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EXPERIMENTAL RESEARCH

Anti-Inflammatory Mechanism of Total Glycosides of Acanthopanax Giraldii

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ABSTRACT **Objective:** To study the anti-inflammatory mechanisms of total glycosides of Acanthopanax Giraldii (TGA). **Methods:** The changes of prostaglandin E_2 (PGE_2), tumor necrosis factor (TNF-α), nitric oxide (NO), and expressions of COX-1 mRNA and COX-2 mRNA in BALB/c mouse macrophages were observed by the radioimmunoassay, ELISA and nitric acid reduction and RT-PCR in the presence or absence of TGA. **Results:** (1) TGA could significantly decrease the production of PGE_2 and NO in mouse peritoneal macrophages. The inhibitory rate to LPS-induced PGE_2 production was 87% (TGA 100 mg/L, $P<0.05$, *vs*. LPS) and 62% (TGA 20 mg/L, $P<0.05$, *vs*. LPS), respectively. The inhibitory rate of NO production in mouse peritoneal macrophages was 49% (TGA 100 mg/L, $P<0.05$, *vs*. LPS) and 21% (TGA 20 mg/L, $P<0.05$ *vs*. LPS), respectively. TGA could not inhibit LPS-induced TNF-α production in mouse peritoneal macrophages. (2) TGA also inhibited the expression of COX-1 and COX-2 mRNA in RAW264.7 cells. The inhibitory rate of TGA to COX-1 mRNA was 22% (TGA 100 mg/L, $P<0.05$, *vs*. blank). The inhibitory rate of TGA to COX-2 mRNA was 55% (TGA 20 mg/L, $P<0.05$, *vs*. LPS) and 100% (TGA 100 mg/L, $P<0.01$ *vs*. LPS), respectively. **Conclusion:** The anti-inflammatory mechanisms of TGA for inhibiting the production of NO and PGE_2 are through inhibiting COX-2 mRNA expression without TNF-α changes.

KEY WORDS total glycosides of Acanthopanax Giraldii, prostaglandin E_2, nitric oxide, tumor necrosis factor, COX mRNA

The rootstock extracts of *Acanthopanax Giraldii Harms* (AGH) have been used for the treatment of rheumatoid arthritis (RA). The main constituents of AGH were sesquiterpenes, organic acid and alkyl hydrocarbons[1]. The results of earlier reports show that total glycosides of *Acanthopanax Giraldii* (TGA), the extract of AGH, could effectively inhibit air-pouch acute inflammation in the rat model[2, 3], prevent and treat adjuvant arthritis in rats[4, 5], and decrease the content of prostaglandin E_2 (PGE_2) in inflammatory tissues induced by a variety of stimuli in rats[6-8]. Although the anti-inflammatory effect of TGA has been indicated, its precise mechanisms, however, have not been completely delineated.

Pathogenesis studies have revealed the primary involvement of cellular immune responses, such as a decrease in T lymphocyte proliferation and functions of suppresser T cells[9], in RA development. It is known that PGE_2 is produced widely in normal tissues and plays an important role in supporting normal physiological functions. Nevertheless, excessive production of PGE_2 has been associated with many pathologic processes and plays a critical role in eliciting the signs and symptoms of inflammation[10,11]. Two isoforms of cyclooxygenase (COX), designated as COX-1 and COX-2, have been known. Both catalyze the biosynthesis of prostaglandins from arachidonic acid. COX-2 expression increases in inflammatory tissues and represents an important step in the inflammatory process[12,13].

Previous studies show that nitric oxide (NO) levels in the synovia are significantly increased in RA patients. Increased expression of inducible nitric oxide synthase (iNOS) is associated with inflammatory responses such as septic shock and RA[14]. TNF-α, mainly generated by macrophages, is another toxic cytokine involved in RA pathological processes. In the nuclear factor-κB (NF-κB) activation pathway, TNF-α degrades the inhibitor of NF-κB, which allows

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the NF-κB heterodimer to migrate to the nucleus and up-regulate the expression of pro-inflammatory factors, including COX-2 gene and iNOS gene[15].

In the present study, the changes associated with a variety of inflammatory mediators were evaluated to delineate the anti-inflammation mechanism of TGA and to provide the proof of the target points, on which TGA exerts its effects.

METHODS

Animals

Female BALB/c mice (20-25 g) were purchased from the Experimental Animal Center, Sun Yat-sen University, Guangzhou, China. Only water was offered to the animals 24 h before the experiment.

Cells

RAW264.7 cells were provided by the Cell Bank, Sun Yat-sen University (Guangzhou, China).

Drugs and Reagents

AGH, collected from Gansu province (China), was purchased from the Zhixin Medicinal Material Co., Ltd. (Guangzhou, China) and identified by Dr. YANG De-po, the School of Pharmaceutical Sciences, Sun Yat-sen University. Trizol, phenol and chloroform extractions were obtained from Invitrogen, USA. Moloney murine leukemia virus-reverse transcriptase (M-MLV-RT), random primers, dimethyl sulfoxide (DMSO) and RPMI-1640 were obtained from Promega, USA. The Griess reaction kit was produced by Jianchen Co., Ltd., Nanjing, China. TNF-α kit and arachidonic acid were obtained from the Jingmei Bio. Tech Co., Ltd., China. Dexamethasone, indomethacin, N-nitro-L-arginine methyl ester (L-NAME) and lipopolysaccharide (LPS) were obtained from Sigma Chemical Co., USA. All other chemicals were of analytical reagent grade. Tested samples were prepared by dissolving them in DMSO and diluted by RPMI-1640 (the final concentration of DMSO was less than 2.5‰). COX-1 and COX-2 primers were synthesized by Bioasia Biotechology Co., China.

Preparation of TGA

Dried powder, obtained from the root-bark of AGH, was extracted with 95% ethanol, filtrated and then concentrated in vacuum at 65 ℃. The residue was extracted in saturated NaCl solution for many times and then the aqueous solution was extracted three times with water-saturated n-butanol. The combined n-butanol phase was washed three times with saturated NaCl solution. Then, it was concentrated in vacuum around 65 ℃ to obtain the crude TGA (yield: 0.16% W/W). It was dried in vacuum and identified as total glycosides by Molish reaction and Libatmann reaction. Eleutheroside E, the richest component in TGA detected by RT-HPLC, was regarded as the quality control.

Cell Culture

Paraffin-elicited macrophages were harvested 3-4 days after intraperitoneal injection of 1 mL sterile paraffin to mice and isolated as reported previously[16]. Shortly, peritoneal cells obtained from 3-4 mice were mixed and centrifugated at 170× g for 10 min at 4 ℃. The cell pellets were washed two times with pre-cold PBS, re-suspended in complete media and incubated for 2 h. Non-adherent cells were then removed by washing the plates three times with PBS, and the adherent cells were cultured in RPMI-1640 without serum. Almost all adherent cells were macrophages as assessed by Giemsa staining. Cell viability was examined by trypan blue dye exclusion.

Mice macrophages and RAW264.7 cells were cultured in RPMI-1640 medium with 10% heat-inactivated fatal bovine serum, 100 U /mL penicillin and 0.1 mg/mL streptomycin sulfate, in an incubator at 37 ℃, 5% CO_2 and 95% humidity. The cytotoxic effects of TGA were evaluated in the absence or presence of LPS by the MTT assay. TGA did not affect the cell viability at the concentrations of 4 mg/L to 250 mg/L regardless of the presence of LPS.

Effects of TGA on LPS-Induced PGE_2 in Peripheral Macrophages

The five groups used in the cell experiments were as follows: the control group, 10 μg/mL LPS group (the final concentration), indomethacin group (the final concentration of 10^{-7} mol/L indomethacin + 10 μg/mL LPS) and TGA groups (20 mg/L TGA +10 μg/mL LPS, 100 mg/L TGA +10 μg/mL LPS).

Mouse peripheral macrophages were plated in 24-well plates (2×10^6 cells per well in a volume of 1 mL), and then 300 μmol/L aspirin was added to irreversibly block the expression of COX-1[17]. After 20-h incubation, plates were washed three

times to remove non-adherent cells and any residual aspirin. The adherent cells (monocytes) were incubated at 37 ℃ with 10 μg/mL LPS in the presence or absence of TGA for 20 h. The 10^{-7} mol/L indomethacin was used as a positive inhibitor. The 10 μmol/L arachidonic acid was added in each well in 30 min before the incubation was finished. After this period, the culture medium was centrifuged at 1 500 g for 3 min at 4 ℃ and the concentration of PGE_2 in the supernatant was measured by a radioimmunoassay method (RIA).

Effects of TGA on COX-1 mRNA and COX-2 mRNA in RAW 264.7 Cells

The experiment of COX-1 mRNA included a marker group, a blank control group and TGA groups (20 mg/L TGA and 100 mg/L TGA, final concentrations). Experiment of COX-2 mRNA was conducted as follows: a marker group, a blank control group, a LPS group (10 μg/mL LPS, the final concentration) and TGA groups (20 mg/L TGA +10 μg/mL LPS, 100 mg/L TGA +10 μg/mL LPS as the final concentrations). The test was repeated for 3 times.

RAW264.7 cells (2×10^6 cells per well in a volume of 3 mL) at different concentrations of TGA or TGA with 10 μg/mL LPS were cultured for 20 h, respectively. Total RNA was isolated for COX-1 mRNA or COX-2 mRNA analysis. Total RNA was extracted from RAW264.7 cells by using the regent Trizol and phenol/chloroform extraction guided by the manufacturer's instructions. The RNA was quantitated and reverse transcribed with 4 μg M-MLV-RT and random primers to prepare cDNA. Reactions were conducted at 37 ℃ for 60 min, and 70 ℃ for 10 min. The polymerase chain reaction (PCR) was carried out on total cDNA with a thermocycling program of 94 ℃ for 5 min for initial denaturation, then 94 ℃ for 60 s, 55 ℃ for 55 s, 72 ℃ for 60 s and 30 cycles totally, and to final extension at 72 ℃ for 7 min.

The sequences of the primers were referred to the references[10,18-20]: COX-1 sense: 5'-AGG AGA TGG CTG CTG AGT TGG-3', COX-1 anti-sense: 5'-AAT CTG ACT TTC TGA GTT GCC-3'; COX-2 sense: 5'-GAT CGA AGA CTA CGT GCA ACA-3'; COX-2 anti-sense: 5'-GCT CAG TTG AAC GCC TTTT-3', GADPH sense: 5'- TCC ACC ACC CTG TTG CTG TAG and GADPH anti-sense: 5'-GAC CAC AGT CCA TGA CAT CACT-3'. GADPH was amplified as an internal standard. The sizes of the PCR products for COX-1, COX-2 and GADPH were 602, 821 and 450 bp, respectively. Relative gray scale=OD value of objective gene/OD value of reference GADPH. Express rates of COX-1/GAPDH mRNA and COX-2/GAPDH mRNA were both 100%. The measurements were performed in triplicate.

Effects of TGA on NO and TNF-α in Mouse Peritoneal Macrophages

The experiment design included a blank group, a LPS group (10 μg/mL), TGA groups (100 mg/L TGA +10 μg/mL LPS, 20 mg/L TGA +10 μg/mL LPS, 4 mg/L TGA +10 μg/mL LPS), a dexamethasone group (1.0 μmol/L dexamethasone +10 μmol/L LPS) as positive control for the TNF-α assay, and a L-NAME group (100 μmol/L L-NAME +10 μmol/L LPS) as positive control for the NO assay.

Mouse peritoneal macrophages were cultured in 24-well plates (2×10^6 macrophages per well in the volume of 1 mL) at 37 ℃ for 24 h. Then, the plates were washed twice by PBS to remove non-adherent cells, and RPMI-1640 was added and cells were incubated for 24 h. The medium was then changed to the one containing LPS (10 μg/mL) in the presence or absence of TGA for 20 h, 100 μmol/L L-NAME and 1.0 μmol/L dexamethasone were used as positive inhibitors of NO and TNF-α, respectively. Nitrite accumulation, an indicator of NO synthesis, was measured in the culture medium by the Griess reaction kit according to the manufacturer's instructions. TNF-α levels in the macrophage culture medium were quantified by the ELISA kit according to the manufacture's instructions. The measurements were performed in triplicate.

Statistical Analysis

Data are expressed as mean ± standard deviation. Differences between groups for continuous variables were evaluated with analysis of variance (ANOVA) and differences between two groups were analysed using unpaired Student's *t*-test. Statistical significance was set as $P< 0.05$.

RESULTS

Effects of TGA on LPS-Induced PGE_2 in Mouse Peritoneal Macrophages

PGE_2 levels were significantly increased in mouse peritoneal macrophages induced by LPS as compared with the control group ($P<0.01$). TGA

at 100 mg/L and 20 mg/L obviously inhibited the up-regulation of LPS-induced PGE_2 production, respectively (P<0.01, Table 1).

Table 1. Effects of TGA on LPS-Induced PGE_2 in Mouse Peritoneal Macrophages ($\bar{x} \pm s$)

Group	n	PGE_2 (ng/L)
Control	6	7.59 ± 3.54
LPS	6	25.28 ± 5.74*
LPS+ indomethacin	6	6.31 ± 2.01△ (107%)
LPS+TGA 20 mg/L	6	14.26 ± 3.69△ (62%)
LPS+TGA 100 mg/L	6	9.87 ± 3.12△ (87%)

Notes: *P<0.01, compared with the control group; △P< 0.01, compared with the LPS group; the data in the parentheses were inhibitory rates

Effects of TGA on COX-1 and COX-2 Expression in RAW264.7 Cells

Compared with the blank group, the COX-1 mRNA expression was suppressed at the concentration of 100 mg/L TGA with the inhibition rate being 22 % (P<0.05), while 20 mg/L TGA did not inhibit the expression of COX-1mRNA (Figure 1).

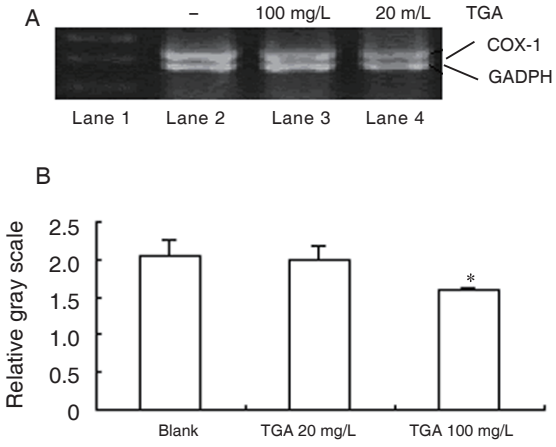

Figure 1. Effects of TGA on COX-1 mRNA Expression in RAW264.7 Cells

Notes: A: RT-PCR analysis; lane 1: DL2000 DNA marker; lane 2: RAW264.7 cell control; lane 3: TGA 100 mg/L; lane 4: TGA 20 mg/L. B: Relative gray scale value profile ($\bar{x} \pm s$, n=3), *P<0.01, compared with the blank group

LPS at 10 μg/mL obviously induced the expression of COX-2 mRNA in RAW264.7 cells as compared with the blank group. The LPS-induced COX-2 mRNA expression was suppressed by TGA at both doses (20 mg/L, P<0.05, and 100 mg/L, P<0.01, Figure 2). The inhibition rate of 100 mg/L and 20 mg/L TGA to COX-2 mRNA was 100% and 55%, respectively. The results were consistent with the inhibitory profile of TGA on PGE_2 release.

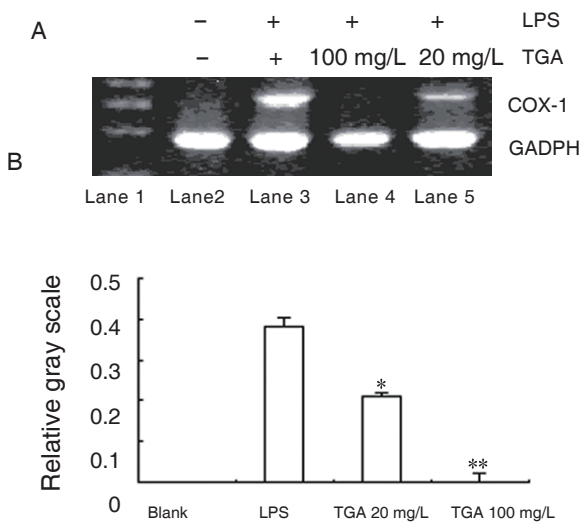

Figure 2. Effects of TGA on COX-2 mRNA Expression in RAW264.7 Cells

Notes: A: RT-PCR analysis; lane 1: DL2000 DNA marker; lane 2: RAW264.7 cell control; lane 3: LPS-induced COX-2 mRNA expression in RAW264.7 cell line; lane 4: TGA 100 mg/L+LPS; lane 5: TGA 20 mg/L+LPS. B: Relative gray scale value profile ($\bar{x} \pm s$, n=3); *P<0.05, **P<0.01, compared with the LPS group

Effects of TGA on LPS-Induced NO and TNF-α Production in Mouse Peritoneal Macrophages

The TNF-α and NO production induced by LPS in mouse peritoneal macrophages increased significantly (P<0.01). Compared with the blank group, the LPS-induced production of NO in mouse peritoneal macrophages was significantly increased by 47% (P<0.01).

Both concentrations of TGA (100 mg/L and 20 mg/L) significantly inhibited the up-regulation of

Table 2. Effects of TGA on LPS-Induced TNF-α and NO in Mouse Peritoneal Macrophages ($\bar{x} \pm s$)

Group	TNF-α (ng/L, n=7)	NO (μmol/L, n=3)
Blank	31.83 ± 2.93	42.18 ± 6.58
LPS	410.85 ± 46.57*	62.18 ± 2.10*
LPS+L-NAME	–	48.24 ± 1.82△△ (70%)
LPS+Dex	94.46 ± 6.97△△	–
LPS+TGA	387.90 ± 42.17	52.48 ± 4.20△ (49%)
LPS+TGA	397.19 ± 25.71	57.94 ± 1.05△ (21%)
LPS+ TGA	45.55 ± 0.06	58.55 ± 5.84 (18%)

Notes: *P<0.01, compared with the blank group; △P<0.05, △△P<0.01, compared with the LPS group; the data in the parentheses were inhibitory rates

LPS-induced NO production as compared with the LPS group (P<0.05). The inhibitory effect of TGA (100 mg/L, 20 mg/L, 4mg/L) on the concentrations of TNF-α was not observed in mouse peritoneal macrophages (Table 2).

DISCUSSION

During early inflammation, the polymorphonuclear neutrophils predominating phase, PGE_2, an arachidonic acid metabolite generated by COX-2, is recognized as a potent, pro-inflammatory mediator, which synergistically exerts its biological activities with NO[17]. As non-stimulated monocytes only express COX-1, LPS-stimulated monocytes will mostly express COX-2 when COX-1 is inhibited by pre-treatment with aspirin[21]. Our experimental results show that the treatment with TGA resulted in a remarkable decrease of PGE_2 in mouse peritoneal macrophages.

To find out the relationship between the inhibitory effects of TGA on PGE_2 production and a modulation of COX-2 induction, COX-2 expression levels were measured by RT-PCR analysis. The LPS-induced COX-2 mRNA expression was suppressed by TGA (P<0.05). TGA at two dose levels (20 mg/L and 100 mg/L) significantly inhibited the COX-2 mRNA production, but only the large dose of TGA (100 mg/L) could inhibit COX-1 expression. The results demonstrated that TGA predominantly inhibited COX-2 mRNA, which was consistent with the inhibitory profile of TGA on PGE_2 release.

The present study extended the findings to show that LPS-induced PGE_2 production was markedly inhibited by TGA in mice peritoneal macrophages. At the same time, the finding that COX-2 was increased in inflammed tissues also strongly indicated that the presence of inducible COX-2 accounts for the pro-inflammatory PG synthesis. Results from RT-PCR analysis further supported that LPS-induced COX-2 mRNA expression in RAW264.7 cells could be significantly blocked by TGA. The capacity of TGA to inhibit the expression of COX-2 mRNA may well explain its marked anti-inflammatory effects *in vivo*, suggesting that TGA may possibly participate in other physiological processes in the organism. Moreover, the inhibition of TGA on COX-1 mRNA may be involved in other physiological functions. The difference between the inhibitory effects on COX-1 and COX-2 allows the hypothesis that TGA has selective inhibition of these two types of COX enzymes. The selected inhibition of TGA on PGE_2 produced by COX needs to be further investigated in the single construction by isolation from AGH.

Chronic inflammation leads to the destruction of normal tissue integrity, such as RA. Production of inflammatory mediators through up-regulation of several inducible genes, such as iNOS, COX-2, and TNF-α, contributes to inflammatory responses and tissue damage[22]. Macrophage activation is a key component of immune and inflammatory responses. The activation of macrophages results in the production of several cytokines including interleukin-1 (IL-1), IL-6 interferon (IFN-γ), IFN-γ and tumor necrosis factor-α (TNF-α) which have been implicated in immune arthritis[15]. LPS is an endotoxin, which activates macrophages to produce inflammatory mediators such as NO, TNF-α, and COX-2, similar to the inflammatory reactions *in vivo*[14].

In the inflammatory reactions in the present study, LPS co-cultured with mouse peritoneal macrophages for 20 h induced a significant increase in TNF-α and NO production. Assays of NO (as nitrite) demonstrated a clear inhibitory influence of TGA on NO production. However, the inhibitory effect of TGA (100 mg/L, 20 mg/L, 4 mg/L) on TNF-α production was not observed in mouse peritoneal macrophages.

In addition to the inhibitiory effect on COX-2-induced PGE_2 release, TGA also potentially inhibited NO production in LPS-treated mouse peritoneal macrophages. Therefore, these results suggested that the inhibition of LPS-induced NO and PGE_2 production may be obtained through the regulation of COX-2 gene expression by TGA. However, we could not rule out the possibility that TGA may directly interfere with COX-2 activity and the expression of iNOS. Moreover, the development of inflammation is thought to be mediated by pro-inflammatory cytokines such as TNF-α[23]. Therefore, we investigated whether TGA could possibly influence the formation of TNF-α in an *in vitro* model. The results demonstrated that TGA did not inhibit TNF-α production. It is reasonable to hypothesize that TGA interfere with the activation of the NF-κB pathway results in a decrease in iNOS and COX-2, but pro-inflammatory stimuli of TNF-α were not affected.

The present study generated evidence that anti-inflammation of TGA is possibly due to remarkable suppression of PGE_2 and NO production. The suppression of PGE_2 was the consequence of down-regulation of COX-2 RNA expression.

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