Glycoprotein Isolated from *Rhus verniciflua* Stokes Inhibits Inflammation-Related Protein and Nitric Oxide Production in LPS-Stimulated RAW 264.7 Cells

Phil-Sun Oh, Sei-Jung Lee, and Kye-Taek Lim*

Molecular Biochemistry Laboratory, Biotechnology Research Institute & Center for the Control of Animal Hazards Using Biotecnology (BK21), Chonnam National University; 300 Yongbong-Dong, Kwangju City, 500–757, South Korea.
Received August 24, 2006; accepted September 30, 2006

***Rhus verniciflua* Stokes (RVS) has traditionally been used for medical purpose, such as healing of inflammatory diseases in South Korea. Glycoprotein (36 kDa) was isolated from RVS fruit, purified and used to evaluate the inhibitory effect on inflammatory-related proteins and nitric oxide (NO) production in lipopolysaccharide (LPS, 200 ng/ml)-stimulated RAW 264.7 (murine macrophage cell line). Our results were showed that RVS glycoprotein has a strong antioxidative activity against lipid peroxyl radicals in cell-free system, and inhibits NO production in LPS-stimulated RAW 264.7 cells. To elucidate the inhibitory effect of RVS glycoprotein on activities of inflammatory-related proteins, we firstly evaluated the amount of intracellular reactive oxygen species (ROS), and expression of intracellular protein kinase C (PKC), nuclear factor (NF)-κB, and activator protein-1 (AP-1). The results in the present study showed that RVS glycoprotein (200 μg/ml) inhibits ROS production and PKCα translocation, and down-regulates the expression of NF-κB and AP-1. Such upstream signals consequently inhibited the levels of inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 expression. Therefore, we speculate that RVS glycoprotein inhibits the inflammatory-related protein and can act as an anti-inflammatory agent.**

Key words nitric oxide; RAW 264.7 cell; *Rhus verniciflua* Stokes (RVS) glycoprotein; inflammatory mediator

Inflammation is response to the pathogens and mechanical alteration in tissues.[1,2] These phenomena are mediated by inflammatory cells such as macrophages. Once macrophages are activated by stimulants, it produces reactive oxygen species (ROS) including nitric oxide (NO), and causes substantial oxidant injury to surrounding tissue.[3]

Nitric oxide (NO) is a short-lived free radical that mediates many biological functions, including neurotransmission, vascular homeostasis, and inflammation.[4] It is synthesized from L-arginine by NO synthase (NOS). The inducible NOS (iNOS) is strongly induced by bacterial endotoxin or inflammatory cytokines, and excessive NO production facilitates the severe injury to host cell and tissue. Like iNOS, COX-2 is also inducible and plays a role in inflammatory cell such as macrophages and endothelial cells.[5] It has reported that the expressions of cyclooxygenase (COX)-2 and iNOS under inflammatory circumstance are elevated through the activation of transcriptional factors in response to pro-inflammatory cytokines and ROS.[6,7]

Nuclear factor (NF)-κB is one of the most ubiquitous transcription factors that regulates gene expressions involved in cellular proliferation, cell adhesion, and inflammatory responses. It exists as a heterodimer of p50 and p65 subunits, and makes complex with inhibitory subunit (IκB) that prevents migration of p50/p65 to the nucleus. The activated NF-κB is then able to translocate into the nucleus, where it binds to specific DNA sequence, thereby controlling their expression.[5,8] Like NF-κB, the AP-1 is a redox-sensitive transcription factor, and is consisted of homo- or heterodimers of the Jun family (c-Jun, JunB, and JunD) and Fos family (c-Fos, FosB, Fral, and Fra2).[9,10] It is well-defined that several binding sites for transcriptional factors are located in the enhancer- or basal promoter region of pro-inflammatory genes including COX-2 and iNOS. Therefore, the inhibition of mediators by an agent is closely related to anti-inflammatory activity, and it might be one of good strategies for prevention of inflammation.

In recent years, many scientists have insisted that phytochemicals in fruits and vegetables have a critical role in preventing reactive oxidants-caused diseases, as antioxidants and functional foods.[11,13,34] *Rhus verniciflua* Stokes (RVS) has been traditionally used to heal inflammation in Korea.[12] Recently, we isolated glycoprotein from RVS fruits (RVS glycoprotein) with an approximate molecular mass of 36 kDa and consisting of carbohydrate (38.75%) and protein (61.25%). In a previous study, our results showed that RVS glycoprotein has an inhibitory effect on hydroxyl radicals-induced apoptotic cell death in normal cells *via* modulation of transcriptional factors. More recently, we found that RVS glycoprotein prevents an increase of plasma lipid levels and improves the antioxidant levels in experimentally induced hyperlipidemic mice.[13,14] However, there is no evidence its anti-inflammatory properties in lipopolysaccharide (LPS)-stimulated macrophage.

Therefore, we examined whether or not RVS glycoprotein modulates the activity of inflammatory-related protein in LPS-stimulated RAW 264.7 cells. We firstly evaluated production of intracellular ROS, expressions of inflammation related-signals (PKCα, NF-κB, and AP-1) and -proteins (iNOS and COX-2) in order to understand ability of RVS glycoprotein on inhibitory inflammation.

MATERIALS AND METHODS

Chemicals Lipopolysaccaride (LPS, L2637), 2′,7′-dichlorofluoresin diacetate (D6883), penicillin G (H0474), and streptomycin (H0447) were obtained from Sigma (St. Louis, MO, U.S.A.). RPMI 1640 and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, U.S.A.). Other chemicals and reagents were of the highest

* To whom correspondence should be addressed. e-mail: ktlim@chonnam.ac.kr

quality available.

Cell Culture RAW 264.7 cells (murine macrophage cell line) were incubated in RPMI 1640 containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C, and 5% CO_2 atmosphere. For stimulation with LPS, cells were seeded into 35 mm culture dishes or 96-well plates at 1×10^5 cells/well and allowed to adhere for 12 h at 37 °C under 5% CO_2. The cell survival was determined by the MTT assay.[15]

Isolation of RVS Glycoprotein RVS glycoprotein was isolated and purified from the fruits of *Rhus verniciflua* Stokes, as described previously.[13,14] Briefly, the fruits were broken into small pieces, and soaked in water for several months in a dark basement. The water extract was filtered through Whatman filter paper (No. 2) and concentrated with a rotary evaporator (Buchi, Flawil, Switzerland). The concentrated solution was dried with a freezer-dryer (Sam Won, Seoul, Korea). Five grams of dried-crude water extract was dissolved in distilled water. The solution was precipitated with 80% ammonium sulfate, dialyzed with a dialysis membrane (Spectra/por, MWCO 6000–8000, Pasadena, CA, U.S.A.) against 20 mM Tris–HCl (pH 7.4) overnight. After dialysis, the sample solution was dried with a freeze dryer and stored at −70 °C. The glycoprotein was verified using Schiff's reagent on the gel after electrophoresis and its purity was more than 95.0%. The dried sample 30.5 mg (0.61% from the original sample) was stored at −20 °C during the experimental period. After verification of high purity of glycoprotein, we treated into the cells for further studies.

Antioxidative Effect of RVS Glycoprotein The antioxidative activity of RVS glycoprotein against lipid peroxidation was measured through ammonium thiocyanate assay as descried previously.[16] For pre-emulsion, a linoleic acid mixed with 3 ml of Tween-20 in 200 ml of 30% (v/v) ethanol. The reaction mixture consisted of 5 ml of pre-emulsion and 0.5 ml of RVS glycoprotein (20—200 μg/ml). The total volume of the reaction was adjusted upto 10 ml with distilled water. The assay was conducted by adding 2.5 ml of 75% ethanol, 0.05 ml of ammonium thiocyanate solution (30% w/v), and 0.05 ml of reaction mixture. The color development in the reaction mixture was measured at 500 nm. In the experiment, α-tocopherol was used as a positive control.

Measurement of Intracellular ROS Amount of ROS measured by using nonfluorescent 2′,7′-dichlorofluorescein diacetate (DCFH-DA). The compound is deacetylated by intracellular esterases to the nonfluorescent DCFH, which is oxidized to the fluorescent compound DCF by ROS. For the measurement of ROS, cells were pre-incubated with 10 μM DCFH-DA for 30 min at 37 °C, and then the cells were co-treated with 200 ng/ml LPS in pre-treatment of RVS glycoprotein (50—200 μg/ml). The fluorescence intensity was measured at excitation wavelength of 485 nm and emission wavelength of 530 nm using fluoresencent microplate reader (Dual Scanning SPECTRAmax, Molecular Devices Corporation, Sunnyvale, CA, U.S.A.). The values were calculated as relative intensity of DCF fluorescence, compared with the control.

Preparation of Protein Extracts (Whole, Cytosol, and Nuclear) The protein extracts were prepared as previously described.[13] For the immunoblotting of iNOS and COX-2, the treated cells were rinsed twice with PBS after removing the medium and scraped in 300 μl of buffer A (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP40, 25% glycerol) containing a protease inhibitor cocktail. After lysis, the cells were centrifuged at 14000×g for 30 min. The supernatant was collected and used as whole cell lysates.[17]

To separate the cytosolic and nucleic protein extracts, the cells were scraped in 500 μl of buffer B (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP-40), followed by centrifugation at 3000×g for 5 min. The supernatant and pellet were designated as cytosolic and nucleic protein extracts separately. The isolated cytosolic protein extracts for the immunoblotting of PKCα were then resuspended in 100 μl of buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1.5 mM $MgCl_2$, 0.5% NP-40) containing a protease inhibitor cocktail (Boehringer, Mannheim, Germany) and centrifuged at 12000×g for 15 min. The pelleted nucleic protein extracts for the immunoblotting of NF-κB and AP-1 were then re-suspended in 200 μl of buffer C, and centrifuged at 12000×g. The amounts of proteins were measured using the Lowry method.[18] The samples of protein were stored at −70 °C.

Western Blot Analysis Intracellular protein extracts were analyzed on a 10% polyacrylamide mini-gel electrophoresis at 100 V for 2 h at room temperature using a Mini-PROTEIN II electrophoresis cell (Bio-Rad). After transfer onto nitrocellulose membranes (Millipore, Bedford, MA, U.S.A.), the membranes were subsequently incubated for 2 h at room temperature with a 1 : 100 dilution of mouse monoclonal antibody (PKC, Ab-2; Oncogene Science, Manhasset, NY, U.S.A.) and a 1 : 3000 dilution of rabbit polyclonal antibodies [NF-κB (p50), c-jun, c-fos, iNOS, COX-2, and α-tubulin, Santa Cruz Biotechnology, CA, U.S.A.] in TBS-T solution. The membranes were incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (1 : 10000; Santa Cruz Biotechnology, CA, U.S.A.). The protein bands were visualized by incubation with nitrobluetetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma Chemical Co., St. Louis, MO, U.S.A.). The results obtained from western blot assay were calculated with the using of the Scion imaging software (Scion Image Beta 4.02, Maryland, U.S.A.) as the relative intensity.

Measurement of Nitric Oxide Production NO production was measured as a function of nitrite (NO_2^-) concentration by the method of Green *et al.*[19] The cells were co-treated with 200 ng/ml LPS in pre-treatment of RVS glycoprotein (50—200 μg/ml) for 24 h in the 96 well multiple plate. The cell culture medium (50 μl) were mixed with 100 μl of 0.1% sulfanilamide and 100 μl 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 2.5% polyphosphoric acid for 5 min. Absorbance was measured at 540 nm with a MicroReader (Hyperion, Inc., Miami, FL, U.S.A.). Nitrite was quantified by using sodium nitrate as a standard.

Statistical Analysis All experiments were done in triplicate, and data were expressed as means±S.D. A one-way analysis of variance (ANOVA) and Duncan test were used significant differences of multiple comparisons (SPSS program, ver 11.0).

RESULTS

Antioxidant Property of RVS Glycoprotein in Cell Free System As shown in Fig. 1, the results showed that RVS glycoprotein has a scavenging activity against the lipid peroxyl radicals generated by the oxidation of linoleic acid. It is well known that the generated radicals play a pivotal role at the initiation stage of inflammation and carcinogenesis. The scavenging activity of RVS glycoprotein was significantly observed at 200 μg/ml of RVS glycoprotein. For example, the scavenging activities increased by 3.7, 7.4, 14.9 and 41.2% at 20, 50, 100, 200 μg/ml of RVS glycoprotein, compared with the control, respectively.

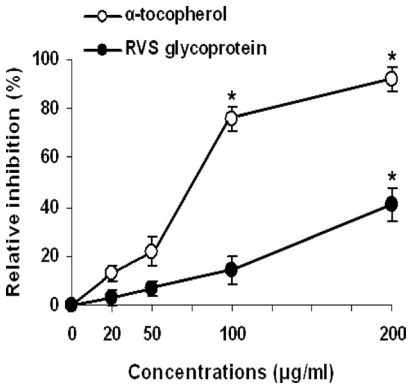

Fig. 1. Antioxidative Effect of RVS Glycoprotein

Antioxidative activities of RVS glycoprotein were represented as relative scavenging activity (%) compared to the control. Data represent the means±S.D. from triplicate experiments (n=3). The activity of RVS glycoprotein was compared with that of α-tocopherol. * Represents significant difference between antioxidative activities. $p<0.05$.

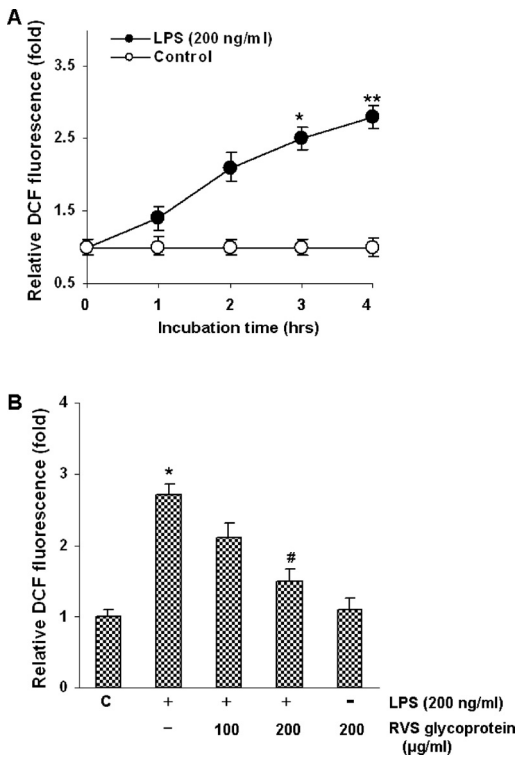

Fig. 2. Effect of RVS Glycoprotein on Intracellular ROS Production in LPS-Stimulated RAW 264.7 Cells

Data represent the means±S.D. from triplicate experiments (n=3). * and ** represent significant differences compared with the control, $p<0.05$ and $p<0.01$, respectively. # represents a significant difference between RVS glycoprotein treatments in the presence of LPS (200 ng/ml) and LPS (200 ng/ml) treatment alone, $p<0.05$.

Intracellular ROS Production by RVS Glycoprotein As shown in Fig. 2A, the relative content of intracellular ROS was gradually increased in treatment with LPS (200 ng/ml) for indicated incubation time. However, the treatment of RVS glycoprotein resulted in dose-dependent inhibition of LPS-stimulated ROS production (Fig. 2B). For instance, the relative amount of ROS significantly increased by 1.7 at treatment of LPS for 4 h, whereas it considerably decreased by 0.5 and 1.2 at treatment with 100 and 200 μg/ml of RVS glycoprotein, compared with the LPS alone.

Effects of RVS Glycoprotein on Expressions of PKCα, NF-κB, and AP-1 As shown in Fig. 3A, results were shown the effects of RVS glycoprotein on expressions of PKCα translocation, NF-κB and AP-1. Here, it should be noted that the relative intensity corresponds to the change of their expression (Fig. 3B). When the cells were treated with LPS alone, intensity of PKCα band reduces at the cytoplasmic level. However, the treatment with RVS glycoprotein in the presence of LPS gradually increased the intensity of PKCα (Fig. 3Aa). For instance, the relative intensities of LPS-stimulated PKCα bands in cytoplasmic fraction were augmented by 0.19 and 0.32 at 100 and 200 μg/ml RVS glycoprotein, respectively, compared to treatment with LPS (200 ng/ml) alone (Fig. 3Ba).

Furthermore, the expression of transcriptional factors (NF-κB, c-jun, and c-fos) were also increased in the treatment

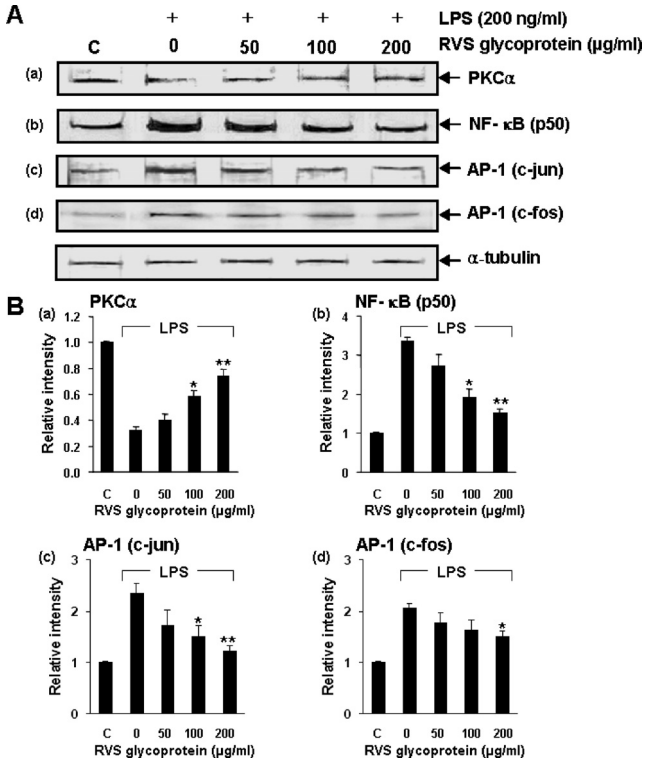

Fig. 3. Effects of RVS Glycoprotein on Activities of PKCα, NF-κB and AP-1 in LPS-Stimulated RAW 264.7 Cells

The relative intensity of bands (A) was calculated using of the Scion Imaging Software (Scion Image Beta 4.02, Maryland, U.S.A.) (B). Data represent the means±S.D. from triplicate experiments (n=3). * and ** represent significant differences between RVS glycoprotein treatments in the presence of LPS (200 ng/ml) and LPS (200 ng/ml) treatment alone, $p<0.05$ and $p<0.01$, respectively. α-Tubulin was used as an internal control. C: control.

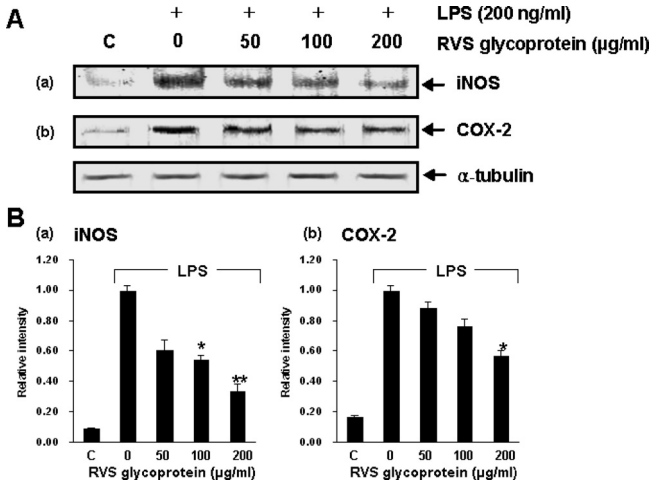

Fig. 4. Effect of RVS Glycoprotein on COX-2 and iNOS Expression in LPS-Stimulated RAW 264.7 Cells

The relative intensity of bands (A) was calculated using of the Scion Imaging Software (Scion Image Beta 4.02, Maryland, U.S.A.) (B). Data represent the means±S.D. from triplicate experiments (n=3). * and ** represent significant differences between RVS glycoprotein treatments in the presence of LPS (200 ng/ml) and LPS (200 ng/ml) treatment alone, $p<0.05$ and $p<0.01$, respectively. α-Tubulin was used as an internal control. C: control.

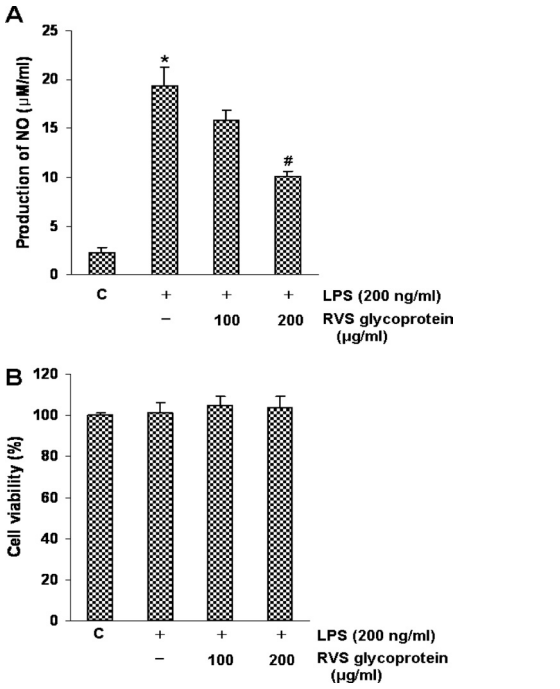

Fig. 5. Effect of RVS Glycoprotein on NO Production in LPS-Stimulated RAW 264.7 Cells

The production of NO was assessed indirectly by measuring the nitrite levels determined by a calorimetric method based on the Griess reaction (A). And, the cell survival was determined by the MTT assay (B). Data represent the means±S.D. from triplicate experiments (n=3). * Represents a significant difference compared with the control, $p<0.05$. # Represents a significant difference between RVS glycoprotein treatments in the presence of LPS (200 ng/ml) and LPS (200 ng/ml) treatment alone, $p<0.05$.

with LPS. However, the treatment of RVS glycoprotein (200 μg/ml) in the presence LPS significantly inhibited the expression of transcriptional factors, although that of c-fos protein did not change significantly (Figs. 3Ab, c, d). For instance, the relative intensities of transcriptional factors (NF-κB, c-jun, and c-fos) were diminished by 1.82, 1.12 and 0.54 at treatment with 200 μg/ml of RVS glycoprotein, compared with the LPS treatment alone, respectively (Figs. 3Bb, c, d).

Effect of RVS Glycoprotein on LPS-Stimulated COX-2 and iNOS In the RAW 264.7 cells without treatment of LPS, the expressions of COX-2 and iNOS protein were not detectable. As shown in Fig. 4Ab, the level of COX-2 protein was markedly augmented in LPS (200 ng/ml)-stimulated cells. However, the additions of RVS glycoprotein in presence of LPS (200 ng/ml) decreased the amount of COX-2 protein in a concentration-dependent manner. Interestingly, the results of the iNOS expression were observed in the similar pattern (Fig. 4Aa). For instance, the relative intensities of COX-2 and iNOS expression were diminished by 0.67 and 0.43 at 100 μg/ml of RVS glycoprotein, respectively, compared with the LPS treatment alone, (Figs. 4Ba, b).

Effect of RVS Glycoprotein on NO Production When the cells were stimulated with LPS (200 ng/ml) for 24 h, the levels of nitrite (a stable oxidized product of NO) significantly increased in the culture medium. For instance, the level of NO production was considerably increased by 6.3, 11.8, and 17 μM at 8, 12, and 24 h in presence of LPS (200 ng/ml). However, the additions of RVS glycoprotein (100, 200 μg/ml) in the presence of LPS (200 ng/ml) gradually decreased the levels of NO, compared with the LPS treatment alone (Fig. 5A). For instance, the levels of NO were significantly diminished by 3.5 and 9.2 μM at 100 and 200 μg/ml of RVS glycoprotein, compared with the LPS treatment alone, respectively. Moreover, the results were confirmed that the inhibitory effect of RVS glycoprotein on NO production was not due to cytotoxicity. In other words, RVS glycoprotein did not observed the cytotoxic effect under the same experimental condition (Fig. 5B). For instance, the cell viability values did not show the any change at 200 μg/ml of RVS glycoprotein, compared with the LPS treatment alone.

DISCUSSION

Initially, we tested the anti-oxidative capacity of RVS glycoprotein against lipid peroxyl radicals that involves in the initiation of inflammation and carcinogenesis. The ammonium thiocyanate method measures the degree of oxidation of Fe^{2+} to Fe^{3+} by peroxyl radicals generated from oxidation of the linoleic acid.[16] Our result showed that the scavenging activity of RVS glycoprotein (200 μg/ml) in the cell free system corresponds to that of α-tocopherol (70 μg/ml) (Fig. 1), suggesting its strong antioxidant property. This radical scavenging activity of RVS glycoprotein is meaningful in biological system, because this activity can directly or indirectly block the damage to bio-macromolecules by inhibiting the superoxide anion (O_2^-)-induced radical formations during inflammation process.[5,20] In the macrophage cell line, RVS glycoprotein was also showed the decreasing effect on intracellular ROS production stimulated by LPS, having an antioxidant activity. LPS (a component of the cell wall of Gram-negative bacteria) is the triggering factor for multiple-organ failure during septic shock. Macrophage activated by LPS generates the ROS and promotes the secretion of pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), PGs, and NO.[21] The increase of intracellular ROS reduced at the addition of RVS glycoprotein in LPS-stimulated RAW 264.7 cells, because of

antioxidative character of RVS glycoprotein. Generally, most plant glycoproteins including RVS glycoprotein have hygroscopic character and high polarity. Therefore, it can act to scavenge reactive radicals as antioxidative compound and easily interact with outer cell membrane receptors due to high polarity.

Among a variety of inflammatory mediators, both NO and PGE_2 have been implicated as important mediators in endotoxemia and inflammatory conditions.[22—24] The overproduced PGE_2 *via* COX-2 activation gives rise to pain, swelling, and stiffness,[25] while the reaction of NO with superoxide anion can modify the free and/or protein-bound amino acid residues, and inhibit the enzymatic activities. Thus, they induce the lipid peroxidation by depleting cellular antioxidant levels.[21,23] This is considerable importance since iNOS-dependent overproduction of NO is considered as pro-inflammatory property. Accordingly, NOS inhibitors for the treatment of NO-mediated inflammatory processes were required high specificity for iNOS. Interestingly, RVS glycoprotein in this study inhibited LPS-induced NO production and iNOS expression without cytotoxicity. This means that RVS glycoprotein is relatively a safe modulator of NO and COX-2 for various pathologic conditions because of inhibition of LPS-stimulated iNOS and COX-2 expression. Although the present investigation cannot be elucidated the mechanism of interaction between NOS and COX expression, the possible mechanism is that RVS glycoprotein is able to inhibit the NF-κB and AP-1 activity, subsequently blocks excessive production of NO resulting from iNOS activation, and then reduces the COX-2 activity in LPS-stimulated RAW 264.7 cells. Indeed, it has reported earlier that NO can stimulate COX expression *via* its reaction with heme component of the COX enzyme.[26—28]

Recently, it has reported that LPS induces the activation of PKC super-family of protein serine-threonine kinases in the macrophages.[7,29] Furthermore, PKCα activation induced by both LPS and IFN-γ can modulate the expression of COX-2 as well as iNOS in macrophages. It means that PKCα expression involves in the regulation of macrophage inflammatory responses.[30,31] Our results showed that the levels of PKCα activation markedly decreased after exposure to LPS in the cytoplasmic fraction. In other words, PKCα was activated by LPS and then it translocates from cytosol to plasma membrane. However, RVS glycoprotein (200 μg/ml) blocked the LPS-induced PKCα translocation. PKCα is required for the activation of specific transcriptional factors (NF-κB and AP-1) regulating iNOS expression.[32—34] Such transcriptional factors are implicated in the inducible expression of a variety of genes in response to oxidative stress,[9] although no common second messenger has yet been identified. As expected, LPS-induced NF-κB activation in this study inhibited at addition of RVS glycoprotein, effectively. Interestingly, increased AP-1 (c-jun) activation was significantly inhibited by RVS glycoprotein, although that of c-fos was inhibited but less than c-jun (Fig. 3). Consequently, the signal of NF-κB inhibition transfers to reduce iNOS expression because of its antioxidants property, inhibition of NO production. From these results, we suggest that the addition of RVS glycoprotein inhibits NO production and the activities of transcriptional factors (especially NF-κB), which relate to factors of inflammation response.[35]

In conclusion, our results showed that RVS glycoprotein has an antioxidative property, and modulates activities of inflammatory related signals (PKCα, iNOS, COX-2, AP-1, and NF-κB) and inhibits NO production in LPS-stimulated RAW 264.7 cells. Taken together, RVS glycoprotein has an anti-inflammatory potential. It still remains the problems which are required the evaluation of pro-inflammatory cytokines, *i.e.* TNF, IFN to elucidate the precise inhibitory ability of RVS glycoprotein in primary cells.

Acknowledgements This study was financially supported by a research fund of Chonnam National University in 2006.

REFERENCES

1) Vane J., Botting R., *FASEB J.*, **1**, 89—96 (1987).
2) Libby P., Ridker P. M., Maseri A., *Circulation*, **105**, 1135—1143 (2002).
3) Adams D. O., Hamilton T. A., *Annu. Rev. Immunol.*, **2**, 283—318 (1984).
4) MacMicking J., Xie Q. W., Nathan C., *Annu. Rev. Immunol.*, **15**, 323—350 (1997).
5) Morham S. G., Langenbach R., Loftin C. D., Tiano H. F., Vouloumanos N., Jennette J. C., Mahler J. F., Kluckman K. D., Ledford A., Lee C. A., Smithies O., *Cell*, **83**, 473—482 (1995).
6) Chandel N. S., Trzyna W. C., McClintock D. S., Schumacker P. T., *J. Immunol.*, **165**, 1013—1021 (2000).
7) Fujihara M., Connolly N., Ito N., Suzuki T., *J. Immunol.*, **152**, 1898—1906 (1994).
8) Karin M., *J. Biol. Chem.*, **274**, 27339—27342 (1999).
9) Gius D., Botero A., Shah S., Curry H. A., *Toxicol. Lett.*, **106**, 93—106 (1999).
10) Aktan F., *Life Sci.*, **75**, 639—653 (2004).
11) Hirota M., Morimura K., Shibata H., *Biosci. Biotechnol. Biochem.*, **66**, 179—184 (2002).
12) Kim T. J., "Korea Resource Plant," II, Seoul University Press, Seoul, 1996, pp. 292—297.
13) Ko J. H., Lee S. J., Lim K. T., *Toxicol. In Vitro*, **19**, 353—363 (2005).
14) Oh P. S., Lee S. J., Lim K. T., *Biosci. Biotechnol. Biochem.*, **70**, 447—456 (2006).
15) Mosmann T., *J. Immunol. Methods*, **65**, 55—63 (1983).
16) Asamari A. M., Addis P. B., Epley R. J., Krick T. P., *J. Agric. Food Chem.*, **44**, 126—130 (1996).
17) Agren M., Kogerman P., Kleman M. I., Wessling M., Toftgard R., *Gene*, **330**, 101—114 (2004).
18) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
19) Green L. C., Wagner D. A., Glogowski J., Skipper P. L., Wishnok J. S., Tannenbaum S. R., *Anal. Biochem.*, **126**, 131—138 (1982).
20) Fridovich I., *Annu. Rev. Pharmacol. Toxicol.*, **23**, 239—257 (1983).
21) Murakami A., Nakamura Y., Tanaka T., Kawabata K., Takahashi D., Koshimizu K., Ohigashi H., *Carcinogenesis*, **21**, 1843—1850 (2000).
22) Guzik T. J., Korbut R., Adamek-Guzik T., *J. Physiol. Pharmacol.*, **54**, 469—487 (2003).
23) Radi R., Beckman J. S., Bush K. M., Freeman B. A., *Arch. Biochem. Biophys.*, **288**, 481—487 (1991).
24) Farias-Eisner R., Sherman M. P., Aeberhard E., Chaudhuri G., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 9407—9411 (1994).
25) Seibert K., Zhang Y., Leahy K., Hauser S., Masferrer J., Perkins W., Lee L., Isakson P., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 12013—12017 (1994).
26) Diaz-Cazorla M., Perez-Sala D., Lamas S., *J. Am. Soc. Nephrol.*, **10**, 943—952 (1999).
27) Mitchell J. A., Kohlhaas K. L., Sorrentino R., Warner T. D., Murad F., Vane J. R., *Br. J. Pharmacol.*, **109**, 265—270 (1993).
28) Posadas I., Terencio M. C., Guillen I., Ferrandiz M. L., Coloma J., Paya M., Alcaraz M. J., *Naunyn. Schmiedebergs. Arch. Pharmacol.*, **361**, 98—106 (2000).
29) Pfannkuche H. J., Kaever V., Resch K., *Biochem. Biophys. Res. Commun.*, **139**, 604—611 (1986).

30) Chen C. C., Wang J. K., Lin S. B., *J. Immunol.*, **161**, 6206—6214 (1998).
31) Giroux M., Descoteaux A., *J. Immunol.*, **165**, 3985—3991 (2000).
32) Kuo C. T., Chiang L. L., Lee C. N., Yu M. C., Bai K. J., Lee H. M., Lee W. S., Sheu J. R., Lin C. H., *J. Biomed. Sci.*, **10**, 136—145 (2003).
33) St-Denis A., Chano F., Tremblay P., St-Pierre Y., Descoteaux A., *J. Biol. Chem.*, **273**, 32787—32792 (1998).
34) Yao J., Mackman N., Edgington T. S., Fan S. T., *J. Biol. Chem.*, **272**, 17795—17801 (1997).
35) Yang F., de Villiers W. J., McClain C. J., Varilek G. W., *J. Nutr.*, **128**, 2334—2340 (1998).