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Original Research Article

Total Glucosides of *Paeonia lactiflora* Pall Suppress Nitric Oxide Production and iNOS Expression in Lipopolysaccharide-Stimulated RAW264.7 Macrophages

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Abstract

Purpose: *To investigate the effect of total glucosides of Paeonia lactiflora (TGPL) on nitric oxide (NO) production and its potential mechanism in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells.*
Methods: *RAW264.7 cells were treated with 10 - 300 μg/ml TGPL and 1 μg/ml LPS. Cell survival was determined by MTT assay. NO level was determined by Griess reaction assay. Inducible NO synthase (iNOS) expression and inhibitor-κBα (IκBα) degradation were determined by Western blot assay. DNA binding activity of NF-κB was determined by ELISA assay using Trans AM™ kit for p65.*
Results: *The concentrations of TGPL (10 - 300 μg/ml) used in this study did not affect cell survival of RAW264.7 cells, which suggest that 10-300 μg/ml TGPL did not show cytotoxic effect on RAW264.7 cells. NO level and iNOS protein expression significantly increased in LPS-stimulated RAW264.7 cells compared to the unstimulated cells. However, 10 - 300 μg/ml TGPL significantly decreased LPS-induced NO level and iNOS protein expression compared to LPS-stimulated RAW264.7 cells alone. Furthermore, 10 - 300 μg/mL TGPL significantly reduced the content of IκBα protein in LPS-stimulated RAW264.7 cells, which suggests that TGPL inhibited LPS-induced degradation of IκBα protein. TGPL remarkably repressed LPS-induced DNA binding activity of P65 in RAW264.7 cells.*
Conclusion: *These findings suggest that TGP inhibits NO production and iNOS expression through suppression of NF-κB activation in LPS-stimulated RAW264.7 cells.*

Keywords: *Total glucosides, Paeonia lactiflora, Nitric oxide, iNOs, Nuclear factor-κB*

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INTRODUCTION

Inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), prostaglandin E2 (PGE2) and nitric oxide (NO), are the prime motivators of inflammation [1]. NO is a short-lived free radical that plays a vital role in diverse physiological processes, including blood vessel tone, neurotransmission, mitochondrial functions, immune regulation and apoptosis. NO is a pro-inflammatory factor in inflammatory condition [2]. NO is synthesized from L-arginine by NO synthases (NOSs): neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). In inflammatory conditions, NO production is catalyzed mainly by iNOS [3]. A significant correlation has been established between NO level and the degree of severity of inflammatory diseases such as rheumatoid arthritis (RA) [4] and systemic lupus erythematosus (SLE) [5] iNOS gene polymorphism has also found to be closely

associated with susceptibility to RA [6] and SLE [7]. These data stand out that iNOS and NO play crucial roles in the development of inflammation.
Nuclear factor (NF)-κB is the key transcriptional factor regulating iNOS gene transcription. NF-κB exists as homo- or hetero-dimeric forms of Rel family proteins such as RelA (p65), RelB, cRel, p50 and p52. NF-κB is present in the cytoplasm in an quiescent state, complexed with the inhibitory κB (IκB) proteins including IκBα, IκBβ, IκBε, p105 and p100 [8]. Lipopolysaccharide (LPS) [9] or cytokines [10] can trigger NF-κB activation through induced IκB degradation, which resulting in the release and nuclear translocation of p65. In the nucleus, p65 binds to the promoter regions of a number of inflammatory genes including iNOS for transcriptional regulation.

Paeonia lactiflora Pall is a widely used Chinese medicinal plant which is claimed to function in replenishing blood and analgesia. Our previous studies have demonstrated that *Shaoyao-Gangao-Tang* (SGT), a traditional Chinese medicine formula that contains *Paeonia lactiflora* Pall and *Glycyrrhiza uralensis* Fisch., can remarkably inhibit the production of inflammatory mediators of adjuvant-induced arthritis [11] or carrageenin-induced pleurisy in rats [12]. However, the effective constituents and potential mechanisms of the anti-inflammatory function of *Paeonia lactiflora* Pall remain unclear. In this study, we investigated the effect of the total glucosides of *Paeonia lactiflora* Pall. on NO production, iNOS expression and NF-κB activation in LPS-stimulated RAW264.7 macrophage cells.

EXPERIMENTAL

Cell culture

RAW 264.7 macrophage cells were purchased from American Type Culture Collection (ATCC; USA), and subcultured to confluence in Dulbecco's modified Eagle's medium (DMEM; ATCC, USA) containing 10 % fetal bovine serum (Hyclone, USA), 100 U/ml penicillin (Life Technologies, USA) and 100 μg/ml streptomycin (Life Technologies, USA) in a humidified 5 % CO_2 atmosphere at 37 °C.

Nitric oxide determination

RAW264.7 cells were incubated with 10-300 μg/ml TGPL (gift from Lansen medicine Co., Ltd, China. TGPL used in this study contained 41.5 % paeoniflorin (w/w) by HPLC analysis) and 1 μg/ml LPS (Sigma, USA) for 18 h. The nitrite accumulation in the supernatant was assayed by Griess reagent (Sigma, USA). Briefly, equal volume of cell-free culture media (100 μl) was reacted with Griess reagent (100 μl), and the absorbance at 540 nm was measured.

MTT assay

RAW 264.7 cells were incubated with 10-300 μg/ml TGPL for 24 h. Then 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) was added for 4 h at the final concentration of 0.5 mg/ml. Subsequently, the culture medium was removed and dimethyl sulfoxide was added to dissolve the formazan crystals, and plates were then read immediately at 570 nm. Wells containing incubation media without cells were used as control. RAW 264.7 cells treated with vehicle only were defined as 100 % viable. Cell survival was defined as the growth of treated cells compared with the untreated.

Western blot

RAW264.7 cells were incubated with 10-300 μg/mL TGPL and 1 μg/mL LPS for 18 h (for iNOS) or pre-treated with 10-300 μg/mL TGPL for 2 h, and then stimulated with 1 μg/ml LPS for 30 min (for IκBα). The cellular protein was extracted by lysis buffer (50 mmol/L Tris-Hcl, pH 8.0, 150 mmol/L NaCl, 1 % NP-40, 0.1 % SDS, 0.5 % sodium deoxycholate) and EDTA-free protease and phosphatase inhibitor cocktail (Roche, Switzerland). The quantity of proteins was measured using the bicinchoninic acid (Pierce, USA) assay. Equal amounts of protein samples were separated by 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred onto PVDF membranes (Millipore, USA). After blocking with 5 % non-fat milk for 1 h at room temperature, the PVDF membranes were incubated with the anti-iNOS antibody, anti-IκBα antibody or anti-β-actin antibody (Santa Cruz, USA) at 4 °C overnight and subsequently with peroxidase-conjugated second antibody at room temperature for 1 h. The protein bands were detected using ECL reagents (Millipore, USA). Chemiluminescent signals were detected and analyzed using the ChemiDoc XRS imaging system (Bio-Rad, USA).

Preparation of nuclear protein extracts

RAW264.7 cells were pre-treated with 10-300 μg/mL TGPL for 2 h, and then stimulated with 1 μg/ml LPS for 1 h. Nuclear protein extracts were

prepared following the manufacturer's instructions (Active Motif, USA). Briefly, cells were gently re-suspended in 500 µL hypotonic buffer and incubated for 15 min on ice. After 25 µL of detergent was added and vortexed, the cells were centrifuged for 30 s at 14000 g at 4 °C. The pellet was used for nuclear fraction collection. Nuclear pellet was resuspended in 50 µL complete lysis buffer, incubated for 30 min on ice and then centrifuged for 10 min at 14000 g at 4 °C. The supernatant (nuclear protein extracts) was collected and stored at -80 °C.

NF-κB DNA-binding activity

The binding ability of NF-κB to DNA consensus sequences was measured by ELISA assay using the Trans AM™ kit for p65 according to the manufacturer's instructions (Active Motif, USA). Briefly, nuclear protein extracts (5 µg) were added to the wells, followed by the primary antibody against p65 and the horseradish peroxidase-conjugated secondary antibody. The optical density was measured at 450 nm.

Statistical analysis

All data are expressed as mean ± SD. Multiple group comparisons were performed using one-way ANOVA and LSD tests using SPSS 19 software. The differences were considered statistically significant at $p < 0.05$.

RESULTS

Effects of TGPL on NO production and cell survival

Firstly, we investigated the effect of TGPL on NO production in LPS-stimulated RAW264.7 cells. The level of NO increased significantly from 8.9 ± 1.6 µM to 64.7 ± 11.1 µM after LPS-stimulation for 18 h ($p < 0.01$, Fig 1A). 10-300 µg/ml TGPL significantly reduced the NO level in a concentration-dependent manner ($p < 0.05$ or $p < 0.01$, Fig 1A). In particular, at 300 µg/mL TGPL reduced the NO level to 29.2 ± 4.1 µM compared to that of 64.7 ± 11.1 µM in LPS alone-stimulated RAW264.7 cells, corresponding to about 55 % inhibition ($p < 0.01$). At the same time, 10-300 µg/ml TGPL did not affect cell survival of RAW264.7 cells compared to the untreated cells (Fig 1B).

Effect of TGPL on iNOS protein expression

Since iNOS is the key enzyme that catalyzes NO production in inflammatory condition, we investigated the effect of TGPL on iNOS protein expression in LPS-stimulated RAW264.7 cells. Trace expression of iNOS protein was detectable in untreated RAW264.7 cells. However, expression of iNOS protein was considerably induced upon exposure to 1 µg/ml LPS for 18 h ($p < 0.01$, Fig 2A, B). 10-300 µg/ml TGPL significantly decreased LPS-induced iNOS protein expression in RAW264.7 cells ($p < 0.01$, Fig 1A, B).

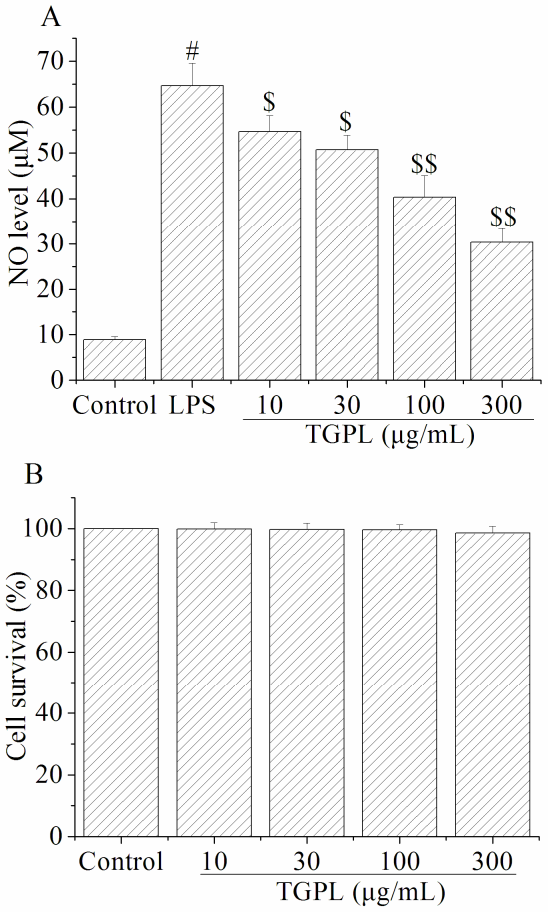

Fig 1: Effects of TGPL on NO production and cell survival in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated with 10 - 300 µg/ml TGP and with or without 1 µg/ml LPS for 18 h or 24 h. A: Secreted NO in the cell-free culture media was analyzed by Griess reaction assay. B: Cell survival was analyzed by MTT assay. Three independent experiments were performed in duplicate; values are mean ± SD. $^{\#}$ $p < 0.01$ vs. Control group; $^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$ vs. LPS group

Effect of TGPL on DNA binding activity of NF-κB

Upon exposure to LPS alone, DNA binding activity of NF-κB was significantly promoted within 1 h in RAW264.7 cells ($p < 0.01$, Fig 3). 10-300 µg/ml TGPL significantly decreased LPS-induced DNA binding activity of NF-κB in a

concentration-dependent manner in RAW264.7 cells, corresponding to approximately 48 % inhibition at 300 µg/ml TGPL ($p < 0.01$, Fig 3).

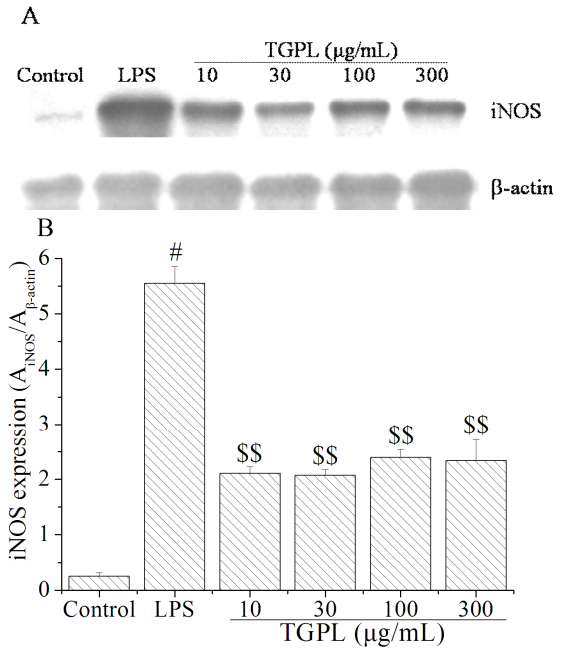

Fig 2: Effect of TGPL on iNOS protein expression in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated with 10 - 300 µg/ml TGPL and with or without 1 µg/ml LPS for 18 h. A: iNOS protein was measured by Western blot assay. B: Bar graphs showed quantitative evaluation of iNOS bands by densitometry. Three independent experiments performed in duplicate; values are mean ± SD. $^{\#}p < 0.01$ vs. Control group; $^{\$\$}p < 0.01$ vs. LPS group

Effect of TGPL on IκBα degradation

Significant reduction of content of IκBα protein occurred within 30 min upon exposure to LPS-stimulation alone ($p < 0.01$, Fig 4). 10-300 µg/ml TGPL markedly increased content of IκBα protein in LPS-stimulated RAW264.7 cells, especially corresponding to approximately 700 % recovery at 300 µg/ml TGPL ($p < 0.01$, Fig 3).

DISCUSSION

A number of inflammatory mediators, including TNF-α, IL-1β, PGE2 and NO, promote inflammation and aggravate tissue damage [13]. Multiple types of inflammatory cells, including synovial fibroblasts, monocytes/macrophages, osteoclasts, endothelial cells and T-lymphocytes, are regulated by NO [14]. At the same time, cytokines, intercellular adhesion molecules, vascular cell adhesion molecules and matrix metalloproteinases, which accelerate the development of inflammation and tissue damage,

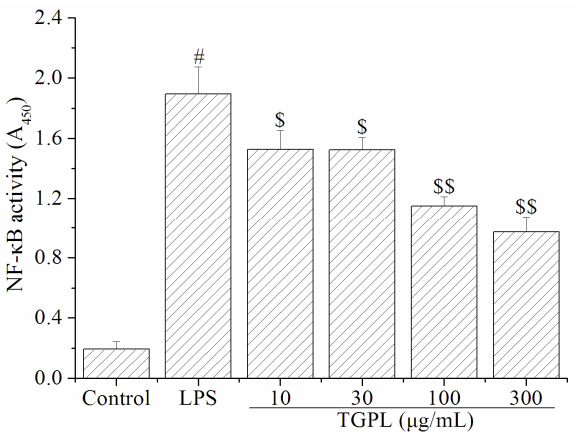

Fig 3: Effect of TGPL on DNA binding activity of NF-κB in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pre-treated with 10-300 µg/ml TGPL for 2 h, and subsequently stimulated with 1 µg/ml LPS for 1 h. Nuclear protein extracts were used to analyze DNA-binding activity of NF-κB by the Trans AM™ kit for p65. Three independent experiments performed in duplicate. $^{\#}p < 0.01$ vs. Control group. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. LPS group

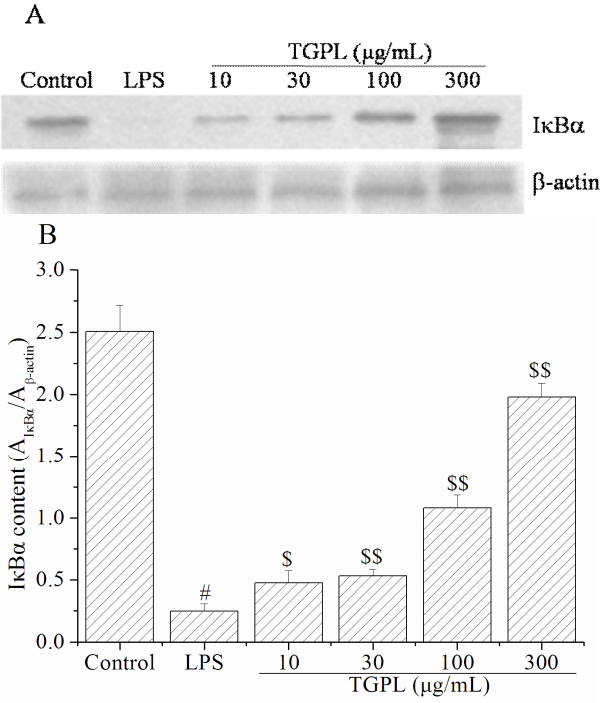

Fig. 4: Effect of TGPL on IκBα degradation in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pre-treated with 10 - 300 µg/ml TGPL for 2 h, and subsequently stimulated with 1 µg/ml LPS for 30 min. A: IκBα protein was measured by Western blot assay. B: Bar graphs showed quantitative evaluation of IκBα bands by densitometry. Three independent experiments performed in duplicate; values are mean ± SD; $^{\#}p < 0.01$ vs. Control group; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. LPS group

can be induced by NO in various types of cells [15]. iNOS is the key enzyme for NO production in inflammatory conditions. In spite of the fact that the iNOS pathway is not as rapid as eNOS or nNOS, it is thought to be capable of generating much larger quantities of NO than constitutive NOS isoforms [16]. These research findings highlight the significance of iNOS and NO in inflammatory diseases. In this study, we found that LPS considerably induced NO production in murine macrophage RAW264.7 cells. However, TGPL, the active fraction of *Paeonia lactiflora* Pall. significantly decreased the level of LPS-induced NO in concentration-dependent manner. Due to the fact that all the concentrations of TGPL used in this study did not show cytotoxic effect on RAW264.7 cells, it seems that TGPL decreased NO level, not as a result of reduction in the quantity of cells, but due to some other mechanism of NO production. Considering that iNOS is the vital enzyme for NO production in inflammatory conditions [3], we investigated the effect of TGPL on LPS-induced NO production in RAW264.7 cells. We found that LPS remarkably induced iNOS protein expression in RAW264.7 cells, and TGPL significantly reduced the LPS-induced iNOS protein expression, which suggests that TGPL was able to regulate the iNOS-NO pathway.

Numerous pro-inflammatory genes, including NO gene, are regulated by a transcriptional factor NF-κB. In the resting state, NF-κB binds to IκB proteins and delays in the cytoplasm [17]. Stimulating factors including LPS and cytokines [9, 10] can induce the degradation of IκB proteins, resulting in the nuclear translocation of NF-κB, where NF-κB can promote the transcription of pro-inflammatory genes. In this study, we investigated the effect and mechanism of TGPL on NF-κB activation. LPS significantly increased DNA binding activity of NF-κB in RAW264.7 cells. TGPL notably and concentration-dependently decreased the LPS-induced DNA binding activity of NF-κB in RAW264.7 cells, suggesting that TGPL was able to inhibit the LPS-induced NF-κB activation. Since IκBα degradation is a prerequisite for the nuclear translocation of NF-κB [17], we investigated the effect of TGPL on IκBα degradation. It was found that LPS reduced the amount of IκBα protein in RAW264.7 cells, suggesting that LPS strongly induced IκBα degradation. TGPL restored the reduction of IκBα protein induced by LPS in a concentration-dependent manner. These results indicate that TGPL could suppress NF-κB activation by the way of inhibition of IκBα degradation.

In the theory of traditional Chinese medicine (TCM), *Paeonia lactiflora* Pall. is thought to possess the outstanding function of “replenishing blood and analgesia”. In the clinical practice of TCM, *Paeonia lactiflora* Pall is frequently applied to treat anemia and a variety of pain in inflammatory diseases. However, the effective constituents and potential mechanisms of the anti-inflammatory function of *Paeonia lactiflora* Pall. were not totally understood till now. In this study, we demonstrated that TGPL could suppress NO production and iNOS protein expression in LPS-stimulated RAW264.7 cells by the way of inhibition of NF-κB activation.

CONCLUSION

This study demonstrates that TGPL can significantly inhibit NO production and iNOS protein expression in LPS-stimulated RAW264.7 cells. The mechanism of this action probably entails suppression of IκBα protein degradation and NF-κB activation in LPS-stimulated RAW264.7 cells. The findings obtained include the effective constituents and potential mechanisms of the anti-inflammatory function of *Paeonia lactiflora* Pall., and thus constitute new contribution to the research on the anti-inflammatory activity of *Paeonia lactiflora* Pall.

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