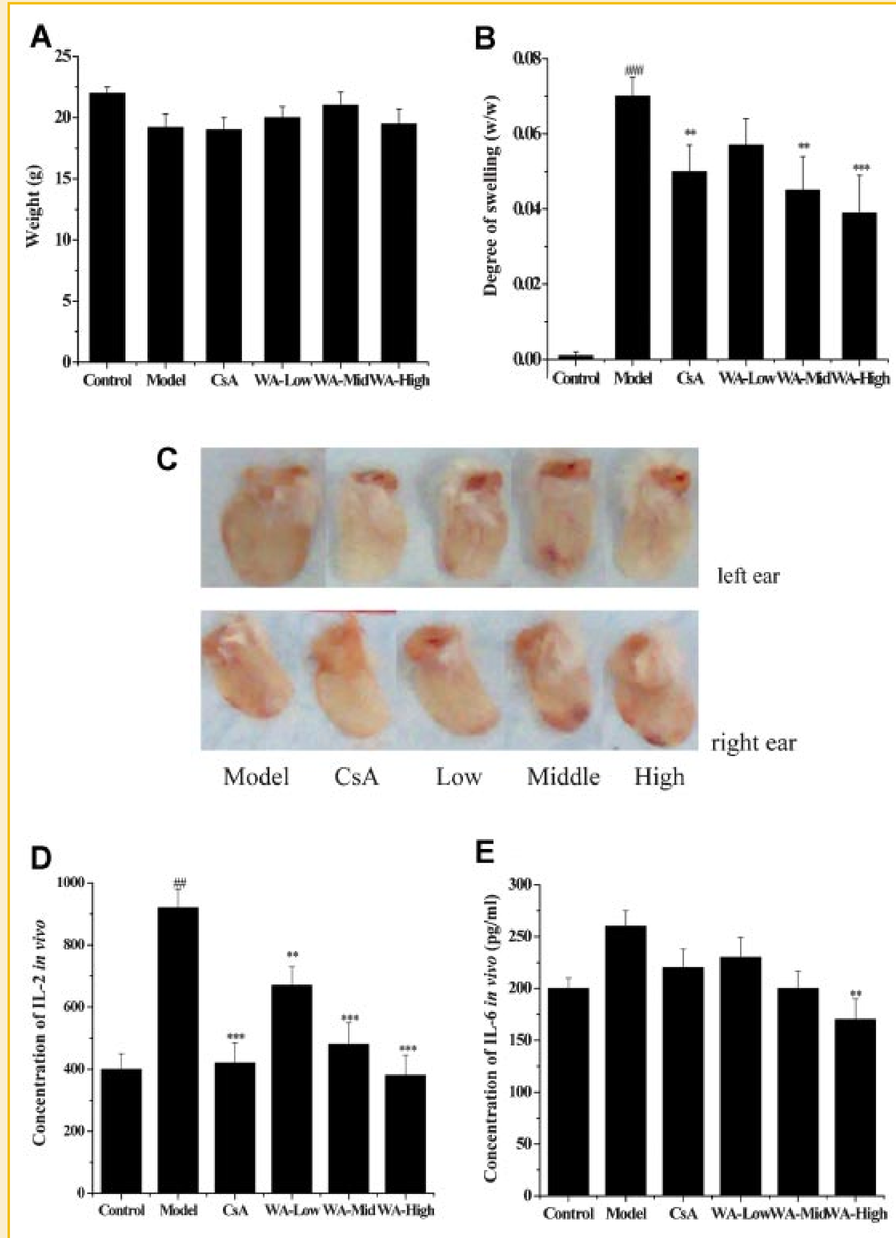

Fig. 7. Effect of Withangulatin A (WA) on mouse xylene-induced ear tumefaction. Mice were treated with WA for 5 days. A: Effect of WA on mice weight. B: Effect of WA on mice ear-swelling. C: Photo of the mice ear treated by WA. D,E: Cytokines IL-2 and IL-6 in the blood serum of experimental animals were detected by ELISA. Data were presented as means $\pm$ SD. Significant differences were indicated by $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with control group and $^{**}P < 0.01$, $^{***}P < 0.001$ compared with model group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

NF-κB nuclear translocation in the LPS-induced mice T lymphocytes (Fig. 5), to correlate with the abolishing COX-2 expression and reducing PGE2 production. These data implicate that MAPKs and NF-κB nuclear translocation seemed to be essentially involved in COX-2 expression in LPS-induced mice T lymphocytes by WA. However, NF-κB nuclear translocation in LPS-stimulated mice T lymphocytes was not reversed by PD98059, SB203580, or SP600125. One possible explanation is that MAPKs possibly regulated some other transcription factors, such as AP-1 and CREB, of course, these hypothesis need further investigation. All of all, our results suggest that the involved mechanisms could be combined and responsible for the anti-inflammatory effects of WA (Fig. 8).

Taken together, WA could inhibit COX-2 expression selectively through p42/p44 MAPK and p38/MAPK pathway and NF-κB p65 nuclear translocation, then to reverse Th1-type polarization; these results are further supported by decreasing the production of IL-2 and IL-6 in mice blood serum in a dose-dependent manner, which could be the mechanism to explain the anti-inflammatory effect of WA in vitro and in vivo. In the light of the present study, WA merits further consideration as an edible phytochemical with potential therapeutic application in pharmacology for drug development.

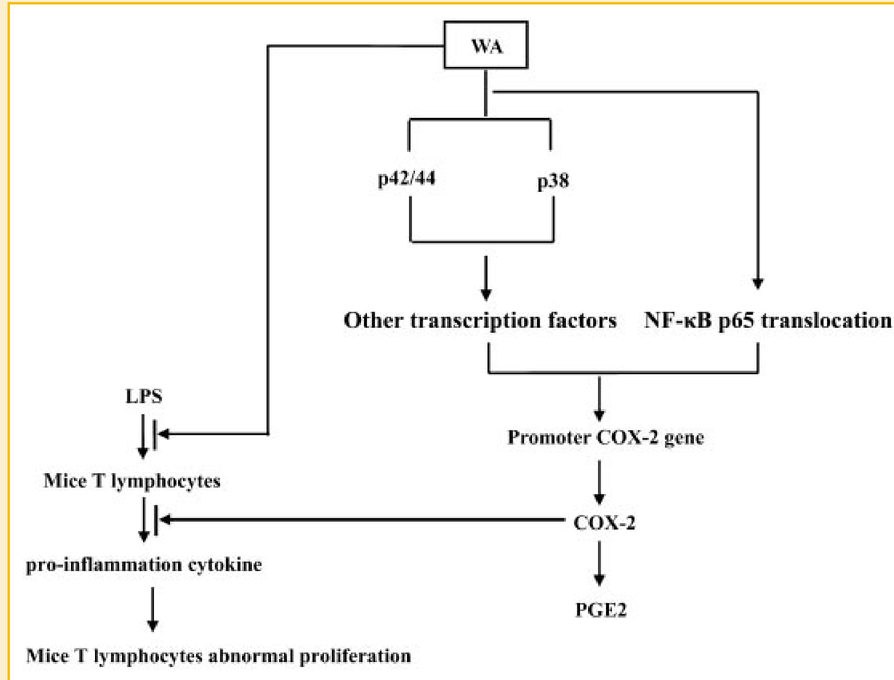

Fig. 8. Hypothetical mechanism of Withangulatin A (WA) in mice T lymphocytes. WA could inhibit the expression of COX-2 through p42/p44/MAPK, p38/MAPK, and inhibited nuclear translocation of NF-κB to restrict mice T lymphocytes from over-expression and decrease the pro-inflammation cytokine to reverse Th1-type polarization.

Furthermore, through the study of WA which is isolated from *P. angulata*, we get a further understanding the possible therapeutic effects of edible *P. angulata* in treatment of inflammatory disorders, such as rheumatoid arthritis, dermatitis, hepatitis, etc.

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