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Protopanaxadiol modulates LPS-induced inflammatory activity in murine macrophage RAW264.7 cells

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Abstract: Protopanaxadiol (PPD) is a mixture of protopanaxadiol type saponins with a dammarane skeleton, from Korean red ginseng (*Panax ginseng* C.A. Meyer; Araliaceae). Korean ginseng is well-known herb to treat almost all kinds of diseases in Oriental medicine. This herb was particularly prescribed for treatment various inflammatory diseases, including rheumatoid arthritis, atherosclerosis, and diabetes mellitus, for centuries. To understand the efficacy of ginseng against inflammatory diseases, we aimed to show anti-inflammatory activities of the PPD in murine macrophage cell line, RAW264.7 cells using nitric oxide (NO) production assay and the expressions of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, and monocyte chemotactic protein-1 (MCP-1). We found that PPD saponin significantly blocked LPS (1 μg/ml)-induced NO production in a dose-dependent manner. In addition, PPD abrogated the expressions of LPS-induced pro-inflammatory cytokines, such as IL-1β and MCP-1. Moreover, cyclooxygenase (COX)-2, a critical enzyme to produce prostaglandin E2 (PGE2), was significantly inhibited by PPD in LPS-activated RAW264.7 cells. Taken together, these results suggested that anti-inflammatory efficacy of Korean red ginseng on inflammatory diseases is, at least, due to the NO inhibitory activity and the inhibition of the expressional level of inflammatory cytokines and/or mediators.

Key word : Protopanaxadiol, Nitric oxide, Inflammatory cytokines, mRNA expression, RT-PCR.

INTRODUCTION

Ginseng, the root of Panax ginseng C.A. Meyer (Araliaceae) is a mild oriental folk medicine that is reported to relieve a variety of ailments[1)]. Ginseng contains many active components such as ginsenosides, polysaccharides, peptides, fatty acids and mineral oils[2)]. Among these components, ginsenosides are believed to be responsible for the most pharmacological and immunological actions[2, 3)]. There are more than 30 kinds of ginsenosides, which are derivatives having the triterpene dammarane structure. These ginseng saponins are divided into two different structural classes, 20(*S*)-protopanaxdiol type saponins (PPD [e.g. Rb1, Rb2, Rc and Rd]) and 20(*S*)-protopanaxtriol type saponins (PPT [e.g. Re, Rf, Rg1 and Rg2])[4, 5)]. To develop ginseng saponins for medical purpose, recently, saponin mixtures (PPD or PPT fractions) but not signal compound level have been actively used to evaluate their biological efficacies to lower their preparation cost. For examples, PPD have been reported to display multiple effects on the immune system[6-8)] although relatively few studies have been reported in cellular mechanism of macrophage cells with PPD saponins[9)].

Macrophages play a central role in inflammatory processes through the release of chemokines (e.g. macrophage inflammatory protein-1 α [MIP-1α] and monocyte chemotactic protein [MCP-1]) and cytokines (e.g. tumor necrosis factor-α [TNF-α], interleukin-1β [IL-1β] and interleukin-6 [IL-6])[10)]. Lipopolysaccharide (LPS) can trigger inflammation and induce the over-expression of various inflammatory mediators, such as MIP-1α and MCP-1, TNF-α, IL-1β and IL-6 and iNOS[4, 11-13)]. These

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mediators are important for the development of new anti-inflammatory drugs and for determining the potential molecular anti-inflammatory mechanisms. In addition, NO is generated by inducible NO synthase (iNOS) and it induces tissue injury at sites of inflammation[14]. iNOS is expressed in response to various inflammatory stimuli, which results in the massive production of NO in macrophages during inflammatory processes[15].

Therefore, in this study, in order to elucidate the anti-inflammatory properties of ginseng saponins, we determined whether PPD displayed anti-inflammatory activity using NO production and expression of inflammatory cytokines such as MCP-1, IL-1β, and TNF-α, in murine macrophage RAW264.7 cells.

MATERIALS AND METHODS

Materials

PPD saponin was provided by KT & G Central Research Institute. RAW264.7 cells were obtained from Korean Cell Line Bank (Seoul, Korea). RT and PCR premix were from Bioneer Co. (Daejeon, Korea). LPS and N-monomethyl-L-arginine (N-MMA) was from Sigma Co. (St Louis, MO). All other reagent were the first grade.

Cell culture

RAW264.7 cells were maintained in RPMI 1640 supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin and a 5% FBS. Cells were grown at 37°C and 5% CO_2 in humidified air.

The measurement of nitrite

To determine the concentration of NO, nitrite (NO_2^-) was measured using the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid), as described previously[16]. Briefly, after the RAW 264.7 cells (1×10^6 cells/ml) were preincubated for 18 h, the cells were incubated with PPD with a LPS (1 μg/ml) for 24 h. One-hundred μl of a supernatant from each well of the culture plates were transferred into 96-well microplates. The supernatant was mixed with an equal volume of Griess reagent at room temperature. The absorbance at 540 nm was determined by a Spectramax 250 microplate reader. The concentrations of nitrite were calculated by a regression analysis using serial dilutions of sodium nitrite as a standard.

Extraction of total RNA

The total RNA from the LPS treated-RAW264.7 cells was prepared by adding Easy blue Reagent (iNtRON Biotechnology Co., Korea), according to the manufacturer's protocol. The total RNA solution was stored at -70 until use.

Semiquantitative RT-PCR amplification

Semiquantitative RT reactions were carried out using a RT premix (Bioneer Co., Korea). Briefly, two μg of total RNA were incubated with oligo-dT_{18} at 70°C for 5 minutes and cooled on ice for 3 minutes, and the reaction mixture was incubated for 90 minutes at 42.5°C after the addition of RT premix. The reactions were suspended at 95°C for 5 minutes due to the inactivation of reverse transcriptase. The PCR reaction was continued using a PCR premix (Bioneer Co., Korea) with appropriate sense and antisense primers for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sense primer, 5'-CAC TCA CGG CAA ATT CAA CGG C-3'; antisense primer, 5'-CCT TGG CAG CAC CAG TGG ATG CAG G-3'), iNOS (sense primer, 5'- CCC TTC CGA AGT TTC TGG CAG CAG C-3'; antisense primer, 5'- GGC TGT CAG AGC CTC GTG GCT TTG G-3', IL-1β (sense primer, 5'- CAG GAT GAG GAC ATG AGC ACC-3'; antisense primer, 5'- CTC TGC AGA CTC AAA CTC CAC-3'), MCP-1 (sense primer, 5'- TCT GTG CCT GCT GCT CAT AGC - 3'; antisense primer, 5'- GGG TAG AAC TGT GGT TCA AGA GG -3'), and TNF-α (sense primer, 5'- TTG ACC TCA GCG CTG AGT TG -3'; antisense primer, 5'- CCT GTA GCC CAC GTC GTA GC-3'), under incubation conditions (a 45-second denaturation time at 94°C, an annealing time of 45°C seconds between 55 and 60°C, an extension time of 45 seconds at 72°C, and a final extension of 10 minutes at 72°C at the end of the cycles. The PCR products were separated on a 1% agarose using a electrophoresis method of BioRad Co. The relative intensity levels were calculated using Eagle eyes image analysis software (Stratagene Co., La Jolla). The resulting density levels of the iNOS, IL-1β, TNF-α, and MCP-1 bands were expressed relative to the corresponding density amounts of the GAPDH bands, which were from the same RNA sample. GAPDH, a housekeeping gene, was used as the RNA internal standard.

Statistical analysis

A one–way ANOVA was used to determine statistically significant differences between values of the experimental and control groups. Data represent the means ± SEM of three experiments, conducted in triplicate. P values of 0.05 or less were considered statistically significant.

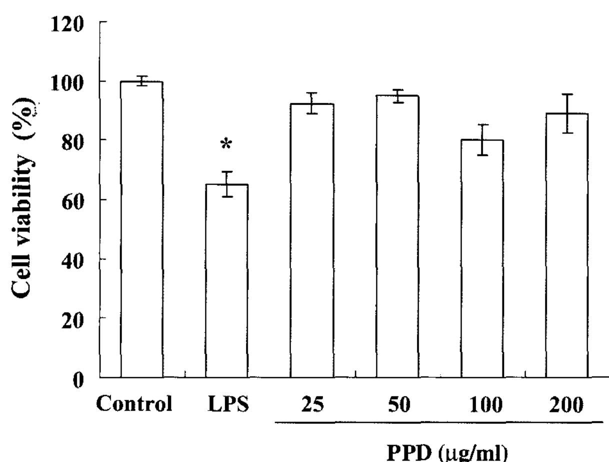

Fig. 1. Effect of PPD saponin on the cytotoxicity of RAW 264.7 cells. RAW 264.7 cells (1×10^6 cells/ml) were incubated either with LPS (1 μg/ml), or PPD saponin (25-200 μg/ml) for 24 h. MTT assay was performed as described in 'Materials & Methods'. Each value is the means ± SEM of three independent experiments performed in triplicate. *P<0.05 versus vehicle control.

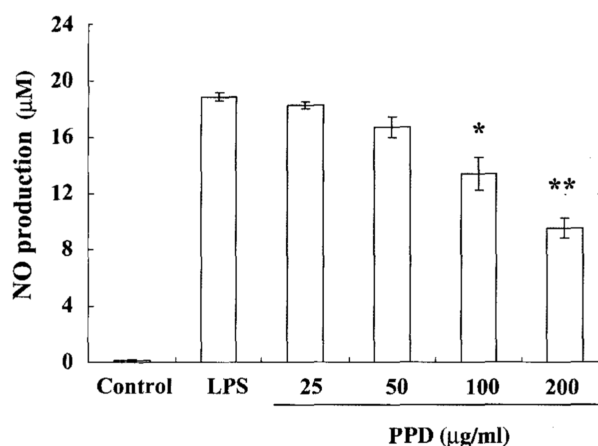

Fig. 2. Effect of PPD saponin on LPS-induced NO production in RAW264.7 cells. The cells were pretreated with PPD (25-200 μg/ml) for 30 min, and after adding a LPS (1 μg/ml), the cells were incubated for a further 24 hrs. The supernatant was removed and the nitrite was determined as described in 'Materials & Methods'. Each value is the means ± SEM of three experiments performed in triplicate. *P<0.05 versus vehicle control.

RESULTS AND DISCUSSION

Effect on the LPS-induced NO production

To exclude the artificial effect due to the cytotoxic activity of PPD on the RAW264.7 cells, we first determined whether running concentrations (between 25 μg/ml and 200 μg/ml) affected the cell viability using MTT assay. As shown in Fig. 1, any concentrations of PPD did not show the cytotoxicity, and we could carry out the following experiments using up to 200 μg/ml of PPD. In contrast, PPT displayed strong cytotoxic activity under the same conditions (data not shown), suggesting its non-selective influence on macrophage functions. PPD dose-dependently inhibited the LPS-induced NO production in RAW264.7 cells (Fig. 2). NO, synthesized by NO synthase from L-arginine, is well known as a major inflammatory mediator in immune cells. NO takes multi-tasting role in cardiovascular system (i.e. anti-hypertensive and anti-platelet activity), in nervous system (i.e. neurotransmitter), and in immune system (i.e. inflammatory mediator), depending on the amount and localization of NO synthesized in limited time[17-19]. Since PPD significantly abrogated the LPS-induced massive NO production, we next examined the expression of iNOS mRNA. As unexpectedly, PPD did not affect the iNOS expression (data not shown). This result possibly indicated that PPD modulated in translational or posttranslational process in the protein synthesis of iNOS, which remained to be examined. To understand which kinds of PPD compounds are involved in significant inhibition of NO production, we evaluated the inhibitory potency of ginsenoside compounds known as PPD class. Rb1, Rc and Rd blocked NO production less than 10% at 50 μM, whereas Rg3 displayed 27% at the same concentration (data not shown). Therefore, our results suggest that PPD's NO inhibitory activity may be derived by Rg3's action. Furthermore, at 50 μM, none of PPT (Rgl, Rg2. Rhl to Re) exhibited weak NO inhibitory effect (0 to 4%). Meanwhile, N-MMA as a control drug, also strongly blocked LPS-mediated NO production with an IC_{50} of 198 μM (data not shown).

Effects on the LPS-induced COX-2 expression

The expression of COX-2 gene was significantly induced by incubation with 1 μg/ml LPS for 24 h. When RAW264.7 cells were stimulated with LPS in the presence of PPD (25-200 μg/ml), a dose-dependent inhibition of COX-2 gene expression was observed (Fig. 3). Since COX-2, an enzyme which catalyzes the generation of prostaglandins from arachidonic acid, also contributes to lesion formation in immune cells, many researcher have carried out to find natural products or synthetic peptides, acting as COX-2 inhibitor [4, 20, 21]. In addition, some laboratories have reported that PPD saponins such as Rb1,

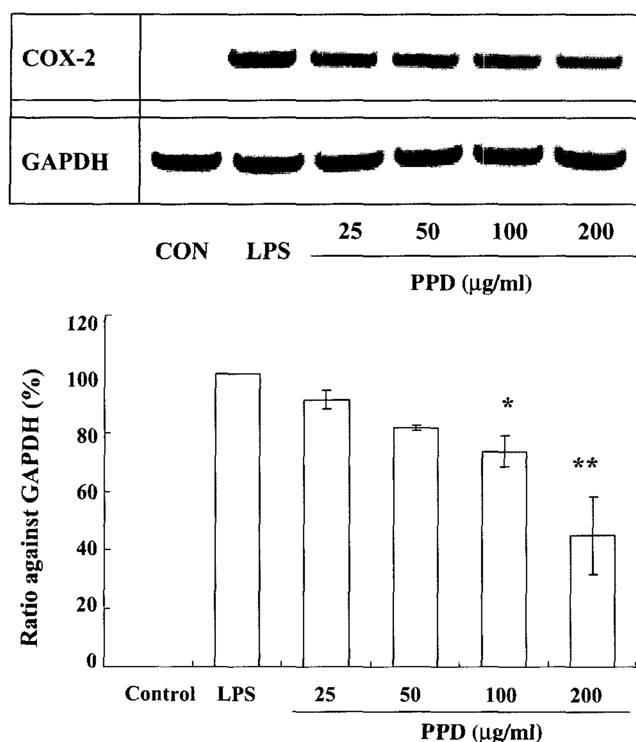

Fig. 3. Effects of PPD saponin on the mRNA expression of COX-2 in LPS-activated RAW264.7 cells. The mRNA levels of COX-2 gene from the RAW264.7 cells were determined by semi-quantitative RT-PCR as described in "Materials & Methods". The figures present the representative results from three separate experiments, which give similar results. *P<0.05 versus vehicle control, **P<0.01 versus vehicle control.

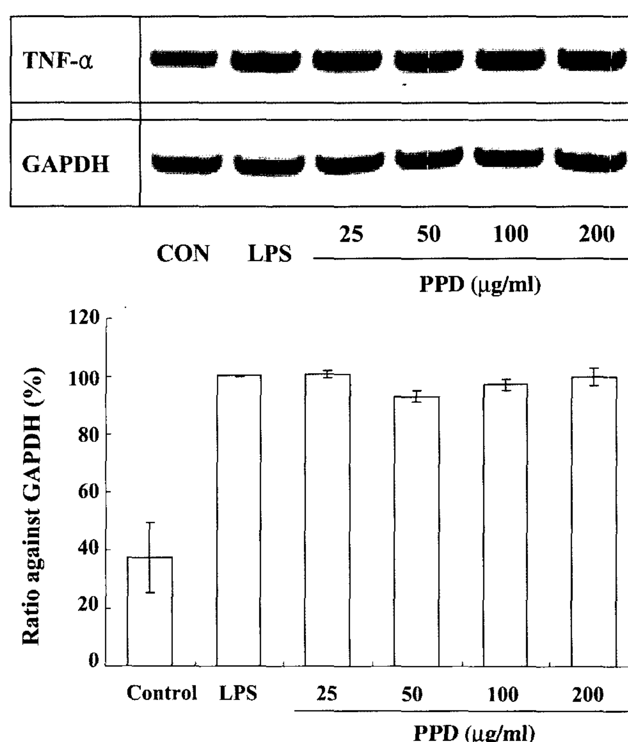

Fig. 4. Effects of PPD saponin on the mRNA expression of pro-inflammatory cytokines in LPS-activated RAW264.7 cells. The mRNA levels of TNF-α from the RAW264.7 cells were determined by semi-quantitative RT-PCR as described in "Materials & Methods". The figures present the representative results from three separate experiments, which give similar results.

Rg3, Rh2 and their metabolites (i.e. compound K) displayed COX-2 inhibiting activity [9, 22, 23]. In this study, we found that PPD did also act as COX-2 inhibitor in murine macrophage RAW264.7 cells, like previously reported in PPT [24]. However, in this study, we did not know whether inhibitory effect of PPD on COX-2 expression was due to the transcriptional modulation of nuclear factor-κB or other transcriptional factors, which remains to be clarified in the future.

Effects on the pro-inflammatory cytokine expression

We next assessed the inhibitory activity of PPD saponin on the expression of LPS-stimulated pro-inflammatory cytokines, including IL-1β, MCP-1, and TNF-α. Cytokines and chemokines (such as IL-1β, IL-6, TNF-α and MCP-1) are known to be pro-inflammatory cytokines that posses a multitude of biological activity linked to normal defense responses as well as the inflammatory diseases such as arteriosclerosis, septic shock and rheumatoid arthritis, and autoimmune diseases[25-27]. Therefore, we examined whether PPD was capable of effectively regulating cytokine expression in murine macrophages (RAW264.7), by using a semi-quantitative RT-PCR method. In unstimulated RAW 264.7 cells, IL-1β and MCP-1 expressions were undetectable, whereas TNF-α expression was noticeably detectable. IL-1β and MCP-1, however, were strongly expressed in response to 1 μg/ml of LPS. As shown in Fig. 4, PPD (25 and 200 μg/ml) did not affect the expression of TNF-α from LPS-activated RAW264.7 cells. This result indicated that PPD did not act at the transcriptional level of TNF-α, but possibly did at the translational level. Indeed, we previously reported that PPD compounds (Rb1, Rb2 and Rc) strongly blocked the production of TNF-α in LPS-activated RAW264.7 cells [28]. This possibility was also driven by the fact that PPD modulated the expression or production of other pro-inflammatory cytokines (i.e. IL-1β and MCP-1). In an accordance with this, we found that some natural products (e.g. *Codonopsis lanceolata* extracts)

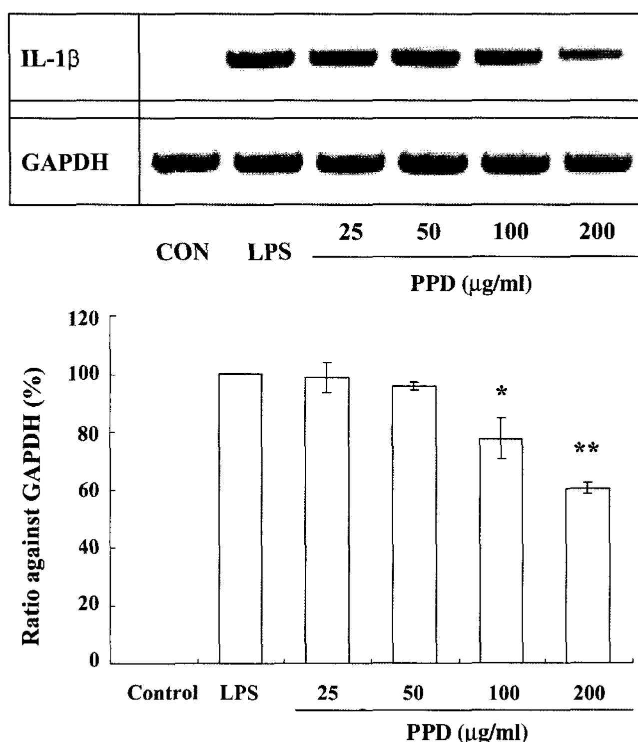

Fig. 5. Effects of PPD saponin on the mRNA expression of pro-inflammatory cytokines in LPS-activated RAW264.7 cells. The mRNA levels of iNOS IL-1β from the RAW264.7 cells were determined by semi-quantitative RT-PCR as described in "Materials & Methods". The figures present the representative results from three separate experiments, which give similar results. $*P<0.05$ versus vehicle control, $**P<0.01$ versus vehicle control.

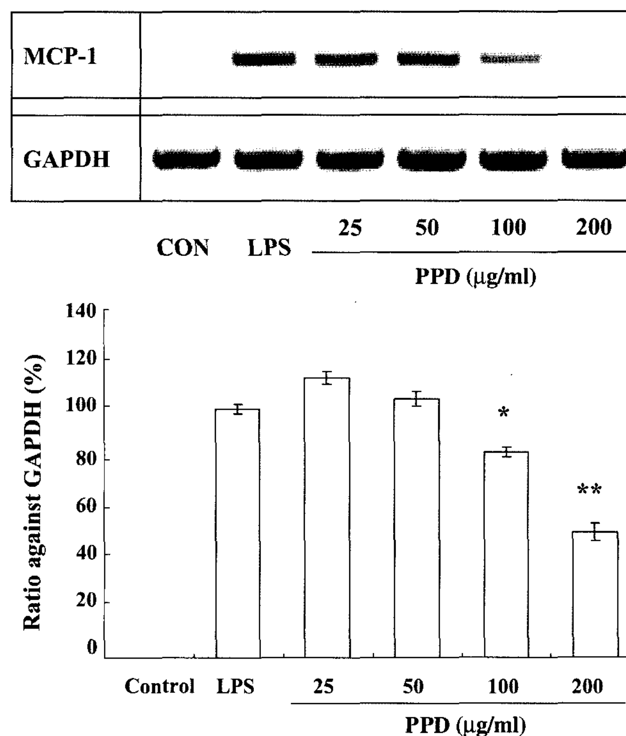

Fig. 6. Effects of PPD saponin on the mRNA expression of pro-inflammatory cytokines in LPS-activated RAW264.7 cells. The mRNA levels of MCP-1 from the RAW264.7 cells were determined by semi-quantitative RT-PCR as described in "Materials & Methods". The figures present the representative results from three separate experiments, which give similar results. $*P<0.05$ versus vehicle control, $**P<0.01$ versus vehicle control.

blocked the LPS-activated TNF-α production using ELISA assay, but they did not affect the expression of TNF-α mRNA (data not shown, submitted). In regulating IL-1β mRNA, PPD did have a significant effect. That is, PPD dose-dependently inhibited the expression of IL-1β mRNA in LPS-activated RAW264.7 cells (Fig. 5). MCP-1, acting through its receptor (i.e., chemokine receptor 2), appears to play an important role in the recruitment of monocytes for atherosclerotic lesions and in the formation of intima thickening after arterial injury [29; 30]. Due to it's critical role in monocyte recruitment in vascular and non-vascular diseases, MCP-1 has become an important therapeutic targets, and efforts are underway to develop potent and specific antagonists for this and related chemokines [29]. As shown in Fig 6, PPD did block the expression of MCP-1 mRNA in a dose-dependent manner. From these results, it can be presumed that PPD has the potential anti-inflammatory activity in LPS-induced RAW 264.7 cells.

In summary, ginseng (the root of *Panax ginseng* C.A. Meyer) was found to show anti-inflammatory and anti-allergic activity in animal models[31-33]. In this study, we found that PPD saponin showed inhibitory effect on LPS-induced inflammatory mediator, NO. In addition, PPD did significantly and dose-dependently inhibit the expression of COX-2 mRNA that catalyzes the generation of prostaglandins from arachidonic acid. The resulting arachidonic acid is a major precursor to convert into many detrimental inflammatory mediators such as thromboxane A2, prostaglandin E2, and leukotrienes. PPD did not affect at the transcriptional level of TNF-α production, while PPD did modulate the expression of IL-1β and MCP-1 mRNA. Taken together, these results suggested that PPD takes, at least, a role in the anti-inflammatory activity of Korean ginseng, and is valuable source to be used anti-inflammatory agents against inflammatory diseases such as arthritis, asthma, and atherosclerosis.

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