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Original Article

Anti-inflammatory effects of four saponins of Rhizoma Paridis via LPS-induced inflammation in RAW 264.7 Macrophages

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Rhizoma Paridis is widely used in Traditional Chinese Medicine. *Rhizoma Paridis* Saponins (RPS), natural compounds purified from *Rhizoma Paridis*, have been found to inhibit cancer growth in vitro and in animal studies. However, which of these compounds is responsible for these effects has not been well investigated. The purpose of this study was to investigate the suppression effect of RPS on the expressions of iNOS and cyclooxygenase 2 (COX-2). Four compounds of RPS were analyzed. Moreover, the effects of RPS on the regulation of lipopolysaccharide (LPS)-induced inflammatory responses in RAW 264.7 macrophages were analyzed. None of the analyzed RPS compounds affected the viability of RAW 264.7 cells at concentrations of 0.5~2.5μM. Furthermore, cell studies showed that COX-2 expression (P<0.05) is best suppressed by compound A. These results suggested that compound A of RPS plays an essential role in cancer chemoprevention.

Keywords. *Rhizoma Paridis* saponins, iNOS, COX-2, MTT

Introduction

Rhizoma Paridis refers to the roots and rhizomes of Paris *polyphylla* var. yunnanensis (Franch.) Hand-Mazz. or Paris *polyphylla* Smith var. chinensis (Franch.) Hara of the family Liliaceae [1]. It is widely used in Traditional Chinese Medicine as an antifebrile, alexipharmic, detumescent, demulcent and hemostatic, as well as in the treatment of hepatopathy [2]. It is the main component of the "Yun-nan-bai-yao" Traditional Chinese Medicine preparation. Many studies have shown that *Rhizoma Paridis* saponins (RPS) possess anti-tumor properties against a variety of malignant cell lines and in a diversity of animal models [3-12]. However, there is little information regarding which compound is responsible for these effects.

Diosgenin is a naturally occurring steroidal saponin present in many medicinal plants including *Dioscorea nipponica*. It has been found to attenuate allergen-induced intestinal inflammation [13]. Considering that RPS structure is similar to that of diosgenin, we used diosgenin as a positive control in this study.

Lipopolysaccharide (LPS), a component of the cell walls of gram-negative bacteria, induces the activation of monocytes and macrophages and involves the production of proinflammatory cytokines [14]. Nitric oxide (NO) is produced

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endogenously by a family of nitric oxide synthases (NOSs) with a wide range of physiological and pathophysiological actions [15, 16]. The inducible form (iNOS), which is expressed in various cell types, including macrophages, is induced in response to proinflammatory cytokines and bacterial LPS [17]. Excessive and prolonged iNOS-mediated NO generation has been linked to inflammation and tumorigenesis. Cyclooxygenase-2 (COX-2) is an inducible enzyme catalyzing the conversion of arachidonic acid to prostaglandins. Aberrant or increased expression of COX-2 has been implicated in many pathological conditions, including tumor promotion and angiogenesis [18, 19]. Diverse evidence from genetic and clinical studies has indicated that overexpression of COX-2 is associated with carcinogenesis [20-23]. These experimental data strongly suggest that inappropriate expression of COX-2 is a critical event in tumorigenesis. One practical implication of these findings is that sustained inhibition of iNOS and COX-2 may be of vital clinical importance in the prevention and therapy of many human cancers.

RPS have been found to inhibit cancer growth in vitro and in animal studies. However, which compound is responsible for these effects has not been well investigated. The purpose of this study was to investigate the suppression effects of RPS on the expressions of iNOS and cyclooxygenase 2 (COX-2). This study focused on the compounds of RPS. We employed Western blotting to analyze the expressions of proteins by RPS in RAW 264.7 macrophages to understand the anti-inflammatory effects of RPS.

Materials and Methods

Chemicals. The mouse macrophage-like cell line RAW 264.7 was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and fetal bovine serum (FBS) was purchased from HyClone (Logan, Utah, U.S.A.). RPMI 1640 medium and medium supplements for cell culture were obtained from GIBCO (Grand Island, N.Y., U.S.A.). LPS and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest quality available.

Preparation of RPS

RPS were purchased from Chengdu Mansite Biotechnology Co., Ltd. (China) and identified by one of the authors according to the China Pharmacopoeia (2005 edition). RPS were dissolved in 95% ethanol or DMSO. The solution was filtered with a 0.45 μm filter.

Cell culture and cell viability assay

RAW 264.7 macrophages (passage levels between 8 and 13) were maintained in RPMI-1640 medium supplemented with 1% penicillin-streptomycin and 10% (v/v) fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO2. Effects of compounds of RPS on cell viability were investigated using MTT [24]. RAW 264.7 macrophages were seeded onto 48-well plates, at 2×10^4 cells per well, and allowed to adhere to the plates overnight, after which the medium was refreshed. Cells underwent 24 h treatment with various concentrations of RPS compounds (0~10μM). All samples were dissolved in 95% ethanol, except compound A which was dissolved in dimethyl sulfoxide (DMSO). The final concentration of ethanol or DMSO added to the medium was below 0.1% (v/v). This concentration showed no influence on the assay system. The LPS (1 μg/mL) added medium was replaced with fresh medium containing 0.5 mg/mL MTT with incubation at 37°C for 4 h. After the reaction, the medium was removed and 100 μL of acidic isopropanol were added to the wells to solubilize the crystals. The optical density of each sample was read at 570 nm against the blank prepared from cell-free wells [25].

Immunoblot analysis

Western blotting was conducted according to the method described by Molina et al. (1999) [26] to measure iNOS and COX-2 protein expressions.

RAW 264.7 macrophages were plated onto 10 cm plates at 2×10^6 cells and allowed to adhere to the plates overnight. The medium was then refreshed. Subsequently, cells were treated with 1 μg/mL LPS and various concentrations of compounds of *RPS* (0~2.5 μM) for 24 h. Cell lysates (20 μg proteins) were resolved on 10% SDS-polyacrylamide gels and electroblotted on a polyvinylidine difluroide (PVDF) membrane. Membrane was blocked at room temperature for 1 h in 5% nonfat dry milk dissolved in TBS buffer (25 mM TRIS, pH 7.4, 150 mM NaCl, 0.3% Tween-20). Blot was incubated with anti-iNOS and anti-COX-2 specific monoclonal antibodies (Upstate Biotechnology Inc., Lake Placid, NY, U.S.A.) at 4°C overnight.

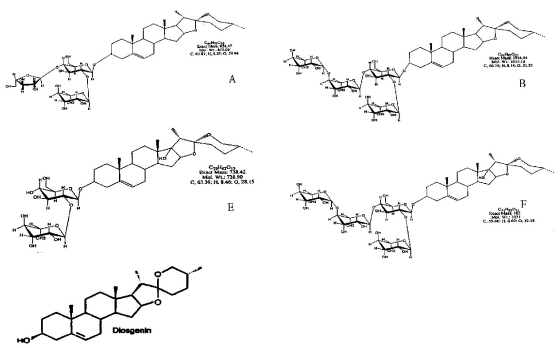

Fig. 1 Chemical structures of RPS.
Molecular structures of identified compounds (named "compounds A, B, E, and F") and diosgenin.

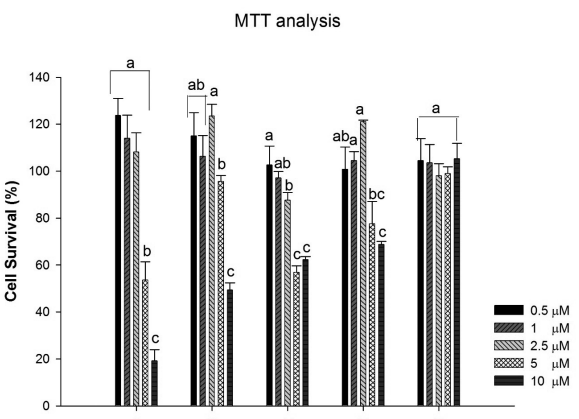

Fig. 2 The cell viabilities of RPS. Values are expressed as mean ±SD. Values within the same group bearing different letters (a, b) are significantly different (P<0.05). Compounds A, B, E, and F of RPS and diosgenin (positive control).

Incubation with appropriate HRP-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO, U.S.A.) was carried out at room temperature for 1 h. The reacted bands were revealed by enhanced chemiluminescence using an ECL commercial kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Blot was stripped and incubated with the anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO, U.S.A.) to ensure equal loading of protein samples and processed as previously described. For quantification, the intensities of protein signals were evaluated with Fujifilm Image Gauge software (ver. 3.46).

Statistical analysis

All results are presented as mean ± SD (n=3). Normality was assessed by the Kolmogorov-Smirnoff test. Differences were determined by tests for homogeneity of variance (Levene's test), one-way ANOVA (SPSS for Windows, version 10.0, SPSS Inc., Chicago, IL, USA), and Tukey's *post-hoc* test for multiple comparisons. Differences were considered significant at $P<0.05$. All reported P values were 2-sided.

Results

As shown in Fig. 1, the chemical structures of RPS (named "compounds A, B, E, and F") were identified as diosgenin based with between two and four bases (-glucosides).

Cell viability

To identify which class of RPS compounds is responsible for its observed biological activity, four RPS compounds and diosgenin were tested on MTT assay. The results showed that none of the samples affected the viability of RAW 264.7 cells at concentrations of 0.5~2.5μM (Fig. 2).

Inhibition of LPS-induced iNOS and COX-2 expressions by RPS

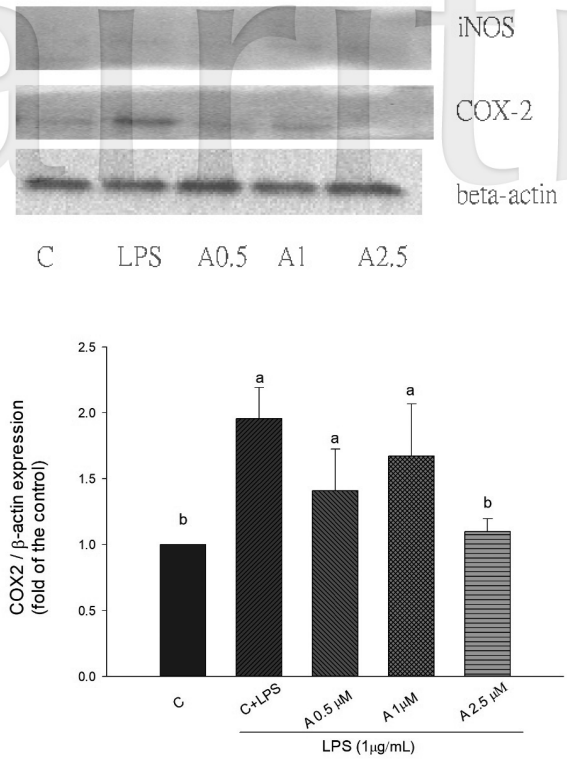

Fig. 3 Effects of compound A of RPS on LPS-induced iNOS and COX-2 expressions. RAW 264.7 macrophages were stimulated with 1 μg/mL LPS and treated with various concentrations of compound A (0~2.5μM) for 24 h. β-actin served as a loading control (n=3).

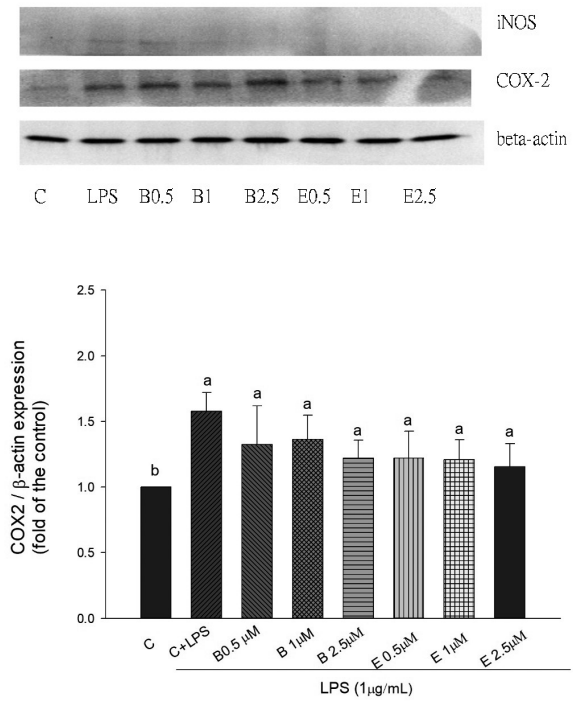

Fig. 4 Effects of compounds B and E of RPS on LPS-induced iNOS and COX-2 expressions. RAW macrophages were stimulated with 1 μg/mL LPS and treated with various concentrations of compounds B and E (0~2.5μM) for 24h. β-actin served as a loading control (n=3).

Immunoblot assay showed that iNOS protein is slightly induced in the presence of LPS in RAW 264.7 macrophages (Fig. 3~5). RPS and diosgenin had no significant effect on LPS-induced iNOS protein expression and iNOS data were not quantified. COX-2 protein was highly induced in the presence of LPS. Co-incubation of RPS with 1 μg/mL LPS inhibited the LPS-mediated induction of COX-2 protein, but not in a dose-dependent manner. Compounds A and F and diosgenin (positive control) showed better suppression effects (P<0.05) than the other compounds. At a concentration of 2.5μM, compound A showed the highest level of suppression.

Discussion

In China, *Rhizoma Paridis*, known as Chong-Lou, is prescribed by herbal practitioners to treat mastitis, sore throat, convulsions, tuberculous meningitis and tumors of the respiratory system, digestive tract, liver, pancreas, urinary bladder and brain, as well as leukemia. Many studies have shown that *Rhizoma Paridis* exhibits anti-tumor activity [27, 28]. However, most have focused on the cytotoxicity of the extract or specific saponin from *Rhizoma Paridis* [29-31]. Although RPS have been identified as the primary components responsible for the anti-tumor effects of this herb [29, 30], there is little information regarding which RPS compound possesses anti-inflammatory effect.

Four RPS compounds were analyzed in this study. We found that compounds of RPS inhibited cell growth in a dose-dependent manner, but did not affect viability of RAW 264.7 cells at concentrations of 0.5~2.5μM (Fig. 2). The overexpression of COX-2 has been implicated in carcinogenesis [32]. In this study, LPS (1 μg/mL) was used to induce COX-2 expression in RAW 264.7 macrophages. The results showed that RPS

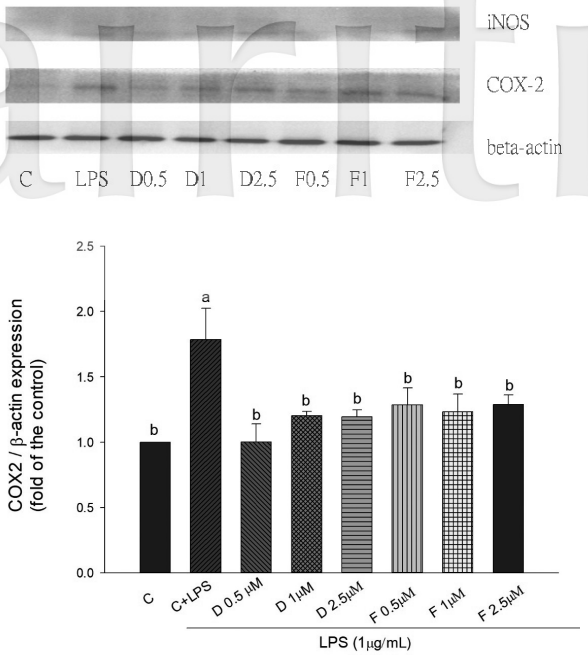

Fig. 5 Effects of compound F of RPS and diosgenin on LPS-induced iNOS and COX-2 expressions. RAW 264.7 macrophages were stimulated with 1 μg/mL LPS and treated with various concentrations of compound F and diosgenin (0~2.5μM) for 24 h. β-actin served as a loading control (n=3).

inhibit COX-2 expression. Compounds A and F showed better suppression of COX-2 than the other compounds. Compounds A and F mainly contain 3 and 4 bases (-glycosides), respectively. Compounds B and E in LPS-stimulated RAW 264.7 cells showed no significant effects (Fig. 1). This suggests that compounds with 3~4 bases (-glycosides) and more OH groups have better bioactivity in terms of suppressing COX-2 expression. This study demonstrated that RPS are active inhibitors of COX-2 expression in RAW 264.7 macrophages. Future animal studies and clinical trials are required to verify the results. In addition, amount of RPS needed to be consumed to prevent inflammatory diseases should be discussed in future studies.

Diosgenin is a naturally occurring steroidal saponin that is abundant in many medicinal plants. Little research has been conducted on the physiological metabolism and biological activity induced by structural differences in diosgenin and diosgenin glycosides. In this study, four diosgenin glycosides with different forms of glycosides and diosgenin with no glycone were investigated for their anti-inflammatory effects and showed sensitivity to the diosgenin constituting the glycoside and with more OH groups, as well as to the structures of the aglycons [33]. The results indicated that diosgenin and compounds A and F suppress COX-2 expression. Other molecular mechanisms and pathways require further investigation.

Conclusions

RPS inhibited LPS-induced COX-2 protein expression in RAW 264.7 macrophages. Compound A of RPS presented stronger effect than the other compounds. The results of this study suggest that RPS compounds with 3~4 bases (-glycosides) and more OH groups are significantly bioactive. However, the actual role of Compound A in chemoprevention requires further investigation.

Conflict of Interests

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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