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# COX-2 as Possible Target for the Inhibition of $PGE_2$ Production by *Rikko-san* in Activated Macrophage

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**Abstract.** *We have previously reported that Rikko-san (RKS) inhibited the lipopolysaccharide (LPS)-stimulated prostaglandin (PG) $E_2$ in mouse macrophage-like RAW264.7 cells without affecting the expression of cyclooxygenase (COX)-2. Here RKS inhibition of the enzyme activity of both COX-1 and COX-2 proteins was investigated. Western blot analysis showed that RKS did not significantly change the S-nitrosylated COX-2 protein level. On the other hand, RKS inhibited the PG production catalyzed by purified COX-2, more effectively than that catalyzed by purified COX-1. These results suggest that RKS inhibits the $PGE_2$ production by selectively inhibiting the COX-2 activity in activated macrophages.*

*Rikko-san* (RKS) is one of the Kampo medicines (Japanese traditional medicines) and is composed of five major ingredients, *Kanzo* (KZ), *Shoma* (SM), *Ryutan* (RT), *Saishin* (SS) and *Bofu* (BF). RKS has been reported to be effective in controlling oral pain caused by dental caries, pulpitis, periodontitis and tooth extraction, but its precise mechanism of anti-inflammatory effect is still unclear (1, 2). In the arachidonic acid (AA) cascade, two enzymes are involved in the production of prostaglandin (PG) $E_2$. PG synthesis begins with the liberation of AA, the prime precursor, from membrane phospholipids by phospholipase $A_2$ ($PLA_2$). Subsequently, cyclooxygenase (COX) catalyze the rate-limiting reactions for PG synthesis comprising the bis-cyclooxygenation of AA to form $PGG_2$ and the peroxidative reduction of this intermediate to $PGH_2$ (3). The $cPLA_2\alpha$ is activated by proinflammatory cytokines or growth factors and catalyzes the AA from the cell surface membrane (4, 5). It is known that COX has two isoforms, COX-1 (constitutive isoform) and COX-2 (inducible isoform) (6). We have previously reported that lower concentrations (0.04-0.4 mg/ml) of RKS stimulated $PGE_2$ production, whereas a higher concentration (4 mg/ml) of RKS inhibited lipopolysaccharide (LPS)-stimulated $PGE_2$ production, suggesting the presence of both stimulator(s) and inhibitor(s) for $PGE_2$ production in RKS (7). The inhibitory effect of the higher concentration of RKS was neutralized somewhat by supplementation of exogenous AA. Among the five major RKS ingredients, only KZ significantly inhibited the LPS-stimulated $PGE_2$ production, whereas the other ingredients (SM, RT, SS, BF) only marginally affected the LPS-stimulated $PGE_2$ production. Western blot analysis demonstrated that RKS and all its ingredients enhanced the intracellular concentration of COX-2 protein, whereas RKS did not significantly affect the expression of $PLA_2$ protein (7).

It has been reported that COX-2 was activated by *S*-nitrosylation caused by iNOS (8). In order to further examine the possible anti-inflammatory effect of RKS, whether or not RKS stimulates the *S*-nitrosylation of COX-2 in RAW264.7 cell was investigated using Western blot analysis, and whether RKS inhibits the enzyme activity of purified COX-1 and/or COX-2 proteins was investigated by measuring the reaction product (8-10).

## Materials and Methods

*Materials*. RKS (No. 2990110010) was obtained from Tsumura Corp., Tokyo, Japan. The five ingredients of RKS and their major components are listed in Table I. The following chemicals and reagents were obtained from the indicated companies. Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp, Carlsbad, CA, USA), fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA, USA) and LPS from *Escherichia coli* (Serotype 0111:B4) (Sigma Chem. Ind., St. Louis, MO, USA).

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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.

*Cell culture*. The RAW264.7 cells were subcultured in DMEM supplemented with 10% heat-inactivated FBS under a humidified 5% $CO_2$ atmosphere and incubated with various concentrations of RKS in the presence or absence of LPS (100 ng/ml).

*S-nitrosylation of COX-2 protein*. The cell pellets were lysed and free thiols were blocked according to the instructions of the *S*-nitrosylated Protein Detection Kit (Cayman Chemical, Ann Arbor, MI, USA). Subsequently *S*-NO was reduced and was labeled by the biotin-switch method, according to the manufacturer's protocols.

*Immunoprecipitation of COX-2 protein*. The biotinylated protein was suspended with rProtein G Agarose (Gibco Industries, Inc., Langley, OK, USA) and was rotated for 60 minutes at 4˚C and centrifuged. The supernatant was mixed with anti-COX-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and was rotated for 60 minutes at 4˚C. Then rProtein G Agarose was added and rotated for 60 minutes at 4˚C. After centrifugation at 2000 ×g for 5 minutes, the precipitate was suspended with 1× SDS-sample buffer, and was boiled for 10 minutes.

*Detection of S-nitrosylated COX-2 protein by Western blot analysis*. The immunoprecipitated protein (equivalent to 25 μg before immunoprecipitation) was applied to the 8% SDS polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore Corp, Bedford, MA, USA). For the detection of the *S*-nitrosylated COX-2 protein, the membranes were blocked with 2% BSA (bovine serum albumin) in phosphate-buffered saline (PBS) for 60 minutes at 4˚C and incubated with *S*-nitrosylation Detection Reagent I (Avidin-HRP) (Cayman Chemical) (1:75) for 60 minutes at room temperature. For the detection of normal COX-2, the membranes were then blocked with 5% skim milk in PBS plus 0.05% Tween 20 for 60 minutes at room temperature and incubated with antibodies against COX-2 (1:2,000) (Santa Cruz Biotechnology) for 90 minutes at room temperature, and then incubated with horseradish peroxidase-conjugated anti-goat IgG (1:2,000) (Santa Cruz Biotechnology, Delaware, CA, USA) for 90 minutes at room temperature. Immunoblots were developed with a Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA, USA). The band density was quantified using an image processing program by National Institutes of Health (NIH).

*Inhibition of purified COXs activity*. Purified COX-1 (ovine) or COX-2 (human recombinant) was incubated with various concentrations of RKS, and the enzyme activity was assayed *in vitro* by determining the concentration of PG [enzyme immunoassay (EIA) kit, Cayman Chemical], a reaction product, according to the manufacturer's instructions. Briefly, the sample and heme were incubated with purified COX-1 or COX-2 for 10 minutes at 37˚C. Then AA was added and incubated for 2 minutes at 37˚C. HCl was used to stop the reaction and $SnCl_2$ was added to generate $PGF_2\alpha$.

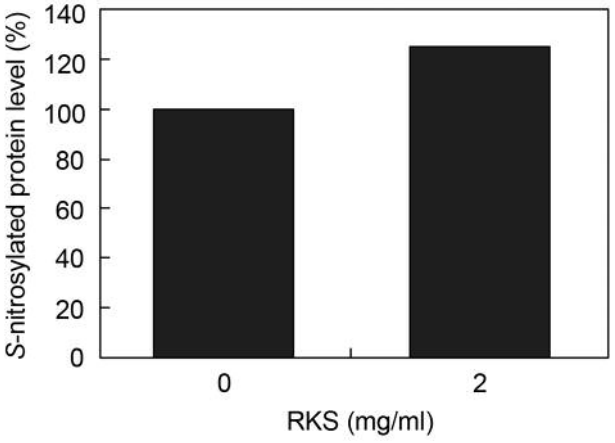


Figure 1. *Effect of RKS on S-nitrosylated COX-2 protein expression in LPS stimulated RAW264.7 cells. RAW264.7 cells were incubated for 24 hours with 100 ng/ml LPS in the presence of the indicated concentrations of RKS. The biotinylated protein was immunoprecipitated and quantified by Western blot analysis. The relative S-nitrosylated COX-2 protein expression was determined by dividing the COX-2 of RKS treated cells by that of control cells. Each value represents mean from two independent experiments.*

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

## Results

*Effect on the expression of S-nitrosylated COX-2 protein*. When RAW264.7 cells were incubated for 24 hours with 2.0 mg/ml RKS, the *S*-nitrosylation of COX-2 protein was not inhibited, but rather slightly increased (Figure 1).

*Inhibition of purified COXs activity*. RKS dose-dependently (0.0625-4.0 mg/ml) inhibited the COX-2 catalyzed PG production from the AA, with 50% inhibitory concentration ($IC_{50}$) of 0.66 mg/ml (Figure 2).

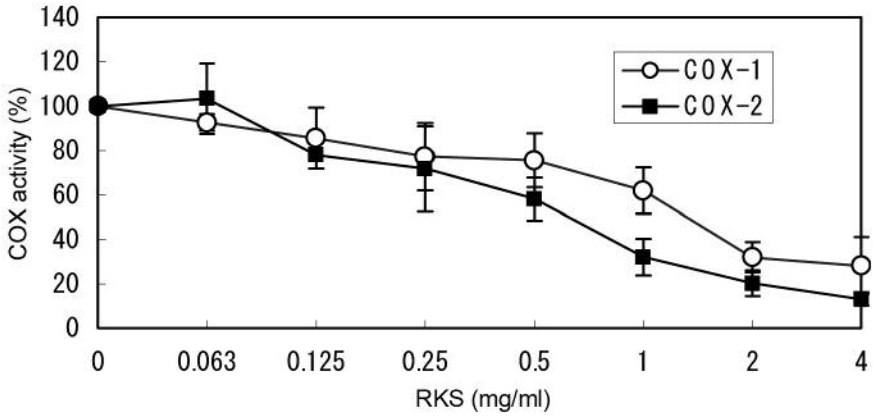


Figure 2. *Effect of RKS on the enzyme activity of purified COX-1 and COX-2 in vitro. COX-1 and COX-2 activity was determined by the amount of product ($PGF_2\alpha$) of enzyme reaction. Each value represents mean±SD from three independent experiments.*

On the other hand, a higher concentration of RKS was required to inhibit the COX-1 catalyzed PG production from the AA, with $IC_{50}$ of 1.40 mg/ml (Figure 2).

## Discussion

During the investigation of the anti-inflammation effect of RKS and its five ingredients, we found that RKS or KZ, one of its five ingredients, significantly inhibited the $PGE_2$ production by LPS-stimulated RAW264.7 cells without significant change in the COX-2 and $cPLA_2$ protein (7). The interaction between iNOS and COX-2 has been shown to be essential for $PGE_2$ production during the inflammatory process (11). It was found that iNOS was bound selectively to COX-2 and activated COX-2 *via S*-nitrosylation, and that NO-mediated activation of COX-2 was inhibited by disrupting the binding of these two enzymes (8). iNOS was indispensable for the activation but not for the increased expression of COX-2 (12). We have previously reported that RKS slightly enhanced the NO production by LPS stimulated RAW264.7 cells at lower concentrations (31-250 μg/ml), whereas it completely inhibited the LPS-stimulated NO production at a higher concentration (1,000 μg/ml). Western blot analysis showed that RKS inhibited the LPS-stimulated expression of iNOS protein (13). In the present immunoprecipitation study of COX-2, the expression of *S*-nitrosylated COX-2 protein was not inhibited by high concentration of RKS, even though the production of $PGE_2$ was inhibited. These results suggested that the action point of RKS ingredients might not be the *S*-nitrosylation site of COX-2 but in other regions. Recently it has been shown that $cPLA_2\alpha$ protein was *S*-nitrosylated, and its activity was enhanced by NO. COX-2 induction or expression markedly enhanced iNOS-induced $cPLA_2\alpha$ *S*-nitrosylation and activation (14). The effect of RKS on nitrosylation of $cPLA_2$ would be worth investigating.

Various natural products have shown inhibition or promotion of COX activity in LPS stimulated cell lines (15, 16) and some of them showed direct inhibition or stimulation of purified COXs activity *in vitro* (9, 17, 18). In Chinese medicine cryptotanshinone has been demonstrated to directly inhibit COX-2 activity without blocking its mRNA and protein expression (19). We previously reported that the reactivity of RKS in RAW264.7 cells was biphasic, at lower concentrations (0.04-0.4 mg/ml) it stimulated the $PGE_2$ production without or with LPS, whereas in higher concentration (4.0 mg/ml) it inhibited the LPS-stimulated $PGE_2$ production (7). The inhibitory effect of a higher concentration of RKS was recovered partly by supplementation with exogenous AA. RKS increased the COX-2 protein level, regardless of the concentration or stimulation of LPS, but did not affect cytosolic $PLA_2$ (7). In the present study RKS dose-dependently inhibited the PG production catalyzed by the purified COX-2 *in vitro* more effectively than that catalyzed by COX-1. RKS at low concentration might act as a potentially selective COX-2 inhibitor in the AA cascade. This is consistent with previous reports that among Kampo medicines *Hange –shashin*-to (<1 mg/ml) has been reported to inhibit COX-2 activity, but not to inhibit that of COX-1 *in vitro*, and glychyrrhizae radix (KZ) (0.1 mg/ml), which is also one of the major ingredients of RKS, and Coptidis Rhizoma, showed an inhibition of COX-2 activity (20). To clarify that RKS is a definite selective COX-2 inhibitor, further investigation using cultured cells expressing COX-1 will be needed.

Recently a dual COX/5-lipooxygenase (5-LOX) inhibitor has been developed. Since 5-LOX synthesize leukotrienes (LTs) which are the other family of AA products and are to some extent more inflammogenic than PGs (21-23), dual COX/5-LOX inhibitors could be expected to have anti-inflammatory effects not only as conventional non-steroidal

anti-inflammatory drugs (NSAIDs), but also as LT inhibitors (24). RKS has traditionally been used in patients with various conditions including asthma and blood diseases without major complications. Further investigations will elucidate the pharmacological effects of RKS on the AA cascade.

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