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In-vitro Anti-inflammatory Activity of *Rubus coreanus* Miq. on Nitric Oxide, Interferon-γ, Cycloxygenase-2, and Tumor Necrosis Factor-α Production in the Macrophage like Cell Line RAW 264.7 Activated by Lipopolysccharide

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ABSTRACT : To search for immunoactive natural products exerting anti-inflammatory activity, we have evaluated the effects of the ethanol extracts of *Rubus coreanus Miq.* (ERC) on lipopolysaccharide-induced nitric oxide (NO), tumor necrosis factor-α (TNF-α), and Interferon-γ (IFN-γ) production by RAW 264.7 macrophage cell line. Our data indicate that this extract is a potent inhibitor of NO production and it also significantly decreased IFN-γ and TNF-α production. Consistent with these results, the protein level of inducible Nitric Oxide Synthase (iNOS) and cyclooxygenase-2 (COX-2) was inhibited by ethanol extracts of ERC in a dose-dependent manner. These results suggest that ERC may exert anti-inflammatory and analgesic effects possibly by suppressing the inducible NO synthase and COX-2 expressions.

Key words : *Rubus coreanus* Miq., nitric oxide, iNOS, TNF-α, IFN-γ

INTRODUCTION

Various in vivo and in vitro experimental models have been set up to assess inhibitory effects of natural products on the inflammatory mediators. Among these, RAW 264.7 mouse macrophage cells are an excellent model for the assessment of pro-inflammatory cytokines and reactive free radical mediators such as tumor necrosis factor (TNF-α), inducible nitric oxide synthase (iNOS), and nitric oxide (NO) (Shin *et al.*, 2004; Jang *et al.*, 2004). These are the major macrophage-derived inflammatory mediators and are also reported to be involved in the development of inflammatory diseases (Freeman and Natanson, 2000). Therefore, the inhibition of the excessive production of TNF-α and/or NO can be a critical point to evaluate anti-inflammatory effects of natural products (Pae *et al.*, 2003).

The most conclusive evidence for NO as a mediator of tissue injury has been obtained from studies on an animal arthritis model, human osteoarthritism, and rheumatoid arthritis (Cochran *et al.*, 1996). In contrast to iNOS, the constitutive epithelial and neuronal forms of NOS are known to contribute relatively little to inflammation and carcinogenesis. Cyclooxygenase (COX) is the enzyme that converts arachidonic acid to prostaglandins (PGs). Like NOS, COX has been found in two isoforms, and COX-2 is an inducible form responsible for the production of large amounts of proinflammatory PGs at the inflammatory site (Weisz *et al.*, 1996).

IFN-γ is an important immunoactivating cytokine which activates macrophages and dendritic cells and promotes Th1 and cytotoxic T cell differentiation from immature CD4 + T and CD8 + T cells, respectively. IFN-γ augments the expression of class I and class II MHC molecules and costimulators on APCs. (Hiromi Takaki *et al.*, 2006)

Furthermore, TNF-α is a potent proinflammatory cytokine that plays an important role in immunity and inflammation.

Rubus is one of a hundred genera in family Rosaceae, subfamily Rosoideae, tribr Potentilleae; there are 250 species of Rubus established across the world. Extracts of the leaves and fruit of Rubus species have been used in various countries as natural remedies to treat diabetes, infections, colic, and burns (Wang *et al.*, 1997). The dried unripe fruits of *Rubus coreanus* Miq.(Rosaceae), well known as 'Bok-bun-ja' in Korea, have been used for centuries as traditional medicine for the treatment of diabetes mellitus and sexual disinclination (Moon *et al.*, 1991).

But there are no data in the literature about anti-inflammatory effects. Thus, as a prelude to determining the underlying mechanisms of the anti-inflammatory effect of ERC, lipopolysaccharide (LPS)-induced NO and IFN-γ TNF-α production in the macrophage cell line RAW 264.7 have been investigated. Considering the use of ERC as an anti-inflammatory drug in the folk medicine, we evaluated the effects of distilled ethanol extract of the ERC on the production of TNF-α, IFN-γ, and NO in RAW 264.7 cells which were stimulated by LPS. The effects of ERC on the expressions of TNF-α, and IFN-γ in

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LPS-activated RAW 264.7 cells have been also studied to investigate the possible mechanisms of action in these studies.

MATERIALS AND METHODS

Preparation of the plant extract

Rubus coreanus Miq. (Compositae) have been bought dried *Rubus coreanus* Miq. fruit in dispensary of Oriental medicine. The air dried *Rubus coreanus* Miq. fruit (70 g) was extracted with 70% ethanol (800 mℓ) for 3 hr. repeated RAW 264.7 cell line and the extract was filtered, and the filtrate was concentrated under reduced pressure.

Reagents

LPS was obtained from Sigma Chemical Co. (St Louis, MO), DMEM medium, and 3-(4,5 dimethythiazol2-yl)-2,5-diphenytetrazoleum (MTT) were obtained from Wako. Fetal bovine serum(FBS) were purchased from Gibco. The antibiotics were from Gibco-BRL (Rockville, MD).

RAW 264.7 cell line and sample treatment

The murine macrophage cell line (RAW 264.7) was obtained from the ATCC (Manassas, VA). The cells were cultured in 10^2 ㎜ dish (Falcon-Becton Dickinson Labwares, Franklin Lakes, NJ) and maintained DMEM containing 10% heat- inactivated FBS, penicillin (100 units/mℓ), and streptomycin sulfate (100 units/mℓ) in a humidified atmosphere of 5% CO_2 at 37℃. The extract was dissolved in PBS and applied to the cell cultures at final concentrations of 0.25, 0.5, 1.0, 2.5 and 5.0 ㎍/mℓ alone or with 1 ㎍/mℓ of LPS.

Assessment of cell viability

Cytotoxicity studies were performed in 96-well plates. RAW 264.7 cells were mechanically scraped, plated at 2×10^5/well in 96-well plates containing 100 ㎕ of DMEM with 10% heat-inactivated FBS and incubated overnight. After overnight incubation, the cells were treated with teat materials, incubated for 24h and washed. Cells were washed once before adding 50 ㎕ of FBS-free medium containing 5 ㎎/mℓ of MTT. After 4 h of incubation at 37℃, the medium was discarded and the formazan blue that formed in the cells was dissolved in 100 ㎕ of DMSO. The optical density was measured at 540 ㎚.

Nitrite oxide determination

The nitrite accumulated in culture medium was measured as an indicator of NO production based on the Griess reaction. Briefly, 100 ㎕ of cell culture medium was mixed with 100 ㎕ of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl], incubated at room temperature for 10 min, and then the absorbance at 540 ㎚ was measured in a microplate reader. Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was measured with the sodium nitrite serial dilution standard curve.

Western blot analysis

Cellular proteins were extracted from control and *Rubus coreanus* Miq.-treated RAW264.7 cells. Cells were collected by centrifugation and washed once with phosphate buffered saline. The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES (pH 7.0), 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM PMSF, 0.5 mM dithiothreitol (DTT), 5 mM Sodium fluoride (NaF), 0.5 mM Sodium orthovanadate) containing 5 ㎍/mℓ each of leupeptin and aprotinin and incubated for 30 min at 4℃. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer's instruction. Fifty micrograms of cellular protein from treated and untreated cell extracts were electroblotted onto a nitrocellulose membrane following separation on 8-12% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4℃, followed by incubation for 1 h with a 1 : 1000 dilution of polyclonal antibodies against IFN-γ and TNF-α (Santa Cruz Biotechnology Inc.). Blots were washed two times with Tween20/Tris-buffered saline (TTBS) and incubated with a 1 : 1000 dilution of horseradish peroxidase conjugated goat-anti rabbit IgG secondary antibody (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Blots were again washed three times with TTBS and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, USA).

Statistical analysis

Data are reported as mean ± S.D. values of three independent determinations. All experiments were performed at least three times, each time with three or more independent observations. Statistical analysis was performed using Student's t-test with one-way analysis of variance.

RESULTS

Effects of *Rubus coreanus* Miq. (ERC) on LPS-Induced NO production.

Treatment of RAW 264.7 macrophages with ERC did not show any cytotoxicity (Table 1). The endotoxin LPS at 1 ㎍/mℓ reduced the viability of RAW 264.7 macrophages. LPS in the presence of ERC did not further affect the viability of RAW

Table 1. Effect of ERC on RAW 264.7 macrophage cells viability.

Sample	Viability (%)
Control	100
LPS	41.21 ± 2.11
L+ERC 0.25 μg/μℓ	65.70 ± 3.10*
L+ERC 0.5 μg/μℓ	66.57 ± 3.21*
L+ERC 1.0 μg/μℓ	87.90 ± 2.35**
L+ERC 2.5 μg/μℓ	63.11 ± 2.22*
L+ERC 5.0 μg/μℓ	95.390 ± 1.22**

Cell viability was measyred by MTT assay. RAW264.7 macrophage cells were treated with LPS (1 μg/mℓ) alone or LPS plus increasing concentrations of ERC (0.25, 0.5, 1.0, 2.5 and 5.0 μg/mℓ) for 24 h. The values are the mean ± S.D. from three independent experiments. *$p<0.05$, **$p<0.005$ *vs.* LPS-treated group.

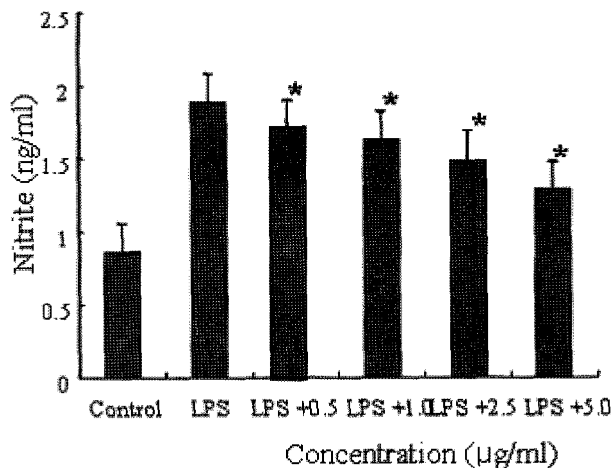

Fig. 1. Concentration-dependent effects of ERC on NO production in RAW267.4 cells. The cells were left untreated or treated with LPS (1 μg/mℓ) and ERC (0.25, 0.5, 1.0, 2.5 and 5.0 μg/mℓ) for 24 h, and the production of NO was evaluated by Griess reaction, respectively. L-N[6]-(1-iminoethyl) lysine (L-NIL) (10 μM) or NS-398 (10 μM) was used in the assay as a positive control (data not shown). The values are the mean ± S.D. from three independent experiments. *$p < 0.05$, **$p < 0.005$ *vs.* LPS-treated group.

264.7 cells. ERC reduced the LPS-induced NO production in a dose-dependent manner (Fig. 1).

Effects of *Rubus coreanus* Miq. on the expression TNF-α and IFN-γ expression.

Western blot analysis was conducted to determine whether the inhibitory effects of ERC on stimulated RAW 264.7 cells related to the modulation of TNF-α, and IFN-γ expression (Fig. 2). In response to LPS, the expression levels of TNF-α, and IFN-γ were markedly increased. Pretreatment of RAW 264.7 cells with ERC significantly decreased TNF-α, and IFN-γ expression in a dose-dependent manner. These results are consistent with the profile of the inhibitory effect of the ERC on TNF-α, and IFN-γ production.

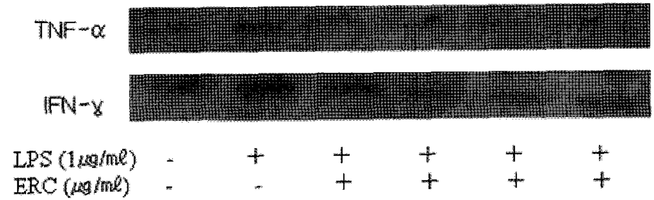

Fig. 2. Concentration-dependent effects of ERC on LPS-induced TNF-α and IFN-γ protein expression in RAW 264.7 cells. Lysates were prepared from control or stimulated cells treated for 24 h with LPS (1 μg/mℓ) alone or in combination with increasing concentrations (0.25, 0.5, 1.0, 2.5 and 5.0 μg/mℓ) of ERC. All lanes contained 200 μg of total proteins. The experiment was repeated three times with similar results.

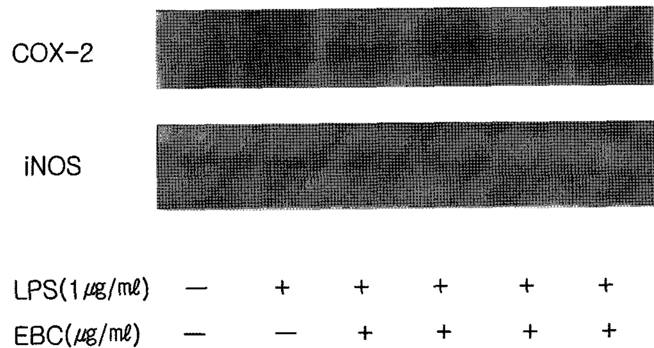

Fig. 3. Concentration-dependent effects of ERC on LPS-induced iNOS and COX-2 expression in RAW 264.7 cells. RAW264.7 macrophage cells were treated with LPS (1 μg/mℓ) alone or LPS plus increasing concentrations of ERC (0.25, 0.5, 1.0, 2.5 and 5.0 μg/mℓ) for 24 h for the assessment of iNOS and COX-2 protein expression, respectively. The experiment was repeated three times with similar results.

Effects of *Rubus coreanus* Miq. on the expression iNOS and COX-2 expression.

Western blot analysis was conducted to determine whether the inhibitory effects of ERC are related to the modulation of iNOS and COX-2 (Fig. 3) expression. A inhibitory effect of ERC was observed on the LPS-induced iNOS and COX-2 protein levels. The expression of housekeeping gene β-actin was not affected by ERC. These results are consistent with the profile of the inhibitory effect of the ERC on NO, and TNF-α production (Fig. 1 and 2).

DISCUSSION

Because the mechanism of the anti-inflammatory effects of ERC has not been reported, we examined the effects of ERC on the release of inflammatory mediators (NO, IFN-γ, and

TNF-α) in LPS-activated macrophages. ERC inhibited LPS-activated NO production and IFN-γ and TNF-α induction in murine macrophage cell in concentration-dependent manners. LPS induces septic shock syndrome and stimulates the production of inflammatory mediator such as NO (Muller *et al.,* 2000). NO possesses cytotoxic properties that are aimed against pathogenic microbes, but it can also have damaging effects on host tissues (Korhonen *et al.,* 2005). Present study showed that ERC could decrease the LPS-mediated production of NO.

The mechanisms of various anti-inflammatory drug actions are related with the induction of PG synthesis, induced by COX (Vane, 1971). Among COX group, COX-2 is considered to be responsible for proinflammatory PG production (Seibert *et al.,* 1994: Masferer, 1994). In our results, ERC significantly inhibited COX-2 expression at protein levels. From these results, it is suggested that ERC may suppress the proinflammatory effect of COX-2 expression.

In the resting murine macrophages, TNF-α expression is not detected at the protein levels. Inflammatory stimulus like LPS strongly induces the translation of TNF-α. Now, our current results indicate that ERC may interfere the translation of TNF-α, which results in the suppression of TNF-α protein synthesis. These results clearly demonstrate the anti-inflammatory effects of ERC as well as the possibility of therapeutic use of ERC. However, further studies on the precise mechanism of action and the isolation/characterization of active chemical constituents are needed.

The cytokine IFN- plays an essential role in innate and adaptive immunity. IFN signaling, which involves a variety of trans-γ and cis-acting factors, is mediated through DNA motifs, IFN-activated sequence, which are found in promoters of IFN-inducible genes.(Chen *et al.,* 1996) So Inflammatory stimulus like LPS strongly induces both the transcription and the translation of IFN-γ. When treated with ERC and IFN-γ was suppressed in the LPS-stimulated RAW 264.7 macrophages.

In conclusion, we present an evidence that ERC inhibits the production of NO, IFN-γ, and TNF-α in the LPS-stimulated RAW 264.7 macrophages at least in part by interfering with iNOS, and TNF-α protein.

Acknowledgements

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