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Concentration-dependent Effect of Rikko-san on the Prostaglandin E_2 Production by Mouse Macrophage-like Cells

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Abstract. *Rikko-san (RKS) and its ingredients were investigated for their activity to stimulate prostaglandin E_2 (PGE_2) production by unstimulated and lipopolysaccharide (LPS)-stimulated mouse macrophage-like Raw 264.7 cells. LPS significantly stimulated the production and extracellular secretion of PGE_2 by Raw 264.7 cells. RKS dose-dependently modified the LPS-stimulated PGE_2 production. A lower concentration (0.04-0.4 mg/ml) of RKS stimulated PGE_2 production without or with LPS, whereas a higher concentration (4 mg/ml) of RKS inhibited the LPS-stimulated PGE_2 production, suggesting the presence of both stimulator(s) and inhibitor(s) for PGE_2 production. The inhibitory effect of a higher concentration of RKS was slightly neutralized by supplementation of exogenous arachidonic acid. Among five RKS ingredients, Kanzo inhibited the LPS-stimulated PGE_2 production to the greatest extent, whereas other ingredients (Shoma, Ryutan, Saishin, Bofu) stimulated PGE_2 production. Western blot analysis demonstrated that RKS and all its ingredients enhanced the intracellular concentration of cyclooxygenase-2, whereas RKS did not significantly affect the phospholipase A_2 protein level. The present study demonstrates that the concentration-dependent effect of RKS on the PGE_2 production by macrophage cannot be explained only by the expression levels of cyclooxygenase-2 or phospholipase A_2.*

Rikko-san (RKS), one of the Kampo medicines, is composed of five major ingredients, *Kanzo* (KZ), *Shoma* (SM), *Ryutan* (RT), *Saishin* (SS) and *Bofu* (BF) and has been widely used for the treatment of oral pain such as dental caries, pulpitis, periodontitis and pain after tooth extraction (1, 2). We previously demonstrated that RKS effectively inhibited the nitric oxide (NO) production by lipopolysaccharide (LPS)-stimulated RAW 264.7 cells, even at non-cytotoxic concentrations (3). Among five RKS ingredients, KZ and SM inhibited the NO production, whereas RT, SS and BF did not show such a clear-cut inhibitory effect, possibly due to the co-existance of both inhibitory and stimulatory substance(s) for NO production. Western blot analysis demonstrated that non-cytotoxic concentrations of RKS and KZ significantly inhibited the LPS-stimulated expression of inducible NO synthase (iNOS). ESR spectroscopy showed that RKS and all its five ingredients scavenged superoxide and NO, possibly by their general reducing activity. These data demonstrated that RKS has both macrophage stimulating and inhibiting factors, which play significant roles in immunological reactions and that the inhibition of NO production might be the result of both the inhibition of iNOS expression and their radical scavenging activity. This suggests that RKS and its components may have some anti-inflamatory action, by modulating the arachidonic acid (AA) pathway.

In AA metabolism, tissue damage or inflammation activates phospholipase A_2 (PLA_2) of the cell membrane, stimulating the release of AA from cell membrane phospholipids. Among several types of PLA_2, $cPLA_2$ is the key enzyme in catalyzing the release of AA (4). AA becomes the substrate for cyclooxygenases (COXs) or lipoxygenase (LOX) to form prostaglandins (PGs) / thromboxane (TXA) or leukotrienes, respectively (5). COX catalyzes the cyclooxygenation of AA to PGG_2 and the peroxidation of PGG_2 to PGH_2. PGH_2 is used as a precursor for many PGs. There are two isoforms of COX, constitutive COX-1 and inducible COX-2. COX-2 is induced by inflammation and major product of COX-2 is PGE_2. PGE_2 augments the effect of bradykinin, peripheral

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Table I. *Five ingredients of Rikko-san (RKS) and their major components.*

Ingredient	Major component	Content[a]
Kanzo (KZ)	glycyrrhizin, glabric acid, liquiritin, licoricone licoflavone	1.5
Shoma (SM)	cimigenol, dahurinol,acerinol, β-sitosterol, cimicifugoside	2.0
Ryutan (RT)	gentiopicroside, trifloraside, swertiamarine, gentisin	1.0
Saishin (SS)	β-pinen, eucarvoe, 1,8-cineol, *l*-sarinin, higenamine	2.0
Bofu (BF)	deltoin, bergapten, psoralen, hamaudol, cimifugin	2.0

[a]expressed as amount (g) per 42.5 g RKS.

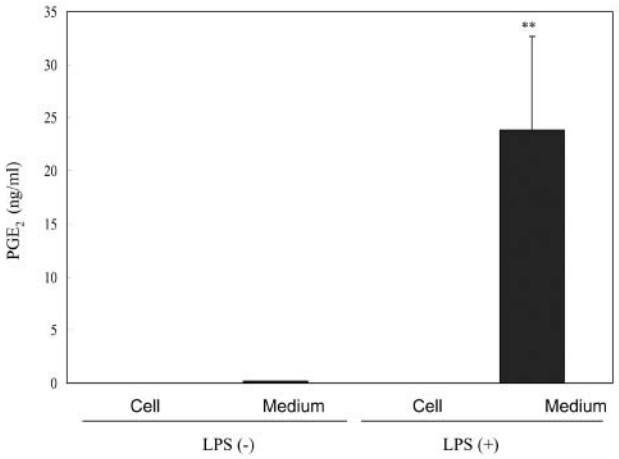

Figure 1. *PGE_2 production by LPS-stimulated and -unstimulated RAW 264.7 cells. RAW 264.7 cells were incubated for 24 h without or with 100 ng/ml LPS and the PGE_2 concentration in the medium and cellular fraction was determined. Each value represents mean±SD of three independent experiments. Statistically significant PGE_2 production (**$p<0.01$), as compared with other groups.*

pain mediators or modulators, by lowering the threshold level of sensation of pain. Although RKS is generally used for oral pain, the anti-inflammatory potential of RKS has not been established yet. Therefore in this article, the effect of RKS and its five ingredients on PGE_2 production and the possible changes in the enzymes involved in the AA metabolism in the LPS-stimulated and -unstimulated macrophage RAW 264.7 cells were investigated.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: RKS (No. 2990110010), its five components [KZ (No. 281013010), SM (No. 281081010), RT (No. 281080010), SS (No. 2001026010), BF (No. 2991031010)] (Tsumura Corp., Tokyo, Japan) [42.5 g RKS contained 1.5 g KZ, 2.0 g SM, 1.0 g RT, 2.0 g SS and 2.0 g BF. (Table I)]; Dulbecco's modified Eagle medium (DMEM) (Invitrogen Corp, Carlsbad, CA, USA), fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA, USA); LPS from *Escherichia coli*. (Serotype 0111:B4), arachidonic acid (Sigma Chem. Ind., St. Louis, MO, USA).

Measurement of PGE_2 production. RAW 264.7 cells were subcultured in 24-well plates and incubated with various concentrations of RKS or its ingredients in the presence or absence of LPS (100 ng/ml). The culture medium supernatant was collected by centrifugation and was determined for the PGE_2 concentration by an EIA kit (Cayman Chemical Co, Ann Arbor, MI, USA). To determine the PGE_2 concentration in the cells, cells were washed twice with cold phosphate-buffered saline without calcium or magnesium (PBS) and lysed by sonication in PBS containing 1% Triton X-100. The PGE_2 in the cell lysate was determined as described above.

Western blot analysis. The cell pellets were suspended in PBS and mixed with an equal volume of 2x sodium dodecyle 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 were boiled for 10 min. The protein in the cell lysate was determined by Protein Assay Kit (Bio Rad, Hercules, CA, USA) and the aliquots equivalent to 20 μg protein were applied to the 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% skim milk in Tris-HCl-buffered saline plus 0.05% Tween 20 overnight at 4˚C and were incubated with antibodies against COX-2 (1:2000) or $cPLA_2$ (1:1000) (Santa Cruz Biotechnology, Delaware, CA, USA) for 90 min at room temperature and then incubated with horseradish peroxidase-conjugated anti-goat IgG (1:2000) (Santa Cruz Biotechnology, Delaware, CA, USA) for 60 minutes at room temperature. Immunoblots were developed with a Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA, USA) and were analyzed using the public domain NIH Image program (National Technical Information Service, Springfield, Virginia, USA, part number PB95-500195GEI).

Statistical treatments. The statistical difference between control and treated-groups was evaluated by Student's *t*-test.

Results

Stimulation of PGE_2 production by activated RAW 264.7 cells. LPS treatment (100 ng/ml, 24 h) significantly stimulated the production of PGE_2 by RAW 264.7 cells ($p<0.01$). Almost all PGE_2 produced by unstimulated or stimulated cells were detected in the medium fraction, while background level of PGE_2 was recovered from the cells (Figure 1). Based on these results, the amount of PGE_2 released into the supernatant from the cells treated for 24 h with LPS was determined in the following experiments.

Bi-modal action of RKS on PGE_2 production. RKS dose-dependently stimulated the PGE_2 production by RAW

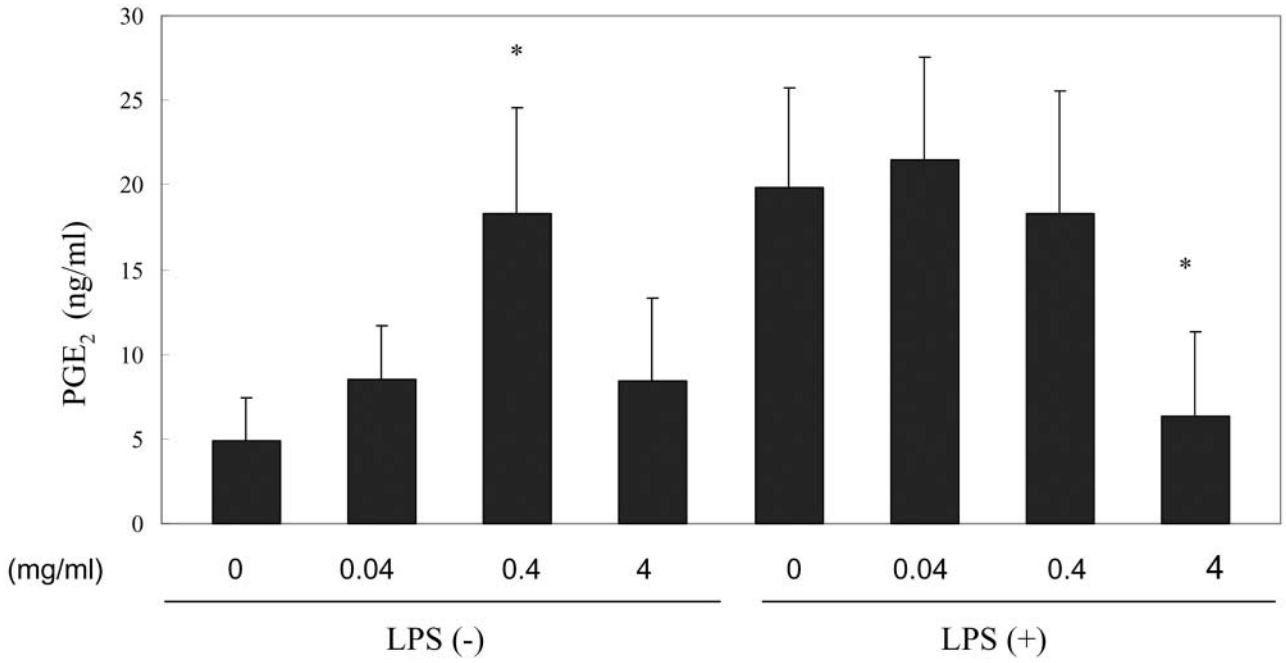

Figure 2. *Effect of RKS on PGE_2 production by LPS-stimulated and -unstimulated RAW 264.7 cells. RAW 264.7 cells were incubated for 24 h without or with 100 ng/ml LPS in the presence of the indicated concentrations of RKS and the PGE_2 concentration in the medium fraction was determined. Each value represents mean±SD from three independent experiments. Statistically significant difference of PGE_2 production (*p<0.05), as compared with each control group without RKS.*

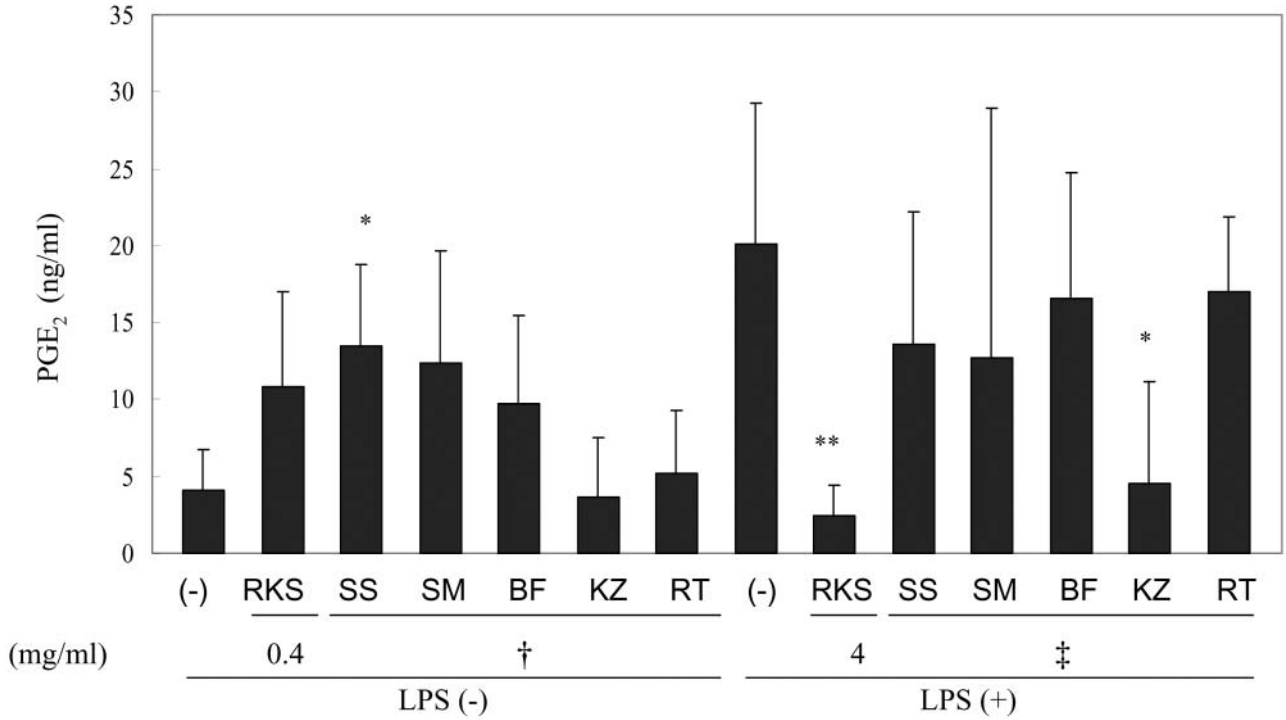

Figure 3. *Effect of the five ingredients of RKS on PGE_2 production by LPS-stimulated and -unstimulated RAW 264.7 cells. RAW 264.7 cells were incubated for 24 h without or with 100 ng/ml LPS in the absence or presence of 0.4 or 4 mg/ml each of RKS or its five ingredients and the PGE_2 concentration of medium fraction was determined. Each value represents mean±SD of three independent experiments. Statistically significant difference of PGE_2 production (*p<0.05; **p<0.01), as compared with each control group without RKS. † Adjusted to the concentration present in 0.4 mg/ml of RKS. ‡ Adjusted to the concentration present in 4 mg/ml of RKS.*

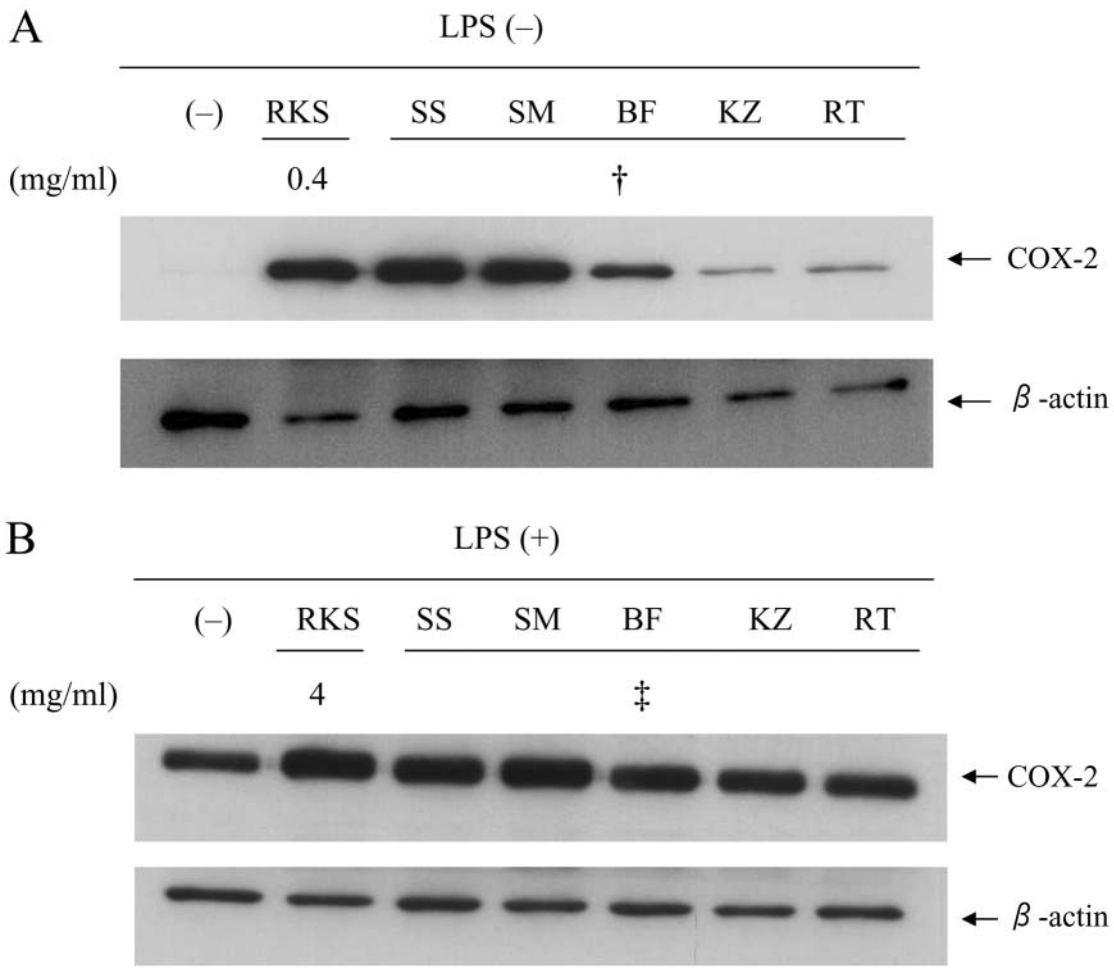

Figure 4. *Effect of RKS and its five ingredients on the intracellular concentration of COX-2 protein in LPS-stimulated and -unstimulated RAW 264.7 cells. RAW 264.7 cells were treated for 24 h without or with 0.4 mg/ml RKS or its five ingredients (A), or without or with 4 mg/ml RKS or its five ingredients in the presence of 100 ng/ml LPS (B). COX-2 protein expression in the cells was assayed by Western blot analysis. †Adjusted to the concentration present in 0.4 mg/ml of RKS. ‡ Adjusted to the concentration present in 4 mg/ml of RKS.*

264.7 cells, with an optimal concentration at 0.4 mg/ml ($p<0.05$), declining at higher concentration (4 mg/ml) (Figure 2). LPS enhanced the PGE_2 production to a level slightly higher than that attained by RKS and this stimulation effect of LPS was significantly inhibited by higher concentration of RKS (4 mg/ml) ($p<0.05$) (Figure 2).

There was a possibility that RKS may have interacted with a component of the PGE_2 assay kit, causing the incorrect calculation of PGE_2 concentration. We found that addition of RKS (0.04, 0.4, 4 mg/ml) only marginally affected the determination of PGE_2 (within 15% fluctuation) (data not shown), reducing this possibility.

Effect of the five ingredients of RKS on PGE_2 production. SS, SM and BF enhanced the production of PGE_2 by unstimulated RAW 264.7 cells, to the extent comparable with that attained by RKS. RT and KZ showed much less stimulation effect. LPS stimulated the PGE_2 production to the greatest extent. The stimulation effect of LPS was inhibited to various extents by BF, RT, SM and SS. The inhibitory effect of KZ was the greatest (Figure 3). The bi-model action of RKS ingredients on PGE_2 production was confirmed.

Effect on the expression of COX-2 and $cPLA_2$ protein. In unstimulated RAW 264.7 cells, RKS, SS, SM and BF (0.4 mg/ml) increased the intracellular level of COX-2 protein. KZ and RT also slightly enhanced the intracellular level of the COX-2 protein (Figure 4A). In the LPS-stimulated cells, RKS (4 mg/ml) and its five ingredients did not inhibit, but rather slightly increased the intracellular concentration of COX-2 (Figure 4B). $cPLA_2$ protein expression was detected at

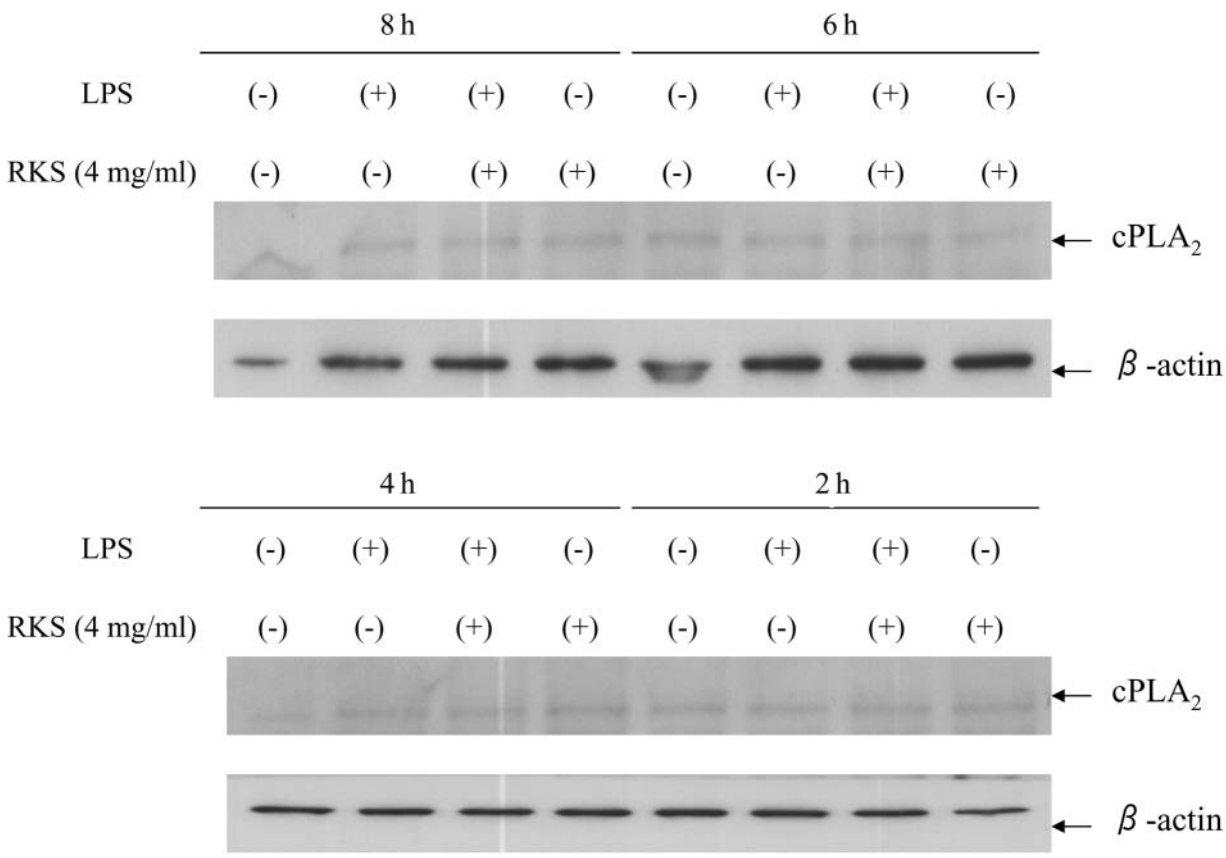

Figure 5. *Effect of RKS on the intracellular concentration of $cPLA_2$ protein in LPS-stimulated and -unstimulated RAW 264.7 cells. RAW 264.7 cells were incubated for 2,4, 6 or 8 h without or with 4 mg/ml RKS and with or without 100 ng/ml LPS. $cPLA_2$ protein expression in the cells was assayed by Western blot analysis.*

comparable levels without or with LPS stimulation and was not significantly changed without or with RKS. (Figure 5).

Reversal effect of arachidonic acid supplementation. The reduction of PGE_2 production of LPS-stimulated RAW 264.7 cells with high concentration (4 mg/ml) of RKS was recovered dose-dependently by the addition of arachidonic acid (Figure 6). The addition of RKS further increased the LPS-stimulated PGE_2 production.

Discussion

RKS, one of traditional Japanese Kampo medicines, has been used as an oral pain reliever, but its precise mechanism of action has not been elucidated yet. The present study demonstrates that RKS not only stimulated, but also inhibited the PGE_2 production by murine macrophage RAW 264.7 cells, depending on its concentration. This suggests that RKS contains both inhibitors and stimulators for PGE_2 production by RAW 264.7 cells. We reported previously that RKS inhibits or stimulates the NO production by RAW264.7 cells, depending on its concentration (3). A similar bi-modal action was recently reported for the extracts of sword brake (6) and *Carlowrightia cordifolia* (Acanthaceae) (7). Likewise, 'heating foods' such as litchi, longan and dried longan, stimulated the production of PGE_2 in RAW264.7 cells, whereas 'cooling foods' such as chrysanthemum flower, bitter gourd and lotus seed plumule, were inhibitory (8). In most of the cases, lower molecular weight components (such as kamebakaurin, wogonin, ginkgetin, 1,2,3,4,6-penta-*O*-galloyl-beta-D-glucose, epigallocatechin gallate) were responsible for the inhibition of NO or PGE_2 production by macrophages (9-12). On the other hand, the higher molecular weight substance(s) in these extracts, including RKS, might stimulate NO/PGE_2 production by RAW264.7 cells. The identification of cell surface receptor(s) for such substances is crucial to clarify the action mechanism of higher molecular weight substance(s).

Among the five ingredients of RKS, KZ only marginally induced the PGE_2 production in the absence of LPS, but

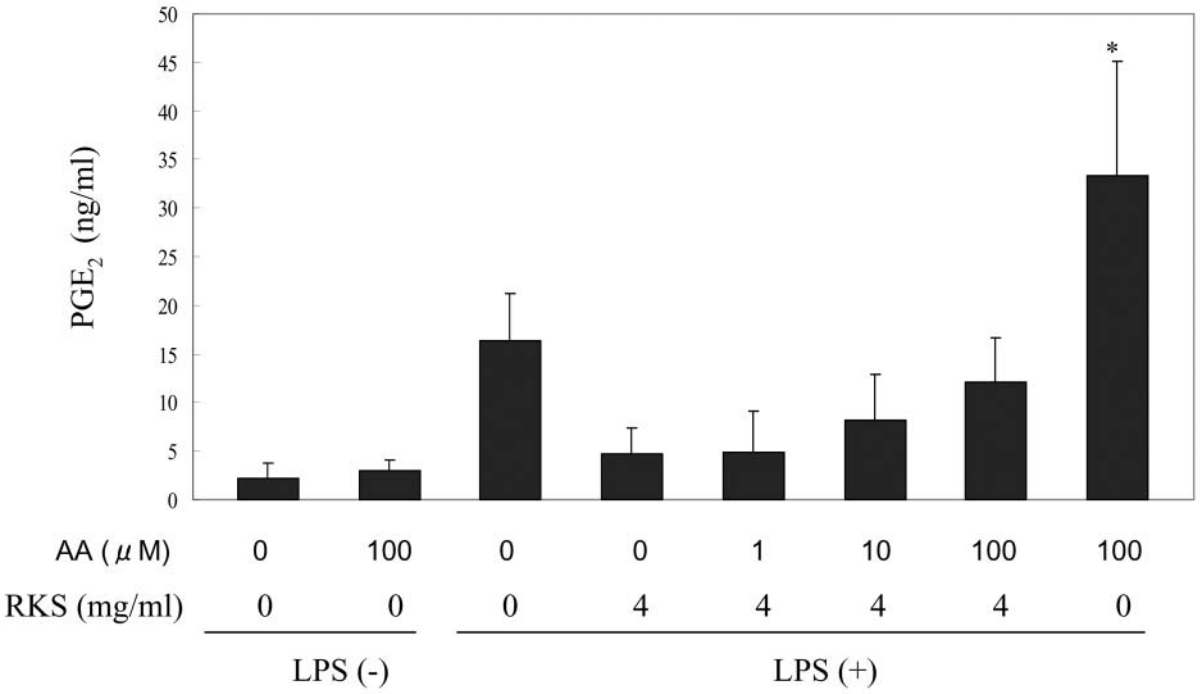

Figure 6. *Effect of addition of arachidonic acid on the PGE_2 production by RKS and LPS-treated RAW 264.7 cells. RAW 264.7 cells were incubated for 24 h without or with 100 ng/ml LPS or 4 mg/ml RKS in the presence of the indicated concentrations of arachidonic acid (AA). The PGE_2 production in the medium fraction was then determined. Each value represents mean±SD from three independent experiments. Statistically significant difference of PGE_2 production (*$p<0.05$), as compared with the group treated with LPS and 4 mg/ml RKS without AA.*

almost completely inhibited the production of PGE_2 by the LPS-stimulated cells. RT induced slightly higher PGE_2 production in the absence of LPS, but inhibited less efficiently the LPS-stimulated PGE_2 production. SS showed higher stimulatory activity of PGE_2 production in the absence of LPS and a higher inhibitory activity of LPS-stimulated PGE_2 production. SM and BF induced the highest PGE_2 production in the absence of LPS, but inhibited the LPS-stimulated PGE_2 production only marginally. Our finding is consistent with the previous report that glycirrhizin, a major component of KZ, inhibited the PGE_2 production by activated rat peritoneal macrophages (13). KZ thus seems to negate the effects of the other four ingredients. Considering these observations together with those of our previous study of NO production by RAW 264.7 cells (3), SS, SM, BF and RT may contain higher amounts of both stimulatory and inhibitory factors of RAW 264.7 cells, while KZ may contain excess amounts of the inhibitory factors.

We found that low concentration of RKS and all its 5 major ingredients induced the expression of COX-2, but the effects of KZ and RT were very weak. It was unexpected that higher concentration of RKS and its all ingredients failed to inhibit the LPS-stimulated COX-2 protein expression. Similarly glycyrrhizin was reported not to show any inhibition of the COXs in cloned mastocytoma cells at concentrations less than 10^{-4} M (14). These results suggest that RKS either exerts its action up-stream of the cyclooxygenation process, or due to the direct inhibition of COX-2 activity.

In the AA cascade, phospholipase A_2 ($cPLA_2$) is a major enzyme of PLA that catalyzes the release of AA from membrane phospholipids (4, 15). LPS was shown to induce the activation of $cPLA_2$ in RAW 264.7 cells (16). We found that RKS did not change the protein level of $cPLA_2$ with or without LPS and that the decline of intracellular PGE_2 level was compensated somewhat by supplementation with exogenous AA. These results suggest that RKS regulates the release and metabolism of AA and phosphorylation of $cPLA_2$, without affecting the protein level of $cPLA_2$ (17). Glycyrrhizin was reported to inhibit PGE_2 production without inhibition of COXs (13, 14). Glycyrrhizin also inhibited the release of AA from the activated rat peritoneal macrophages (13), and inhibited the PLA_2-induced carboxyfluorescein release from D,L-dipalmitoyl phosphatidylcholine liposomes (18). Glycyrrhizin in RKS may be responsible for the inhibition of $cPLA_2$ activation by RKS.

In conclusion, RKS was suggested to have different biological activities that inhibit and stimulate the PGE_2 production by RAW 264.7 cells, at higher or lower concentrations, respectively. It remains to be investigated

whether RKS and its ingredients abolish the phosphorylation of the inhibitor-κB and inhibit the expression of NF-κB target genes such as iNOS, COX-2 and TNF-α.

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