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Scoparone from *Artemisia capillaris* Inhibits the Release of Inflammatory Mediators in RAW 264.7 Cells upon Stimulation Cells by Interferon-γ Plus LPS

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Scoparone is a major component of the shoot of *Artemisia capillaris* (Compositae), which has been used for the treatment of hepatitis and biliary tract infection in oriental countries. In the present study we observed that, scorparone exhibited no cytotoxic effect in unstimulated macrophages, but reduced the release of nitric oxide (NO) and prostaglandin E_2 (PGE_2) upon stimulation by IFN-γ/LPS or LPS. The inhibitory effects were found to be in conjuction with the suppression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in IFN-γ/LPS stimulated RAW 264.7 cells. Moreover, scoparone also attenuated the production of tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 in LPS-stimulated RAW264.7 cells. These results suggest that scoparone decreases the production of the inflammatory mediators such as NO and PGE_2 in macrophages by inhibiting iNOS and COX-2 expression.

Key words: Scoparone, *Artemisia capillaries,* Nitric oxide, PGE_2, iNOS, COX-2

INTRODUCTION

Nitric oxide (NO) and prostaglandin E_2 (PGE_2) are well known to be involved in the development of inflammation (Lee *et al.*, 1992; Moncada *et al.*, 1991; Nathan, 1997; Sautebin, 2000; Wheeler and Bernard, 1999). These two inflammatory mediators are associated with the expression nitric oxide synthase (NOS) and cyclooxygenase (COX). The three distinct isoforms of NOS have been identified as neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Michel *et al.*, 1995). Like NOS, two types of COX, constitutive cyclooxygenase-1 (COX-1) and inducible cyclooxygenase-2 (COX-2), have been isolated and characterized (Smith *et al.*, 1996). Amongst these isoforms, iNOS and COX-2 are induced by cytokines and outer bacterial toxins such as lipopolysaccharide (LPS) and lipoteichoic acid (Penglis *et al.*, 2000; Yamashita *et al.*, 2000). Besides, pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 are interlinked with the production of small inflammatory mediators like NO and PGE_2, and thus contribute in eliciting inflammatory response (Dinarello, 1999; Feldmann *et al.*, 1996; Hagan *et al.*, 1993; Mannel and Echtenacher, 2000; Straub *et al.*, 2000). Accordingly, agents that block bacterial toxin-induced production of NO, PGE_2 or pro-inflammatory cytokines might be beneficial in the treatment of inflammatory responses.

Scoparone (6,7-Dimethoxy coumarin) (Fig. 1) is a major component of the shoot of *Artemisia capillaris* Thunb. (Compositae), which has been used as antipyretic, anti-l flammatory, diuretic and choletica for the treatment of hepatitis and bilious disorder (Chang and But, 1987). Several studies have demonstrated the free radical scavenging, immunosuppressive and vasodilator activities of scoparone (Huang *et al.*, 1991, 1992b). Moreover,

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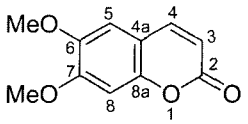

Fig. 1. Chemical structure of scoparone isolated from *Artemisia capillaris*

scoparone has been shown to reduce IL-1 and IL-2 production and IL-2 receptor expression in human peripheral blood mononuclear cells (Huang *et al.*, 1992a). However, the action of scoparone on interferon-β (IFN-β) plus LPS-induced NO, PGE_2 and pro-inflammatory cytokines production is still undefined. Hence, we in the npresent studt we have investigated the effect of scoparone on the production of NO, PGE_2, and pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and the expression of iNOS and COX-2 in IFN-γ/LPS or LPS stimulated RAW 264.7 cells.

MATERIALS AND METHODS

Chemicals and reagents

Dulbeco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and antibiotics were purchased from GIBCO BRL (Grand Islang, NY). Interferon-β and N^G-monomethyl-L-argine (N^GMMA) were obtained from Genzyme (Cambridge, MA). Rabbit anti-iNOS and rabbit anti-COX-2 antibodies were obtained from Santacruz Biotechnology (Santa Cruz, CA). Tween 20, LPS (phenol extracted *Salmonella entritidis*), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl-thiazol-2-yl)2-,5-diphenyltetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Co. (ST Louis, MO). Immunoassay kits (Quantikine™) for TNF-α, IL-1β, IL-6 and PGE_2 were purchased from R&D System (Minneapolis, MN, USA). Ninety-six well tissue culture plate and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD). All other reagents were tested for their LPS content with the use of a colorimetric Limulus amoebocyte lysate assay (detection limit, 10 pg/mL; Whitaker Bioproducts, Walkersville, MD).

Plant materials

The shoots of *Artemisia capillaris* Thunb were purchased from the herbal medicine co-operative association of Jeonbuk Province, Korea, in October 2003. A voucher specimen (no. WP344) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea).

Extraction and isolation

Dried shoots of *A. capillaris* (1 kg) were extracted with hot water (10 L) for 2 h. Water extract was concentrated and partitioned with *n*-BuOH to obtain *n*-BuOH soluble fraction (28.0 g). This fraction was suspended in 60% aqueous MeOH and partitioned with CH_2Cl_2 to give 10.6 g of CH_2Cl_2 soluble fraction, which was chromatographed on a silica gel (500 g) column with *n*-hexane: acetone (3:1) as mobile phase to obtain three subfractions (Fr. A-C). Fraction B (3.24 g) was further separated by a silica gel column (eluent: *n*-hexane:acetone, 8:1) to yield scoparone (180 mg; w/w% = 0.018). The obtained scoparone was identified by the comparison of its spectral data (MS, ^{1}H- and ^{13}C-NMR) with the reported data (Valenciennes *et al.*, 1999). The spectral data of scoparonr was: ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.76 (3H, s, 7-OCH_3), 3.84 (3H, s, 6-OCH_3), 6.42 (1H, J = 9.6, H-3), 7.03 (1H, s, H-8), 7.54 (1H, s, H-8), 7.54 (1H, s, H-5), 7.63 (1H, d, J = 9.6, H-4); ^{13}C NMR (125 MHz, $CDCl_3$): δ 56.2 (7-O<u>C</u>H$_3$), 56.4 (6-O<u>C</u>H$_3$), 108.9 (C-5), 114.2 (C-8), 114.4 (C-4), 121.1 (C-4a), 137.4 (C-8a), 140.8 (C-4), 142.6 (C-7), 144.2 (C-6), 161.2 (C-2); ESI-MS: *m/z* 207 (100%, M + H$^+$).

Cell culture

Murine macrophage RAW 264.7 cell line, obtained from American Type culture Collection (ATCC, TIB 71, Maryland, USA), were maintained at 1×10^6 cells/mL cultures in DMEM supplemented with 10% heat inactivated FBS, penicillin G (100 IU/mL), streptomycin (100 mg/mL), and L-glutamine (2 mM), and were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. On the following day, the medium was replaced with fresh DMEM, and the cells were then stimulated with IFN-γ (10 U/mL) plus LPS (100 ng/mL) or LPS alone (100 ng/mL) in the presence or absence of scoparone (dissolved in 100% DMSO and then diluted with medium to a final concentration of = 0.1%) for the as indicated time periods.

MTT assay for cell viability

Murine macrophage cells were plated at a density of 2×10^5 cells/mL into 96 well plate containing 100 mL of new media. After the addition of 1-50 mg/mL of scoparone, the plates were incubated for 24 h. The MTT assay was performed as the procedure described previously (Jang *et al.*, 2004).

Nitrite assay

Accumulated nitrite, an oxidative product of NO, was measured in the culture medium by Griess reaction (Becherel *et al.*, 1997; Kang *et al.*, 1999) according to the method described previously (Jang *et al.*, 2004).

PGE_2, TNF-α, IL-1β and IL-6assay

Murine macrophage cells (1×10^6/mL) were pre-incubated with scoparone for 2 h and were further cultured for 6 or 18 h with IFN-γ (10 U/mL) plus LPS (10

ng/mL) or LPS (100 ng/mL) alone in 24-well plates. Supernatants were removed at the allotted times and PGE_2, TNF-α, IL-1β and IL-6 levels were quantified by immunoassay kits according to the manufacture's protocols, respectively.

Western blot

Cellular proteins were extracted from the control and scoparone-treated RAW264.7cells. The washed cell pellets were resuspended in hypotonic buffer (10 mM HEPES, 10 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, pH 7.4) and incubated for 15 min at 4°C. Nuclei and cell debris were removed by microcentrifugation followed by quick freezing of the supernatants. Fifty micrograms of cellular protein from treated and untreated cell extracts were electroblotted onto nitrocellulose membrane and were separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4°C, followed by incubation with appropriate dilutions of primary antibodies (against rabbit anti-iNOS, rabbit anti-COX-2) for 4 h. Blots were washed twice with PBS and were incubated with 1: 5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h at room temperature. Subsequently, the blots were washed thrice in Tween 20/Tris-bufferd saline, developed with 10 mL of a 1:1 mixture of solutions of ECL detection system for 1 min, dried quickly, and exposed to a film for 2-20 min. Protein concentration was determined by Bio-Rad protein assay reagent according to the manufacture's instruction.

Data analysis

All values were expressed as the mean ± S.D. of three independent determinations. All experiments were done at least three times, each time with three or more independent observations. Statistical analysis was performed with analysis of variance (ANOVA) and Student's *t*-test.

RESULTS AND DISCUSSION

Scoparone, a main constituent of the shoot of *Artemisia capillaris*, has been studied for its anti-inflammatory effect. It has been well known that NO, PGE_2 and pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are involved in the development of inflammation. Initially, we investigated the effects of scoparone on the release of two inflammatory mediators, NO and PGE_2. When compared with the untreated control, IFN-γ (10 U/mL) plus LPS (100 ng/mL) induced an enormous increase in nitrite production in RAW 264.7 cells. The IFN-γ/LPS-induced NO accumulation was markedly suppressed by a NO synthesis inhibitor, N^GMMA, at a concentration of 2 mM (Fig. 2A). Scoparone also apparently inhibited the production of NO in the macrophages activated with IFN-γ/LPS in a concentration dependent manner (Fig. 2A). However, scoparone did not interfere with the reaction between nitrite and Griess reagents at a concentration of 50 mg/mL (data not shown). Similar results were obtained on the effects of scoparone (1-50 μg/mL) on PGE_2 synthesis in IFN-γ/LPS-stimulated RAW264.7 cells (Fig. 2B). These results clearly indicated that scoparone had a concentration dependent inhibitory effect on NO and PGE_2 production in response to IFN-γ/LPS without

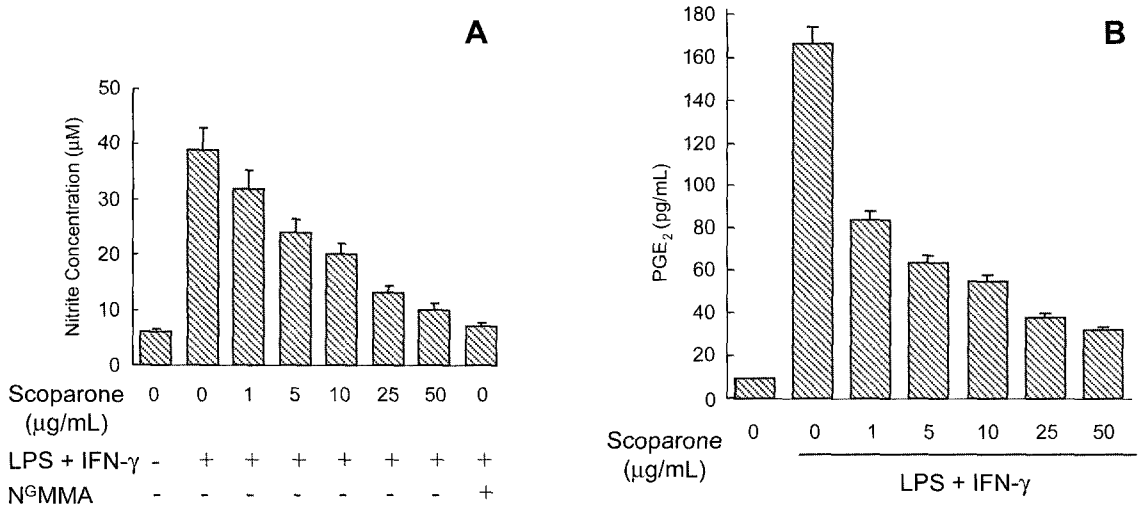

Fig. 2. Effect of scoparone on IFN-γ/LPS-induced NO and PGE_2 production in RAW 264.7 cells. Cells were incubated with or without IFN-γ (10 U/mL) plus LPS (100 ng/mL) for 18 (NO) or 6 h (PGE_2) in the presence or absence of scoparone at indicated concentrations. Nitrite accumulation (A) and PGE_2 production (B) in the culture medium were determined as described in Materials and Methods. Each column represents the mean±S.D. from three independent experiments. ** $P<0.01$ indicates significant differences from the IFN-γ/LPS treated group.

exhibiting any cytotoxicity.

To elucidate the inhibitory mechanism of scoparone, we further investigated its effect on iNOS and COX-2 expression. In response to IFN-γ/LPS, the expression level of iNOS was significantly reduced by scoparone in a concentration-dependent manner (Fig. 3A). A similar behavior was observed from the study on the effect of scoparone on the IFN-γ/LPS-induced COX-2 expression. Thus, the COX-2 expression induced by IFN-γ/LPS was significantly inhibited in the cells treated with scoparone

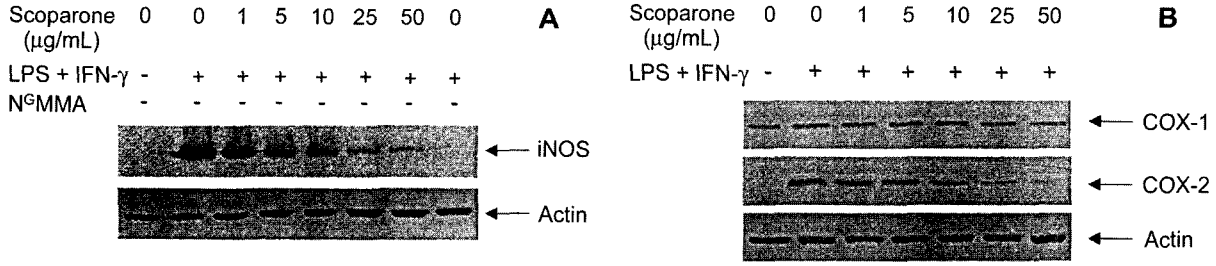

Fig. 3. Effect of scoparone on the expression of iNOS (A) and COX-2 (B) in RAW 264.7 cells. Cells were incubated with or without IFN-γ (10 U/mL) plus LPS (100 ng/mL) inn the presence or absence of scoparone at indicated concentrations. Western blot analysis was carried out as described in Materials and Methods.

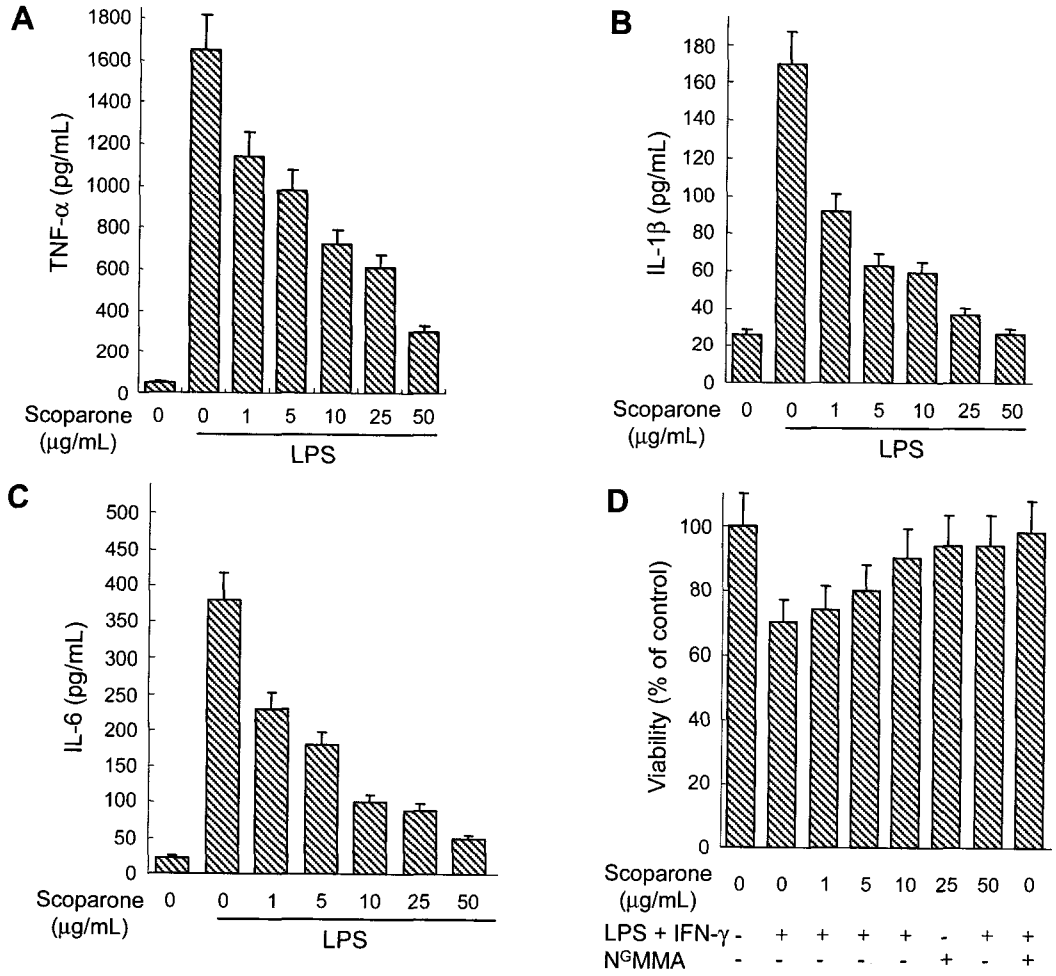

Fig. 4. Effect of scoparone on LPS-induced cytokine production and on IFN-γ/LPS induced cytotoxicity in RAW 264.7 cells. Cells were incubated with or without 100 ng/mL LPS for 6 (TNF-α and IL-6) or 12 h (IL-1β) or with 10 U/mL IFN-γ plus 100 ng/mL LPS for 24 h (cytotoxicity) in the presence or absence of scoparone at indicated concentrations. The productions of TNF-α (A), IL-β (B) and IL-6 (C), and the cell viability (D) in the macrophages were determined as described in Materials and Methods. Each column represents the mean ±S.D from three independent experiments. **P< 0.01 indicates significant differences from the IFN-γ/LPS treated group.

(Fig. 3B). These findings indicate that scoparone has inhibitory effect on IFN-γ/LPS-induced NO and PGE_2 generation in RAW 264.7 cells by suppressing iNOS and COX-2 protein expression.

A body of evidence has indicated that pro-inflammatory cytokines such as IL-1, TNF-α and IL-6 control inflammation both *in vitro* and *in vivo* (Dinarello, 1999; Feldmann *et al.*, 1993; Harada *et al.*, 1994; Mannel and Echtenacher, 2000; Straub *et al.*, 2000). These cytokines appear to be interlinked in a cascade which is produced serially by macrophages during an inflammatory response. It has been recently reported that exposure to LPS can cause inflammatory liver damage and septic shock due to the high production levels of cytokines (Mojena *et al.*, 2001). The present study showed that the production of major macrophage-derived pro-inflrmmatory cytokines like TNF-α, IL-1β and IL-6 was significantly inhibited by scoparone in a concentration dependent manner in LPS-activated RAW264.7 cells (Fig. 4A-4C).

In addition, the cytotoxic effect of scoparone was evaluated in the absence or presence of IFN-γ/LPS. When treated alone, scoparone did not affect the cell viability at the employed concentration range (1-50 μg/mL) (data not shown). However, scorparone increased cell viability of IFN-γ/LPS activated macrophages upon comparison to IFN-γ/LPS activated cells (Fig. 4D).

On the other hand, nuclear factor-$_k$B (NF-$_k$B) response elements have been indicated to be essential for the expression iNOS and COX-2, which are involved in inflammatory process by producing NO and PGE_2, respectively (Barnes and Karin, 1997; Kotake *et al.*, 1998; Roshak *et al.*, 1996; Xie *et al.*, 1994). Therefore, further investigations need to be carried out to confirm whether scoparone suppresses NF-$_k$B activation and components leading to NF-$_k$B activation.

In summary, we have demonstrated that scoparone, isolated from *Artemisia capillaris*, inhibits the production of NO, PGE_2, TNF-α, IL-1β, IL-6 and affects the expression of iNOS and COX-2 in IFN-γ/LPS or LPS stimulated RAW 264.7 cells. From these observations, it is expected that scoparone may contribute to the anti-inflammatory activity of the shoot of *Artemisia capillaris*, which would be useful for the treatment of inflammatory diseases.

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