Effect of *Sasa senanensis* Rehder Extract on NO and PGE$_2$ Production by Activated Mouse Macrophage-like RAW264.7 Cells

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Abstract. *Alkaline extract of Sasa senanensis Rehder (SE) has shown diverse biological activity. As an extension, whether SE affects the function of activated macrophages was investigated. SE inhibited the nitric oxide (NO) production by lipopolysaccharide (LPS)-activated mouse macrophage-like RAW264.7 cells. Western blot and RT-PCR analyses demonstrated that this was due to the inhibition of inducible NO synthase (iNOS) expression at both protein and mRNA levels. ESR spectroscopy shows that SE dose-dependently scavenged the NO radical produced by NOC-7. In order to confirm the anti-inflammatory potency, possible effects on prostaglandin (PG) E$_2$ production and expression of enzymes involved in the arachidonic acid pathway were next investigated. It was found that SE effectively inhibited the PGE$_2$ production by LPS-stimulated RAW264.7 cells, although the extent of inhibition of PGE$_2$ was slightly less than that of NO production. SE inhibited cyclooxygenase (COX)-2 expression at both protein and mRNA levels, but to much lesser extents as compared with those for iNOS expression. SE contained much lower concentration of arginine, precursor of NO, as compared with the culture medium. These data suggest that SE exerts a weak anti-inflammatory activity.*

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Alkaline extract of *Sasa senanensis* Rehder extract (SE) has been reported to show antiseptic (1), membrane stabilizing (2), anti-inflammatory and phagocytic (3) and radical scavenging activity (4). It has recently been reported that SE shows slightly higher cytotoxic activity against normal human oral cells (gingival fibroblast, pulp cell, periodontal ligament fibroblast) than against human tumor cell lines (oral squamous cell carcinoma, glioblastoma, myelogenous and T-cell leukemia), antibacterial activity, the inhibition of the bacterial production of volatile sulfur compounds, anti-HIV activity, anti-influenza virus activity and vitamin C-synergized radical scavenging activity (5). Since SE contained relatively higher amount of lignin carbohydrate complex (1.76 g per 100 mL of SE, measured after acid precipitation, dialysis against distilled water and lyophilization), the higher anti-HIV activity of SE might be ascribed to lignin carbohydrate complex present in the extract. Since SE displayed potent radical scavenging activity against superoxide anion and hydroxyl radical (5), it is possible that SE might show anti-inflammatory activity. At present, information of the anti-inflammatory activity of SE is limited (3), and, thus, the molecular basis for anti-inflammatory action of SE is unclear. Therefore, the effect of SE on nitric oxide (NO) and prostaglandin E$_2$ (PGE$_2$) production by activated macrophages was investigated, as well as whether SE might inhibit the inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) expression that regulate the amount of NO and PGE$_2$, respectively. For this purpose, mouse macrophage like cells RAW264.7 were used, this cell line has been established from the peritoneal fluid of BALB/c mice and has shown the phenotypes characteristics of monocytes and macrophages transformed by Abelson murine leukemia virus (6). RAW264.7 cells activated by lipopolysaccharide (LPS) have been used by

thousands of investigators for the study of the signal transduction pathway during the activation process of macrophages (7, 8) and the search for the substances that modify the macrophage function (9).

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, KS, USA); lipopolysaccharide (LPS) from *Escherichia coli* (Serotype 0111:B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chem Co., St Louis, MO, USA); dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA) (Wako Pure Chem. Ind., Osaka, Japan); 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy1-3-oxide (carboxy-PTIO; a spin trap agent), 1-hydroxy-2-oxo-3-(*N*-3-methyl-3-aminopropyl)-3- methyl-1-triazene (NOC-7; an NO generator) (Dojin, Kumamoto, Japan).

SE was prepared and supplied by Daiwa Biological Research Institute Co., Ltd., Kawasaki, Kanagawa, Japan. SE contained 58.2 mg solid materials/mL (5), and higher amounts of serine (839 μM), glycine (2606 μM), alanine (915 μM) and glutamic acid (452 μM), and lower amount of arginine (14 μM) than the culture medium (DMEM+10% FBS) (serine 390 μM, glycine 411 μM, alanine 96 μM, glutamic acid 106 μM, arginine 366 μM).

Cell culture. RAW264.7 cells (kindly supplied by Prof Ohmori, Meikai University, Japan) were cultured as an attached monolayer culture in DMEM supplemented with 10% heat-inactivated FBS under a humidified 5% CO_2 atmosphere (9, 10). When the cells were grown to confluency, they were detached by splashing the medium to them through the pipette, and seeded at lower cell density. The cells were not collected by scraping them out from the plate with a rubber policeman, since the scraping procedure damaged the cells (unpublished data).

Determination of viable cell number. RAW264.7 cells were inoculated at 1.5×10^6/mL (100 μL) in a 96-microwell plate (Becton Dickenson, Labware, NJ, USA) and incubated for 1-2 hours. Near-adherent cells were replaced with fresh culture medium and treated for 24 hours with the indicated concentrations of SE in phenol red-free DMEM supplemented with 10% FBS in the presence or absence of LPS (0.1 μg/mL). Viable cell number was then determined by MTT methods. In brief, cells were incubated for 30 minutes with 0.2 mg/mL of MTT. After removal of the medium the cells were lysed with DMSO, and the absorbance at 540 nm was recorded using microplate reader (9, 10).

Assay for NO production. RAW264.7 cells were inoculated at 1.5×10^6/mL (100 μL) in a 96-microwell plate and incubated for 1-2 hours. Near confluent cells were treated for 24 hours with the indicated concentrations of SE (0-25%) in phenol red-free DMEM supplemented with 10% FBS in the presence or absence of LPS (0.1 μg/mL). The NO production by RAW264.7 cells was quantified by Greiss reagent, using the standard curve of NO_2^-. To eliminate the interaction between sample and Greiss reagent, the NO concentration was also measured in the culture medium without the cells, and this value was subtracted from that obtained with the cells. Then

concentration that inhibited the LPS-stimulated NO production by 50% (50% inhibitory concentration: IC_{50}) was determined from the dose-response curve (9). The efficacy of inhibition of NO production was evaluated by the selectivity index (SI), which was calculated using the following equation: $SI = CC_{50}/IC_{50}$.

Assay for prostaglandin E_2 (PGE_2) production. Raw264.7 cells were inoculated at 1.5×10^6/mL in a 96-mirowell plate and incubated for 1-2 hours. Near-adherent cells were treated for 24 hours with various concentrations of SE (0-3.2%) in the presence of LPS (0.1 μg/mL). The culture supernatant was collected by centrifugation, and determined for the PGE_2 concentration by EIA kit (Cayman Chemical Co, Ann Arbor, MI, USA) (11).

Assay for iNOS protein expression. RAW264.7 cells were inoculated at 1.5×10^6/mL in 96-well plates (Becton Dickinson) and incubated for 1-2 hours. Near-adherent cells were treated for 24 hours with various concentrations of SE (0-6.2%). The concentrations were selected by CC_{50} in the presence or absence of LPS (100 ng/mL). The cell pellets were lysed with 50 μL of lysis buffer [10 mM Tris-HCl (pH 7.6), 1% Triton X-100, 150 mM NaCl, 5 mM EDTA-2Na and 2 mM PMSF] for 10 minutes on ice. The cell lysates were centrifuged at 16,000×g for 20 minutes at 4°C to remove the insoluble materials and the supernatant was collected. The protein concentrations of supernatant were measured by Protein Assay Kit (Bio Rad, Hercules, CA, USA). Equal amount of the protein from cell lysates (10 μg) was mixed with 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], boiled for 10 minutes, and applied to the SDS-8% polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% non-fat skim milk in phosphate-buffered saline [PBS (–)] plus 0.05% Tween 20 for 90 minutes and incubated for 90 minutes at room temperature with anti-COX-2 or anti-iNOS (dilution: 1:2,000 and 1:1,000, respectively, Santa Cruz Biotechnology, Delaware, CA, USA) or anti-actin antibody (1:6,000, Sigma), and then incubated with horseradish peroxidase-conjugated anti-goat, anti-rabbit or anti-mouse IgG for 60 minutes at room temperature. Immunoblots were developed with a Western Lighting™ Chemiluminescence Reagent plus (9).

Assay for iNOS and COX-2 mRNA expression. RAW264.7 cells were inoculated at 1.5×10^6/mL in 24-well plates and incubated for 1-2 hours. Near-confluent cells were treated for 24 hours with various concentrations of SE(0-3.1%) in the presence or absence of LPS (0.1 μg/mL). The expression levels of iNOS and COX-2 mRNA were measured by RT-PCR. The primer sequence and size is: (i) iNOS: PCR products size (496 bp), sense primer (5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'), antisense primer (5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'), (ii) COX-2: Product size (371bp), sense primer (5'-TTTGTTGAGTCATTCACCAGACAGAT-3'), antisense primer (5'-CAGTATTGAGGAGAACAGATGGGATT-3'), (iii) G3PDH: Products size (452 bp), sense primer (5'-ACCACAGTCCATGCCATCAC-3'), antisense primer (5'-TCCACCACCCTGTTGCTGTA-3') (9).

NO radical scavenging activity. The radical intensity of benzocycloheptaoxazines was determined at 25°C, using electron spin resonance (ESR) spectroscopy (JEOL JES REIX Tokyo, Japan,
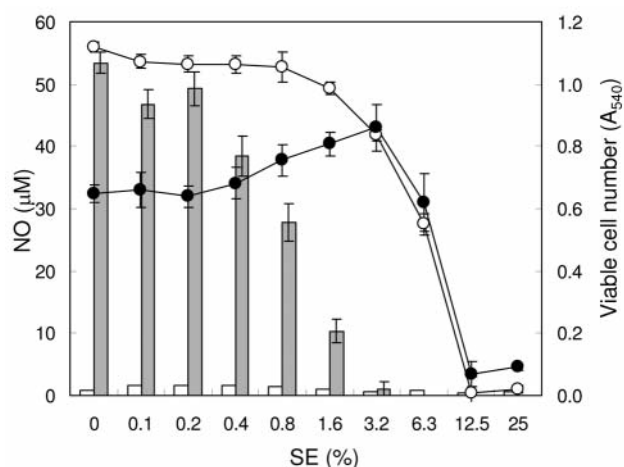
Figure 1. *Inhibition by SE of LPS-stimulated NO production. RAW264.7 cells were incubated for 24 hours with the indicated concentrations of SE in the presence (gray or black) or absence (white) of 0.1 μg/mL LPS and the viable cell number and extracellular concentration of NO were then measured. Each value represents mean ±SD from three independent experiments.*
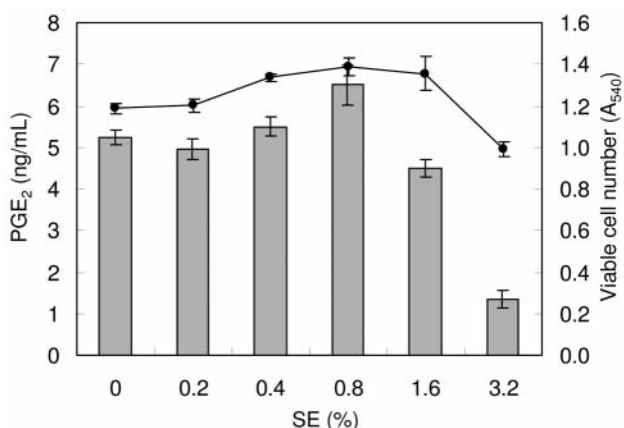
Table I. *Inhibition of NO and PGE$_2$ production by SE in activated macrophages.*

	CC$_{50}$	IC$_{50}$	SI
NO production			
Exp. 1	9.4	0.85	11.0
Exp. 2	5.9	0.58	10.2
Exp. 3	6.0	0.62	9.7
mean±S.D.	7.1±2.0	0.68±0.15	10.3±0.67
PGE$_2$ production			
Exp. 1	7.1	2.6.	2.7
Exp. 2	7.1	1.0	7.1
mean	7.1	1.8	4.9
NO scavenging		0.21	

RAW264.7 cells were incubated for 24 hours with various concentrations of SE in the presence of 0.1 μg/mL LPS, and then the viable cell number and extracellular NO and PGE$_2$ were determined. From the dose-response curve, the 50% cytotoxic concentration (CC$_{50}$) and 50% inhibitory concentration of NO production (IC$_{50}$) and PGE$_2$ were determined. Selectivity index (SI) was determined by dividing CC$_{50}$ value by IC$_{50}$ value.

Figure 2. *Inhibition by SE by LPS-stimulated PGE$_2$ production. RAW264.7 cells were incubated for 24 hours with various concentrations of SE in the presence of 0.1 μg/mL LPS, and then the viable cell number (circle) and extracellular PGE$_2$ concentration (bar) were determined. Each value represents mean SD from three independent experiments.*

X-band, 100 kHz modulation frequency) (9). The radical intensity of NO, produced from the reaction mixture of 20 μM carboxy-PTIO and 60 μM NOC-7, was determined in 0.1 M phosphate buffer, pH 7.4 in the presence of 30% DMSO (microwave power and gain were changed to 5 mW and 400, respectively). When NOC-7 and carboxy-PTIO were mixed, NO was oxidized to NO$_2$ and carboxy-PTIO was reduced to carboxy-PTI, which produces seven-line signals. Samples were added 3 minutes after mixing. The NO radical intensity was defined as the ratio of the signal intensity of

the first peak of carboxy-PTI to that of MnO, and expressed as the ratio to the height of MnO, an external marker. The concentration that reduced the NO radical intensity by 50% (IC$_{50}$) was determined from the dose response curve (9).

Results

Inhibition of NO and PGE$_2$ production. It has been previously reported that LPS (0.1 μg/mL) enhanced the NO (9) and PGE$_2$ (11) production by RAW264.7 cells. It was first confirmed that the 0.1 μg/mL of LPS was the optimum concentration for the induction of NO production by RAW264.7 cells (data not shown). SE did not inhibit the cell proliferation in the range of 0.1-1.6% (measured by MTT method). SE dose-dependently inhibited the LPS-stimulated NO production (Figure 1). A total of three independent experiments demonstrated that SE at lower cytotoxic range (0.4-1.6% , CC$_{50}$=7.1±2.0%) effectively inhibited the LPS-stimulated NO production (IC$_{50}$=0.68±0.15%), yielding a selective index of 10.3±0.67 (Table I).

It was found that untreated RAW264.7 cells produce a back ground level of PGE$_2$ (0.054 ng/mL) (data not shown), and the addition of 0.1 μg/mL LPS enhanced the PGE$_2$ production up to 5.3 ng/mL (Figure 2). SE reproducibly inhibited the LPS-stimulated PGE$_2$ production (Figure 2), yielding a selectivity index of 4.9 (Table I).

Inhibition of iNOS and COX-2 expression. Whether the inhibition of NO and PGE$_2$ production by SE is due to the reduced expression of iNOS and COX-2 proteins, respectively, was then investigated. Western-blotting
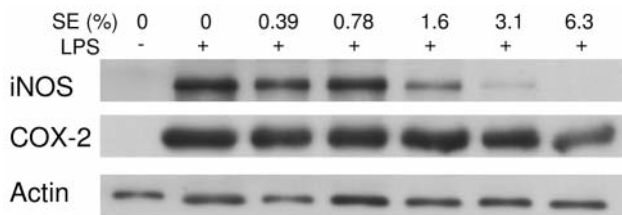
Figure 3. *Inhibition of iNOS and COX-2 protein expression by SE in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated for 24 hours with the indicated concentrations of SE in the presence or absence of LPS (0.1 μg/mL) and processed for western blot analysis for iNOS, COX-2 and actin (internal marker) protein expression. Representative pattern of western blot analysis of three independent experiments are shown. Reproducible results were obtained in the experiments performed by one other investigator.*
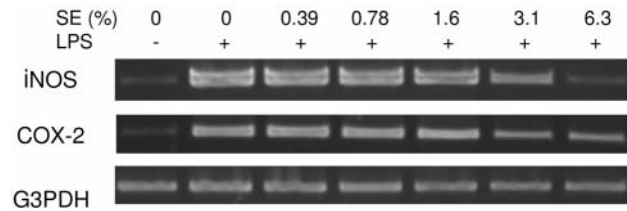

Figure 4. *Inhibition of iNOS and COX-2 mRNA expression by SE in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated for 24 hours with the indicated concentrations of SE in the presence or absence of LPS (0.1 μg/mL) and processed for RT-PCR analysis for iNOS, COX-2 and G3PDH (internal marker) mRNA expression. Similar data were obtained in another two experiments. Reproducible results were obtained in the experiments performed by one other investigator.*

analysis showed that this was the case (Figure 3). It is apparent that the inhibitory effect of SE on the expression of iNOS protein was more obvious than that of COX-2 protein.

RT-PCR experiments demonstrated that the reduced expression of iNOS and COX-2 proteins was due to the inhibition of mRNA expression for both of these proteins (Figure 4). It is again apparent that inhibitory effect of SE on the iNOS mRNA expression was more evident than that of COX-2 mRNA expression.

NO scavenging activity of SE. The amount of NO is regulated by both synthesis and degradation. The possibility that the reduced amount of NO recovered from the LPS-stimulated RAW264.7 cells in the presence of SE might be due to the direct scavenging activity of SE against NO was then investigated. *In vitro* experiments with ESR spectroscopy show that this was the case: SE dose-dependently reduced the radical intensity of NO, generated from NOC in the presence of carboxy-PTIO (Figure 5).

Discussion

The present study demonstrated for the first time that SE inhibited the NO production in LPS-activated RAW264.7 cells, and this was due to the combination of the inhibition of iNOS expression (at both protein and mRNA levels) by SE and its NO scavenging activity. It was also found that SE inhibited the LPS-induced PGE_2 production and COX-2 mRNA expression, although the extent of inhibition was much less than that of NO and iNOS inhibition. These data demonstrated that SE has some anti-inflammatory activity. There was a possibility that arginine (a precursor of NO and citrulline) in SE might have overestimated the present results. Amino acid analysis of SE showed that SE contained
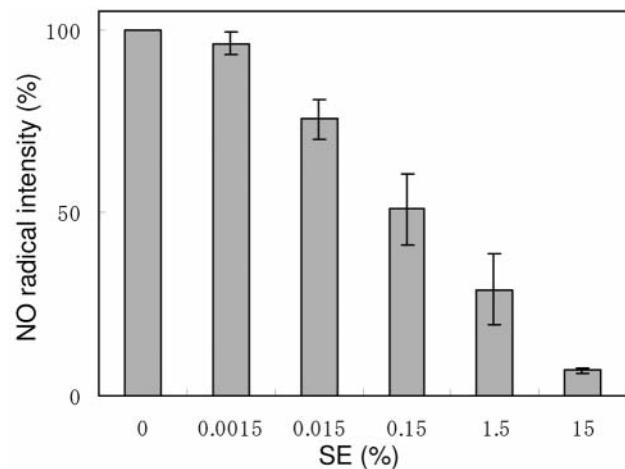

Figure 5. *NO radical scavenging activity of SE. Each value represents mean±S.D. from three independent experiments.*

lower concentration of arginine (14 μM), as compared to the culture medium (DMEM+ 10% FBS) (366 μM), reducing this possibility.

The other component in SE that has to be considered is lignin carbohydrate complex. It has been previously reported that lignin carbohydrate from pine cone extract (12) and cacao husk (13) stimulated the NO production by RAW 264.7 cells, an opposite action of the crude SE extract. It remains to be investigated whether lignin carbohydrate complex from SE might display similar stimulation effect on NO production by RAW 264.7 cells. Although SE contains many components that may positively or negatively affect the NO/ PGE_2 production by RAW264.7 cells, the anti-inflammatory action of SE should be further pursued.

The present study, along with a recent paper (14), demonstrated the considerable difference between the extents of inhibition of NO production by SE and that of PGE_2, indicating that determination of inhibition of NO production alone by the activated macrophage may not be sufficient to accurately evaluate the anti-inflammatory activity. However, NO (including iNOS) has been reported to slow the growth of *Mycobacterium tuberculosis* (*Mtb*); genetic inactivation of iNOS increase the susceptibility of mice to *Mtb* (15). Also autophagy is linked to a mechanism that inhibits the survival of *Mtb* within host cells independent of NO (16). Since RAW264.7 cells are easily committed to autophagy under nutritionally starved conditions (10), the effect of lignin, NO generator, on the induction of autophagy should thus be investigated.

Acknowledgements

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