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Anti-Inflammatory Mode of Isoflavone Glycoside Sophoricoside by Inhibition of Interleukin-6 and Cyclooxygenase-2 in Inflammatory Response

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Soy, high dietary intake for the oriental population, is a main source of isoflavonoids. Sophoricoside (SOP) an isoflavone glycoside was isolated from immature fruits of *Sophora japonica* (Leguminosae family) and its inhibitory effect on chemical mediators involved in inflammatory response was investigated in this study. SOP inhibited the interleukin (IL)-6 bioactivity with an IC_{50} value of 6.1 μM whereas it had no effects on IL-1β and TNF-α bioactivities. SOP was identified as a selective inhibitor of cyclooxygenase (COX)-2 activity with an IC_{50} value of 4.4 μM, but did not show inhibitory effect on the synthesis of COX-2. However, SOP had no effect on the production of reactive oxygen species including superoxide anions and nitric oxide. These results revealed that *in vitro* anti-inflammatory action of SOP is significantly different from that of genistein known as a phytoestrogen of soy products. This experimental study has documented an importance of dietary soy isoflavonoids as multifunctional agents beneficial to human health, and will help to clarify protective mechanisms of SOP against inflammatory conditions.

Key words: *Sophora japonica*, Sophoricoside, Anti-inflammation, Interleukin-6, Cyclooxygenase

INTRODUCTION

Flavonoids, regular constituents of the diet, were first identified as vitamin P, and together with vitamin C, they were found to be important in the maintenance of capillary wall integrity and capillary resistance (Detre *et al.*, 1986; Gabor, 1988). Interest has recently increased on the flavonoids because they show broad pharmacological activities including antihypertensive, antiarrhythmic, anti-inflammatory, antiallergic, hypochlolesterolemic, antihepatotoxic and antitumor properties (Rao *et al.*, 1994; Harbone and Williams, 2000; Wenzel *et al.*, 2000; Duarte *et al.*, 2001).

Soy is a main source of isoflavonoids, and its daily intake is very high to oriental population (Wu *et al.*, 1998). Genistein (Fig. 1) is well known as a phytoestrogen of soy products, and was reported to prevent cancer and osteoporosis (Wu *et al.*, 1998; Messina, 1999; Wiseman, 2000). Sophoricoside (SOP) known as an isoflavone glycoside (Fig. 1) was isolated from *Sophora japonica*, a plant of Leguminosae family in our previous work (Min *et al.*, 1999). In this study, anti-inflammatory action of SOP on chemical mediators in inflammatory response has been demonstrated.

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MATERIALS AND METHODS

Materials

Lipopolysaccharide (LPS, *E. coli* 0127:B8) was purchased from Difco (Detroit, USA), human interleukin (IL)-6 from Wako (Osaka, Japan), and fetal bovine serum (FBS) from HyClone (Logan, USA). An ELISA kit for TNF-α was purchased from Amersham-Pharmacia (Piscataway, USA), a Fast RNA kit from Qbiogene (Carlsbad, USA), and a RNA PCR kit from Takara (Shiga, Japan).

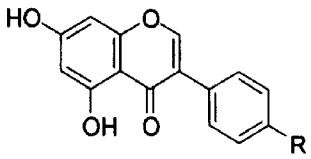

Fig. 1. Chemical structures of sophoricoside and genistein. Substituents are a glucose moiety (OGlc) and a hydroxyl group (OH).

SOP was isolated from *Sophora japonica* in our previous work (Min *et al.,* 1999) and its purity is >97%. All other reagents were obtained from Sigma-Aldrich (St. Louis, USA).

Bioassay of proinflammatory cytokines

IL-1β bioassay was carried out as a parameter of antiproliferation of human melanoma A375.S2 by the cytokine. Briefly, 100 μL of the A375.S2 cells (2×10^4 cells/mL) in DMEM containing 10% FBS were dispensed into a 96-well culture plate, and added with 50 μL of human IL-1β (2.5 ng/mL) and 50 mL of the medium with or without SOP. After incubation at 37°C with 5% CO_2 for 96 h, viability of the cells was determined by optical density at a wavelength of 450 nm using the WST-1 method (Huhtala *et al.,* 2003). IL-6 dependent proliferation of hybridoma MH60/BSF-2 or TNF-α dependent antiproliferation of human fibrosarcoma WEHI-164 was carried out as other cytokine bioassays (Yun *et al.,* 2000).

ELISA of TNF-α

Two hundred μL of murine macrophages Raw264.7 (1×10^5 cells/mL) in DMEM containing 10% FBS were dispensed into a 96-well culture plate and incubated at 37°C with 5% CO_2 for 48 h. After washing with PBS, the cells were added with 100 μL each of LPS (2 μg/mL) and SOP, and then incubated at 37°C with 5% CO_2 for 24 h. After centrifugation at 700×g for 30 min at 4°C, supernatant was used to determine its TNF-α content with an ELISA kit according to the supplier's instruction (Amersham-Pharmacia).

Assay of COX activity

Microsomal fraction of bovine seminal vesicles or LPS-activated murine macrophages Raw264.7 was used as the cyclooxygenase (COX)-1 or COX-2 source, respectively (Cadenas & Sies, 2000). COX activity was measured by chemiluminescence emitting for 30 s in total 200 μL reaction mixture of 0.1 M Tris-HCl (pH 8.0) containing enzyme source, 100 μM arachidonic acid as substrate and 25 μM luminol as enhancer.

Measurement of NO production

Nitrite content in the culture medium was analyzed as an index for nitric oxide (NO) production. Two hundred μL of murine macrophages Raw264.7 (1×10^6 cells/mL) in DMEM containing 10% FBS were dispensed into a 96-well culture plate and incubated at 37°C with 5% CO_2 for 24 h. After washing twice with PBS, Raw264.7 cells were added with 100 μL each of LPS (2 μg/mL) and SOP, and then incubated at 37°C with 5% CO_2 for 24 h. After centrifugation at 700×g for 30 min at 4°C, 100 μL of the supernatant was reacted with the same volume of the Griess reagent and then nitrite content was measured by absorbance at a wavelength of 540 nm. Sodium nitrite was used as a standard.

RT-PCR of COX-2 and iNOS transcripts

Two mL of murine macrophages Raw264.7 (5×10^5 cells/mL) were dispensed into a 6-well plate, and then incubated at 37°C with 5% CO_2 for 48 h. After washing with PBS, the cells were added with 1 mL each of LPS (2 μg/mL) and SOP, and then incubated at 37°C with 5% CO_2 for 6 h. Raw264.7 cells were collected to purify total RNA by using a kit according to the supplier's instruction (Qbiogene). The total RNA was subjected to RT-PCR for obtaining amplified COX-2 and inducible nitric oxide synthase (iNOS) transcripts by using a RNA PCR kit according to the supplier's instruction (Takara). Briefly, total cellular RNA (2 μg) was used for RT with Avian myeloblastosis virus reverse transcriptase at 50°C for 25 min. The resulting cDNA samples were PCR amplified for 25 cycles, one cycle with 30 s denaturation at 94°C, 30 s annealing at 65°C, and 2 min extension at 72°C. The PCR primers were designed as follows; COX-2 (583 base pairs) with forward 5'-ACTCACTCAGTTTGTTGAGTCATTC -3' and reverse 5'-TTTGATTAGTACTGTAGGGTTAATG-3', iNOS (457 base pairs) with forward 5'-GTCAACTGCA AGAGAACGGAGAAC-3' and reverse 5'-GAGCTCCTC CAGAGGGTAGGCT-3', and β-actin (745 base pairs) as an internal control with forward 5'-CACCACACCTTCTACAAT GACCTGC-3' and reverse 5-GCTCAGGAGGAGCAATGA TCTTGAT-3'. RT-PCR products were resolved on 1.5% agarose gel by electrophoresis, and quantified by scanning densitometry (Kodak).

Measurement of reactive oxygen species

Human monocytes were isolated from venous blood of healthy adult donors by centrifugation with a histopaque (d=1.08). Fifty μL of the monocytes (2×10^6 cells/mL) and 50 μL of 0.1 mM lucigenin were added to a white 96-well microplate. After incubation for 15-30 min, 50 μL of unopsonized zymosan (*S. cerevisiae*, 1.2 mg/mL) or phorbol myristate acetate (PMA, 0.4 μg/mL) and 50 μL of SOP were added to the cells. Chemiluminescence emitted from each of the wells was immediately measured as relative light units at 37°C in the dark for 120 min at 5-min intervals using a luminometer (EG & G Berthold).

Chemiluminescence response was obtained as an integrated area below the resulting chemiluminescence curve.

Neutrophils were collected from peritoneal lavages in 1% casein-treated male rats of Sprague-Dawley strain, and then subjected to sonication followed by centrifugation to obtain a supernatant as a source of myeloperoxidase (MPO). The MPO activity was determined as the initial rate of increase in absorbance at a wavelength of 485 nm in total 200 μL reaction mixture of 10 mM sodium phosphate buffer (pH 7.4) containing 40 μL each of 44 mM hydrogen peroxide, the enzyme source, 0.1% *o*-phenylenediamine and SOP.

Statistical analysis

Data were collected as control %, means±SEM of three independent tests assayed in triplicate or duplicate. Statistical significance was analyzed by using the unpaired, two-tailed *t* test. A probability of less than 0.001 was considered significant.

RESULTS

Effect of SOP on proinflammatory cytokine

SOP showed differential inhibitory effects on proinflammatory cytokines. SOP did not inhibit IL-1β and TNF-α bioactivities up to 50 μM concentration (Table I). SOP showed inhibitory effects with 61.0±2.5% of the control (100%) at 3.1 μM, 49.9±3.8% at 6.3 μM, 36.0±2.5% at 12.5 μM, 6.3±2.7% at 25 μM, and 3.0±2.1% at 50 μM on IL-6 bioactivity (Fig. 2A). As a positive control, madindoline A showed an IC_{50} value of 8.5 μM on the IL-6 bioactivity (data not shown). However, SOP at 12.5-50 μM concentrations did not inhibit TNF-α release in LPS-stimulated murine macrophages Raw264.7, significantly (Fig. 2B). The resting Raw264.7 cells released 3.2±4.7 ng/mL of TNF-α as a basal level in the medium, and increased to 53.8±6.5 ng/mL of TNF-α after stimulation with LPS. When treated with 12.5 μM or 50 μM SOP plus LPS, the macrophages Raw264.7 showed 48.3±4.5 ng/mL or 47.5 ±4.3 ng/mL of TNF-α in the medium, respectively.

Table I. Effects of sophoricoside on IL-1β and TNF-α bioactivities

Parameter	Inhibition (%)	
	50 μM	12.5 μM
IL-1β bioactivity	8.5±2.4	0.5±2.9
TNF-α bioactivity	5.7±0.4	2.7±4.4

IL-1β bioactivity was measured as antiproliferation of melanoma A375.S2 by human IL-1β, and TNF-α as antiproliferation of fibrosarcoma WEHI-164 by human TNF-α.

Each data represents mean±SEM of three independent tests assayed in triplicate.

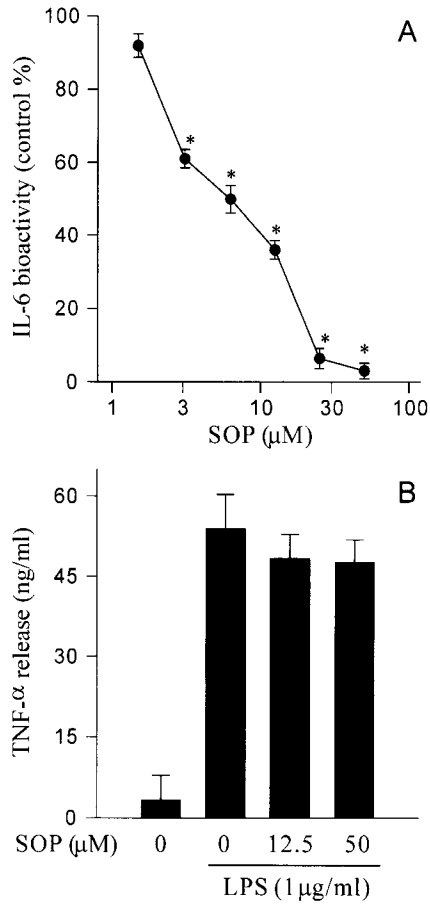

Fig. 2. Effects of sophoricoside on IL-6 bioactivity and TNF-α release. Inhibitory effect of sophoricoside (SOP) on IL-6 bioactivity using hybridoma MH60/BSF-2 is represented as control %, mean±SEM of three independent tests assayed in triplicate (A). Significant difference from the control is $p<0.001$ (*). Inhibitory effect of SOP on TNF-α release in LPS-stimulated murine macrophages Raw264.7 was measured using an ELISA kit (B). Amount of TNF-α released in the supernatant is represented as mean±SEM of three independent tests assayed in duplicate.

Effect of SOP on COX isozyme

SOP showed dose-dependent inhibitory effects on COX-2 activity with 66.4±2.0% of the control (100%) at 1.6 μM, 39.0±3.6% at 6.3 μM, 15.2±2.5% at 25 μM, and 5.7±2.2% at 100 μM (Fig. 3A). However, SOP showed very weak inhibitory effect on COX-1 activity with 78.1± 2.2% of the control (100%) at 500 μM and 64.5±2.4% at 1000 μM (Fig. 3A). As a positive control, NS-398 showed an IC_{50} value of 0.8 μM on COX-2 activity (data not shown). SOP at 12.5-50 μM concentrations did not show significant inhibitory effects on synthesis of COX-2 transcript in LPS-activated murine macrophages Raw264.7, which was analyzed by RT-PCR (Fig. 3B). A density ratio of COX-2 versus β-actin signal was 3.5% at the resting Raw264.7 cells, and was increased to 77.1% at LPS-activated Raw264.7 cells. The density ratio of COX-2 versus β-actin signal was 70.6-73.7% at Raw264.7 cells

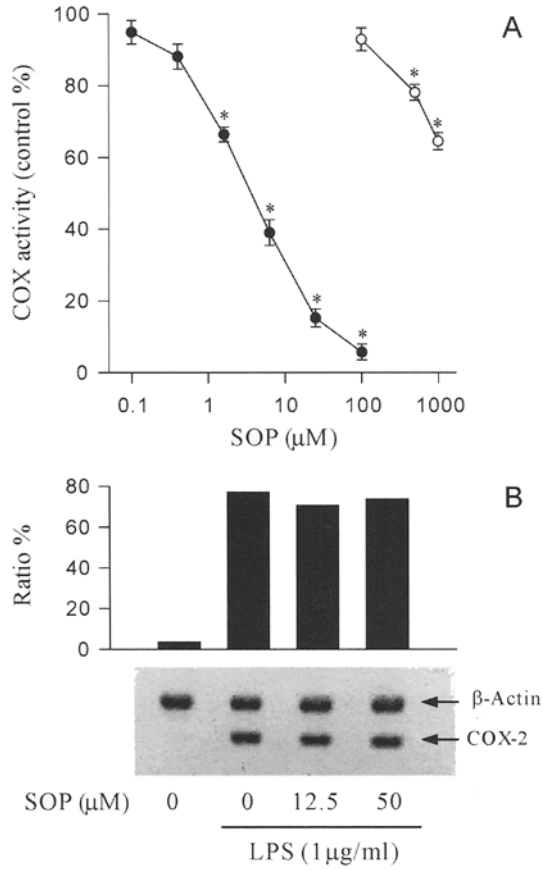

Fig. 3. Effects of sophoricoside on COX isozymes. Inhibitory effects of sophoricoside (SOP) on COX activity (A) and COX-2 synthesis (B) are represented. In panel A, effects on cell free COX-1 (open circle) and COX-2 (solid circle) activities are represented as control %, mean±SEM of three independent tests assayed in triplicate, and significant difference from the control is $p<0.001$ (*). In panel B, RT-PCR product of COX-2 in LPS-stimulated murine macrophages Raw264.7 and density ratio % of COX-2 versus β-actin signal as an internal standard are represented.

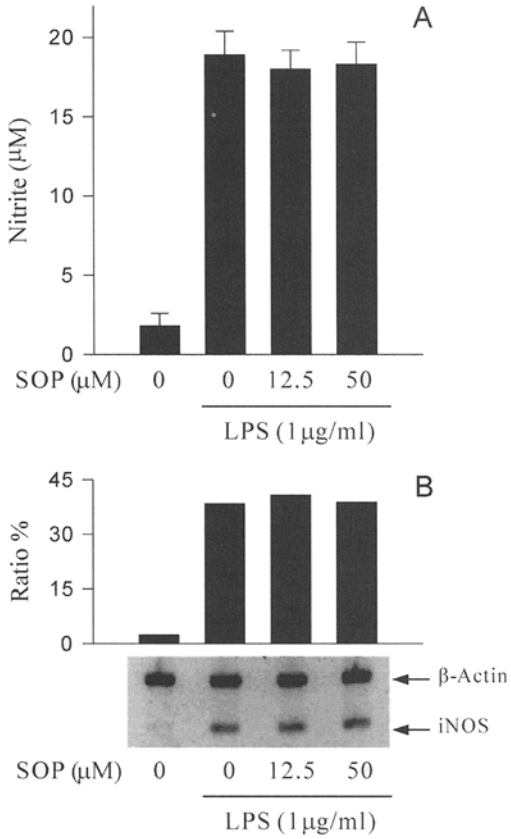

Fig. 4. Effects of sophoricoside on NO production and iNOS synthesis. Inhibitory effects of sophoricoside (SOP) on NO production (A) and iNOS synthesis (B) are represented. In panel A, nitrite content in the supernatant of LPS-stimulated murine macrophages Raw264.7 was determined as an index of NO production by using the Griess reagent, of which sodium nitrite was used as a standard. Data are represented as mean±SEM of three independent tests assayed in triplicate. In panel B, RT-PCR product of iNOS in LPS-stimulated murine macrophages Raw264.7 and density ratio % of iNOS versus β-actin signal as an internal standard are represented.

treated with LPS plus 12.5 μM or 50 μM SOP.

Effect of SOP on NO production

The resting macrophages Raw264.7 cells, after 24 h of culture, produced very low nitrite content of 1.8±0.8 μM (Fig. 4A). After stimulation with LPS, the nitrite content of macrophages was increased about 10-fold (18.9±1.5 μM). SOP at 12.5-50 μM concentrations did not show significant inhibitory effect on NO production in LPS-stimulated Raw264.7 cells. Furthermore, SOP did not show significant inhibitory effects on synthesis of iNOS transcript in LPS-activated murine macrophages Raw264.7, which was analyzed by RT-PCR (Fig. 4B). A density ratio of iNOS versus β-actin signal was 2.4% at the resting Raw264.7 cells, 38.4% at LPS-activated Raw264.7 cells, and 38.9-40.7% at Raw264.7 cells treated with LPS plus 12.5 μM or 50 μM SOP.

Effect of SOP on production of reactive oxygen species

Unopsonized zymosan or PMA was used as a challenger, and lucigenin as an enhancer to detect the superoxide anions by chemiluminescence. When treated with less than 0.001 mg/mL of unopsonized zymosan or less than 0.001 μg/mL of PMA, human monocytes showed similar amount of superoxide anions with the basal level. Maximal production of superoxide anions was observed when human monocytes were stimulated with 0.3 mg/mL of unopsonized zymosan or 0.1 μg/mL of PMA. SOP at 12.5-50 μM concentrations did not show significant inhibitory effects on the production of superoxide anions in unopsonized zymosan or PMA-activated human monocytes (Table II). Furthermore, SOP at 12.5-50 μM concentrations did not inhibit MPO activity from rat neutrophils significantly (Table II).

Table II. Effect of sophoricoside on production of oxygen radicals

Parameter	Inhibition (%)	
	50 μM	12.5 μM
Superoxide anions		
Human monocytes (zymosan)	16.8±4.5	7.5±1.8
Human monocytes (PMA)	6.3±1.7	8.8±2.6
Hypohalides		
MPO activity	10.7±3.6	9.0±2.4

Superoxide anions produced in zymosan or PMA-activated human monocytes were measured by chemiluminescence assay. MPO activity was spectrophotometrically measured where enzyme source was prepared from neutrophils collected from peritoneal lavages of Sprague-Dawley rats.
Each data represents mean±SEM of three independent tests assayed in triplicate.

DISCUSSION

SOP (Fig. 1) used in this study was isolated from immature fruits of *Sophora japonica*, a plant of Leguminosae family. It was reported that flower buds of the plant contained a great deal of rutin, a flavonol glycoside, while a major polyphenol of the immature fruits was determined as SOP, an isoflavone glycoside (Liu and Sheu, 1989). Very few biological activities of SOP have been reported to show anti-inflammatory effect on croton oil-induced ear edema, and to inhibit the IL-5 and IL-3 bioactivities (Gabor and Razga, 1991; Min *et al.*, 1999; Yun *et al.*, 2000).

To understand the anti-inflammatory action, inhibitory effects of SOP on chemical mediators in inflammatory response have been investigated in this study. Several cytokines are known to play important roles in inflammatory diseases. SOP did not show inhibitory effects on IL-1β and TNF-α bioactivities (Table I). However, the compound showed dose-dependent inhibitory effects on IL-6 bioactivity with an IC_{50} value of 6.1 μM (Fig. 2A). SOP was reported to show inhibitory effects on bioactivities of IL-3 and IL-5 involved in allergic inflammation (Yun *et al.*, 2000). Receptors of IL-3, 5 and 6 are members of the cytokine receptor superfamily type I, but those of IL-1β and TNF-α are classified as the immunoglobulin receptor superfamily and TNF/nerve growth factor receptor superfamily, respectively (Haworth *et al.*, 1998). Thus, SOP showed selective inhibitory effects on bioactivities of proinflammatory cytokines with the cytokine receptor superfamily type I.

A major pharmacological target of nonsteroidal anti-inflammatory drugs was known to inhibit the activity of COX, which in turn results in a diminished synthesis of prostanoids. COX was identified to exist in two distinct isoforms. COX-1 is constitutively expressed as a housekeeping enzyme mostly in all tissues, and mediates physiological responses such as gastrointestinal mucosa protection, renal blood flow and platelet aggregation (Smith *et al.*, 1998). On the other hand, COX-2 is rapidly up-regulated in response to cytokines and growth factors, and produces prostanoids involved in pathological processes, such as acute and chronic inflammatory states (McMurray & Hardy, 2002). Accordingly, many of side effects including gastrointestinal ulceration and bleeding due to nonsteroidal anti-inflammatory drugs are ascribed to an inhibition of COX-1 activity (Tanaka *et al.*, 2002). SOP showed potent inhibitory effect on COX-2 activity with an IC_{50} value of 4.4 μM but very weak inhibitory effect on COX-1 activity (Fig. 3A). However, SOP did not suppress the synthesis of COX-2 transcript in LPS-stimulated murine macrophages Raw264.7 (Fig. 3B). Consequently, SOP was identified as a selective inhibitor of COX-2 activity, which can show therapeutic actions similar to those of nonsteroidal anti-inflammatory drugs without causing the unwanted side effects.

Leukocytes can increase their production of reactive oxygen species, such as NO, superoxide anions and hypohalides, in pathological conditions including inflammation (Dalgren and Karlsson, 1999). SOP did not inhibit the NO production in LPS-stimulated murine macrophages Raw264.7 (Fig. 4A). Furthermore, the compound did not show inhibitory effect on not only production of superoxide anions in unopsonized zymosan or PMA-stimulated human monocytes but also MPO activity from rat neutrophils (Table II). Consistently, SOP was previously reported not to show antioxidant effect on *t*-butyl hydroperoxide-initiated chemiluminescence of mice liver homogenates (Fraga *et al.*, 1987).

In chemical structure, genistein and SOP contain common isoflavone skeleton but are different from each other in the absence or presence of a glucose moiety at 4' position (Fig. 1). Genistein was reported to inhibit not only the synthesis of COX-2 in various cell types stimulated by LPS, TNF-α, IL-1β or growth factors but also the NO production, through suppression of iNOS synthesis, in LPS-activated murine macrophages Raw264.7 (Akarasereenont *et al.*, 1994; Corbett *et al.*, 1996; Sheu *et al.*, 2001). Previously, we demonstrated inhibitory effect of genistein on production of reactive oxygen species in the respiratory burst of leukocytes (Yun *et al.*, 2001). Therefore, genistein and SOP seem to display different *in vitro* anti-inflammatory action each other. However, removal of sugar moiety in glycosylated polyphenols is usually necessary for passive diffusion across the small intestine brush border to occur (Hollman *et al.*, 1999). Glycosidase activities can occur in the cells of gastrointestinal mucosa or can be secreted by the colonic microflora (Day *et al.*, 1998; Hollman *et al.*, 1999). Thus, SOP might be converted to genistein to show anti-inflammatory activity *in vivo* even though more

pharmacokinetic studies must be carried out to verify.

In the present study, we have demonstrated that SOP showed inhibitory effects on IL-6 bioactivity and COX-2 activity. These findings expand the importance of dietary soy isoflavonoids as multifunctional agents, and will help to clarify protective mechanisms of SOP, an isoflavone glycoside, against inflammatory conditions.

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