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Effect of *Scutellariae* Radix Ingredients on Prostaglandin E_2 Production and COX-2 Expression by LPS-activated Macrophage

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Abstract. *We previously reported that Sairei-to concentration-dependently modified lipopolysaccharide (LPS)-stimulated prostaglandin E_2 (PGE_2) production in mouse macrophage-like RAW264.7 cells. Among twelve major ingredients of Sairei-to, Scutellariae radix inhibited the LPS-stimulated PGE_2 production to the greatest extent, followed by Zingiberis rhizoma, Glycyrrhizae radix, Atractylodis lanceae rhizoma and Pinelliae tuber. Scutellariae radix contained several major flavonoids such as baicalin, baicalein and wogonin. We investigated the effect of these flavonoids on PGE_2 production and COX-2 expression by LPS-activated RAW264.7 cells. Wogonin inhibited PGE_2 production most efficiently, followed by baicalein and then baicalin, in the same order as their membrane permeability. It was unexpected that wogonin and all other compounds would fail to inhibit the expression of COX-2 at both protein and mRNA levels, suggesting the importance of re-evaluating the point of action of wogonin.*

Sairei-to, one of the Kampo medicines, is composed of 12 major ingredients (*Bupleuri* radix, *Pinelliae* tuber, *Scutellariae* radix, *Zizyphi* fructus, *Ginseng* radix, *Glycyrrhizae* radix, *Zingiberis* rhizoma, *Cinnamomi* cortex, *Atractylodis lanceae* rhizoma, *Hoelen*, *Polyporus* and *Alismatis* rhizoma), and has been used for the treatment of dermatitis or mucositis such as systemic lupus erythematosus, an autoimmune disease.

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We previously proposed that Chinese medicines can be separated into two groups (Groups I and II), based on their actions on nitric oxide (NO) production by activated macrophages. Sho-saiko-to, Hange-shashin-to and Sairei-to (tentatively classified as Group I) significantly reduced the extracellular concentration of NO in the lipopolysaccharide (LPS)-stimulated mouse macrophage-like RAW264.7 cells at their non-cytotoxic concentrations. On the other hand, higher concentrations of Byakko-ka-ninjin-to, Hochu-ekki-to, Juzen-taiho-to and Ninjin-yoei-to (tentatively classified as Group II) are required to exert similar magnitude of the inhibitory effects, due to the presence of both the inhibitors and stimulators for NO production in these preparations (1).

Cyclooxygenase (COX) catalyzes the cyclooxygenation of arachidonic acid (AA) to prostaglandin G_2 (PGG_2) and the peroxidation of PGG_2 to prostaglandin H_2 (PGH_2). PGH_2 is used as a precursor for many prostaglandins (PGs). Two isoforms of COX, constitutive cyclooxygenase-1 (COX-1) and inducible cyclooxygenase-2 (COX-2), are known to catalyze the biosynthesis of PGs from AA. COX-2 is induced by inflammation and the major product of the enzyme reaction catalyzed by COX-2 is PGE_2.

Our previous Western blot analysis demonstrated that Sairei-to unexpectedly enhanced the expression of COX-2 protein without affecting significantly phospholipase A_2 (PLA_2) protein expression, suggesting the importance of the activity, but not the amount of COX-2 in the concentration-dependent stimulation of PGE_2 production by Sairei-to (2).

Various analytical methods, such as high performance liquid chromatography (HPLC), thin–layer chromatography (TLC) and mass spectrometry (MS) have been used to identify or quantify the marker components for the quality control and standardization purposes of medicinal plants (3). These methods are useful to ensure their consistent pharmacological, biological activity and stability. *Scutellariae* radix, a

commonly used traditional Chinese herb, possesses a broad spectrum of biological activities (4-6). Comprising one of the 12 major ingredients of Sairei-to, *Scutellariae* radix was found to contain several major flavonoids such as baicalin, baicalein and wogonin (structure shown in Figure 1) in addition to 40 other components. However, the biological significance of these flavonoids in *Scutellariae* radix is unclear. We investigated the potency of baicalin, baicalein and wogonin to inhibit LPS-stimulated PGE_2 production in conjunction with their membrane permeability assessed by HPLC.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Sairei-to (No. 114, Tsumura Corp., Tokyo, Japan); baicalin, baicalein, wogonin (Wako Pure Chemical Industries, Ltd., Osaka, Japan); Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, NY, Grand Island, USA); fetal bovine serum (FBS), LPS from *Escherichia coli* (serotype 0111:B4) (Sigma Chem. Ind., St. Louis, MO, USA).

Cell culture. RAW264.7 cells that had been established from the peritoneal fluid of BALB/c mice and shown the phenotype characteristics of monocytes and macrophages (7) (supplied by Professor Ohmori, Meikai University) were cultured in DMEM supplemented with 10% heat-inactivated FBS, under a humidified 5% CO_2 atmosphere.

Measurement of PGE_2 production. RAW264.7 cells were subcultured in 24-well plates and incubated for 24 hours without or with *Scutellariae* radix (75 μg/ml) or its ingredients (baicalin, baicalein or wogonin; 1, 5, 10 μg/ml) in the presence or absence of LPS (100 ng/ml). The culture medium supernatant was collected by centrifugation and determined for the PGE_2 concentration by EIA kit (Cayman Chemical Co, Ann Arbor, MI, USA).

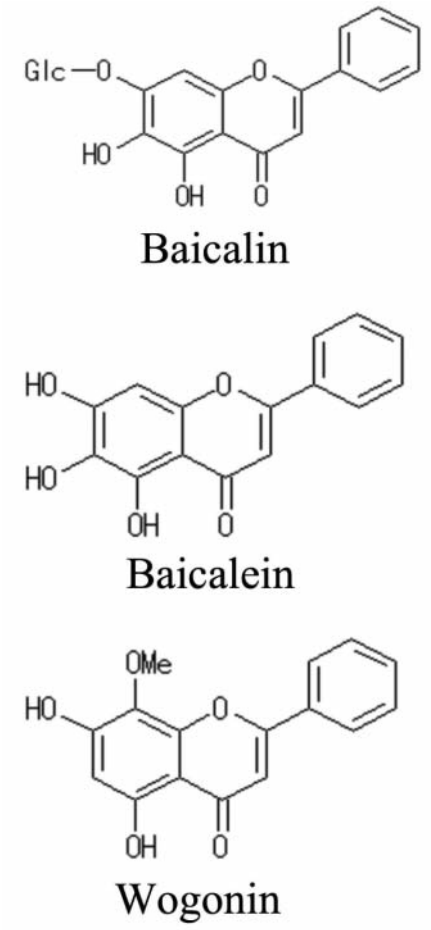

Figure 1. *Chemical structures of baicalin, baicalein and wogonin.*

HPLC analysis. RAW264.7 cells were incubated for 3 hours with each flavonoid. The cells were then washed three times with phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(–)], lysed with lysis buffer [10 mM Tris-HCl (pH 9.6), 1% Triton® X-100 150 mM NaCl, 5 mM EDTA-2Na] and deproteinized with an equivalent amount of acetonitrile. After centrifugation for 5 minutes at 10,000×*g*, the supernatant was collected and stored at –40˚C until HPLC determination. Ten μl of supernatant were injected into an HPLC system (ODS-HG-5; 4.5 mm × 150 mm, Develosil, Nomura Chemical Co., Ltd., Aichi, Japan), eluted with a mobile phase of 50% acetonitrile in water containing 0.2% phosphate and at a flow rate of 1 ml/min, and then detected with UV at 274 nm.

Western blot analysis. RAW264.7 cells were treated under the same condition for the measurement for PGE_2 production. The cell pellets were suspended in lysis buffer and mixed with an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer [0.1 M Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.01% bromophenol blue, 1.2% 2-mercaptoethanol], and boiled for 10 minutes. The protein in the cell lysate was determined by Protein Assay Kit (Bio-Rad, Hercules, CA, USA) and aliquots equivalent to 20 μg protein were applied to 8% SDS polyacrylamide gel electrophoresis and then transferred to PVDF membrane (Immobilon-P; Millipore Corp, Bedford, MA, USA). The membranes were then blocked with 5% skimmed milk in PBS(–) containing 0.05% Tween 20 overnight at 4˚C, incubated with antibodies against COX-2 (1:2,000) (Santa Cruz Biotechnology, Delaware, CA, USA) or Actin (1:2,000) (Sigma) for 90 minutes at room temperature and then incubated with horseradish peroxidase-conjugated anti-goat IgG (1:2,000) (GE Healthcare UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire, England) for 60 minutes at room temperature. Immunoblots were developed with a Western Lightning™ Chemiluminescence Reagent Plus system (Perkin Elmer Life Sciences, Boston, MA, USA).

Assay for mRNA expression. Total RNA was isolated by ISOGEN (NIPPON GENE CO., Tokyo, Japan) protocol. RAW264.7 cells were treated under the same condition for the measurement for PGE_2 production. The cells were lysed in 500 μl ISOGEN, and mixed with 100 μl chloroform. After centrifugation at 12,000×*g* for 15 minutes, the supernatant was collected and mixed with 250 μl isopropanol. After centrifugation at 12,000×*g* for 10 minutes, the pellet was washed in 500 μl 75% ethanol, air dried and dissolved in DEPC-treated H2O. A reverse transcriptase (RT) reaction was performed with 1.0 μg of total RNA, using Rever Tra Ace (Toyobo Co., LTD, Osaka, Japan) and oligo $(dT)_{20}$ primer. Single strand

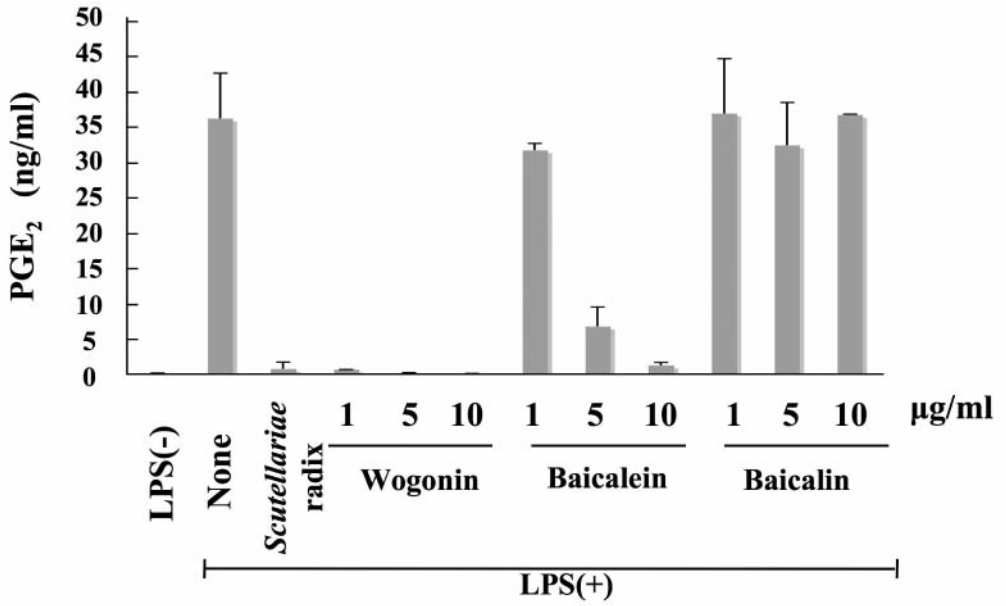

Figure 2. *Inhibition of LPS-stimulated PGE_2 production by Scutellariae radix extracts. RAW264.7 cells were incubated for 24 hours with or without 100 ng/ml LPS in the presence of Scutellariae radix (75 μg/ml), baicalin, baicalein or wogonin (1, 5, 10 μg/ml), and the PGE_2 concentration in the culture medium was then determined. Each value represents mean±SD from three independent experiments.*

cDNA obtained by RT reaction was amplified, using KOD plus (Toyobo), COX-2 specific primer or β-actin specific primer. RT-PCR products were applied to 2% agarose gel, and after separation, the gels were stained with ethidium bromide and then photographed under UV light.

Results

Inhibition of LPS- stimulated PGE_2 production. LPS significantly enhanced the PGE_2 production by RAW264.7 cells. *Scutellariae* radix (1 μg/ml) and wogonin (1 μg/ml), reduced LPS-stimulated PGE_2 production nearly completely. Baicalein concentration-dependently reduced the LPS-stimulated PGE_2 production, but to a slightly lesser extent. On the other hand, baicalin did not inhibit the LPS-stimulated PGE_2 production at the concentrations of 1-10 μg/ml (Figure 2).

Intracellular uptake of Scutellariae component. When RAW264.7 cells were incubated for 3 hours with baicalin, baicalin or wogonin, only wogonin was detected intracellularly (Figure 3).

Effect on COX-2 expression. Western blot analysis demonstrated that LPS (100 ng/ml) induced COX-2 protein expression from an undetectable level to a significantly higher level in RAW264.7 cells. Sairei-to (1 mg/ml), *Scutellariae* radix (75 μg/ml), baicalin, baicalein or wogonin (1-10 μg/ml) did not reduce, but rather slightly increased the LPS-stimulated COX-2 protein expression (Figure 4).

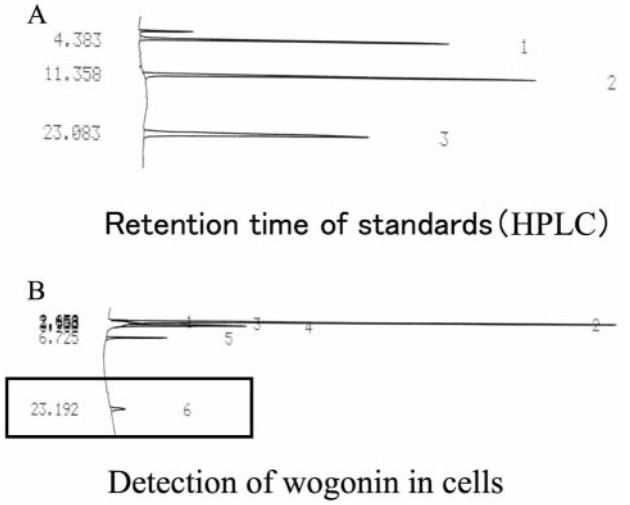

Figure 3. *Comparison of the membrane permeability of wogonin, baicalin and baicalein. RAW264.7 cells (5×10^6) were treated for 3 hours with 1 μg/ml of baicalin, baicalein or wogonin, and the intracellular uptake was determined by HPLC. A: 1, Baicalin; 2, baicalein; 3, wogonin. B: Only wogonin was actually found.*

RT-PCR analysis demonstrated that LPS (100 ng/ml) also enhanced COX-2 mRNA expression from an undetectable level to a significantly higher level in RAW264.7 cells. Sairei-to (1 mg/ml), *Scutellariae* radix (75 μg/ml), baicalin, baicalein or wogonin (1-10 μg/ml) did not apparently affect the LPS-stimulated COX-2 mRNA expression (Figure 5).

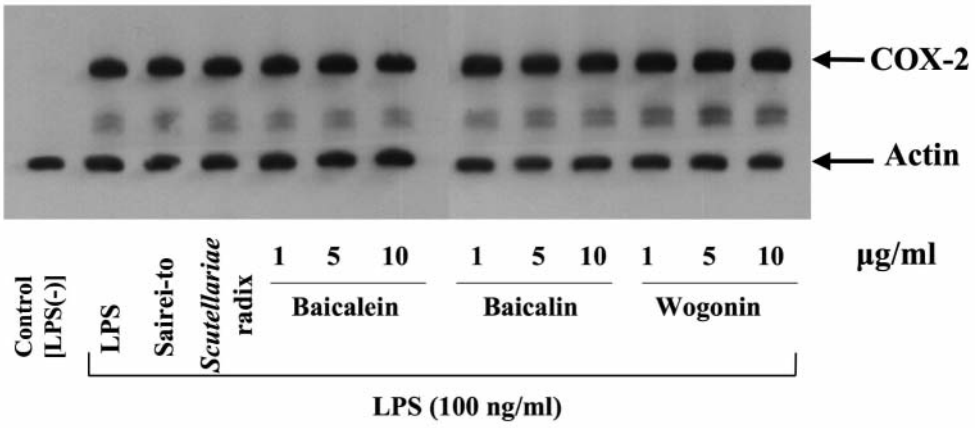

Figure 4. *Effect of Sairei-to and Scutellariae radix extracts on COX-2 protein expression. RAW264.7 cells were treated for 24 hours with Sairei-to (1 mg/ml), Scutellariae radix (75 μg/ml), baicalin, baicalein or wogonin (1, 5 or 10 μg/ml) in the presence or absence of 100 ng/ml LPS, and the COX-2 and Actin protein expression in the cells was assayed by Western blot analysis.*

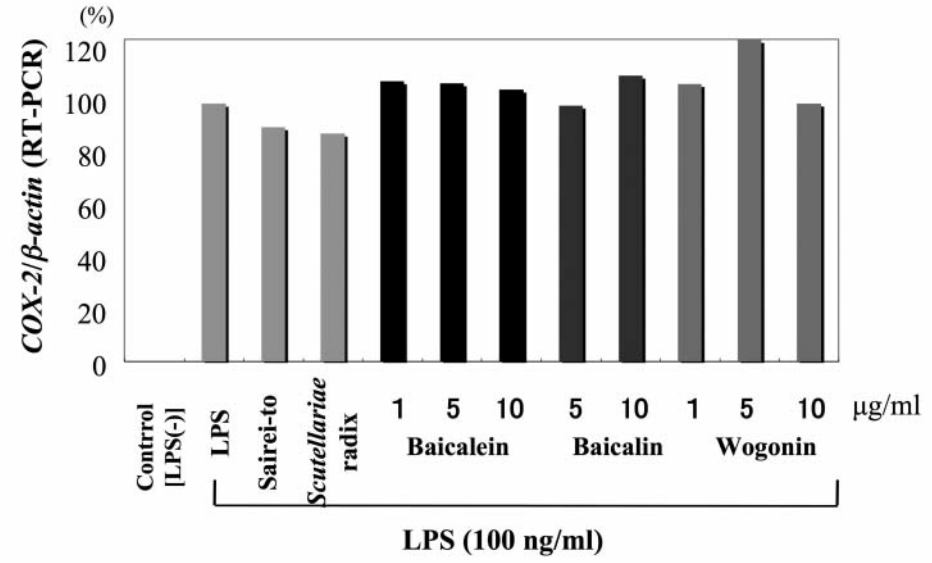

Figure 5. *Effect of Sairei-to and Scutellariae radix extracts on COX-2 mRNA expression. RAW 264.7 cells were incubated with 24 hours with Sairei-to (1 mg/ml), Scutellariae radix (75 μg/ml), baicalin, baicalein or wogonin (1, 5, 10 μg/ml) in the absence or presence of 100 ng/ml LPS. RNA was then isolated and the RT-PCR product was applied to agarose gel electrophoresis. Expression of COX-2 mRNA was plotted as a ratio to β-actin mRNA expression.*

Discussion

Scutellariae radix, the root of *Scuttellaria baicalensis* has long been used as a component of Chinese herbal medicines. It contains baicalin, baicalein and wogonin as the mainly active constituents. Baicalin and baicalein have wide-ranging pharmacological activities including anti-inflammatory, anti-allergic, antiviral, antiproliferative, antitumor, hepato- and lung protective activity (8-15). Previous study has shown that both baicalin and baicalein inhibited both PGE_2 production and *COX-2* gene expression (16), supporting and contradicting our present data that these compounds inhibited LPS-stimulated PGE_2 production, but failed to inhibit the LPS-stimulated COX-2 expression at both protein and mRNA levels.

It has been reported that baicalin could be obtained by water extraction from *Scuttellaria baicalensis* at higher yield, whereas baicalein was recovered as the main constituent by

ethanol extraction (17). This result can be explained by the higher hydrophilicity of baicalin (which is a glycoside) than baicalein and wogonin (which are aglycones). Baicalin is transformed into baicalein by intestinal bacterial glucuronidase, absorbed in the rat tissue and restored there as the original form (18). In monolayer Caco-2 cells, baicalein was incorporated into the cells and then conjugated with glucuronic acid to yield baicalin (18). All of the *Scuttellaria baicalensis* extracts inhibited PGE_2 production in SCC-25 cells (18). Baicalein from *Scutellariae* radix potently blocked the mitogen-activated protein kinase (MAPK) cascade, thus inhibiting PGE_2 release from astrocytes in C6 rat glioma. Baicalein has anti-inflammatory action possibly through the inhibition of the release of AA (19).

We investigated whether *Scutellariae* radix extracts antagonize PGE_2 production by LPS-stimulated RAW264.7 cells. We found that wogonin inhibited PGE_2 production most potently, followed by baicalein and then baicalin, in the same order as their membrane permeability (wogonin>baicalein> baicalin). This may be due to the higher lipophilicity (or membrane permeability) of wogonin, with its OCH_3 group (Figure 1) and suggests its possible anti-inflammatory potency. However, none of these compounds significantly affected the COX-2 expression at either the protein or mRNA level (Figures 4 and 5), nor did they affect $cPLA_2$ protein expression (2). *Scutellariae* radix contains baicalein and wogonin, which affect the AA cascade through the inhibition of MAPK cascade which in turn activates $cPLA_2$. The inhibition of PGE_2 production by *Scutellariae* radix in LPS-stimulated RAW264.7 cells may be mediated by the inhibition of the MAPK cascade and the release of AA. Further experiments are necessary to identify the points of action of this compound (such as the inactivation step of NF-κB signaling, activation step of COX-2 *etc*.).

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