Anti-inflammatory Effect of *Quercus Salicina* in IFN-γ/LPS-stimulated Mouse Peritoneal Macrophage

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Quercus salicina has been widely used as a traditional medicine for the treatment of various diseases. In macrophages, nitric oxide (NO) is released as an inflammatory mediator and has been proposed to be an important modulator of many pathophysiological conditions in inflammation. In the present study, the inhibitory effect of methanolic extracts of *Q. salicina* (QSM) on NO production in LPS-stimulated mouse (C57BL/6) peritoneal macrophages was investigated. QSM suppressed NO production without notable cytotoxiciy. QSM also exhibited down-regulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression via attenuation of NF-κB translocation to nucleus in rIFN-γ and LPS stimulated mouse peritoneal macrophages. The present study strongly suggest that *Q. salicina* may be beneficial in diseases which related to macrophage-mediated inflammatory disorders.

Key words : *Quercus salicina*, nitric oxide, Anti-inflammatory

Introduction

Inflammation is characterized by redness, heat, swelling, pain and dysfunction of the organs. It is a complex physiological and pathological process mediated by variouse cytokines produced by immune cells including neutrophils, macrophages, mast cells, platelets[1]. Many types of autoimmune diseases and allergies such as asthma, rheumatoid arthritis and multiple sclerosis are example of excessive inflammatory responses[2].

Macrophages play a central role in host defense and maintenance as a major immune cell in inflammation, since they are concerned in not only innate immunity but specific acquired immunity[3,4]. Under inflammatory conditions, the activated macrophages may generate large amount of nitric oxide (NO).

NO, a toxic free radical, is synthesized from L-arginine, by a family of nitric oxide synthase (NOS). Nanomolar concentration of NO generation by constituent NOS (cNOS) act as neurotransmitter and vasodilator[5]. However, it is well known that inducible NOS (iNOS) mediated mass NO production can cause cytotoxicity and tissue damage[6].

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Cyclooxygenase-2 (COX-2), an inducible enzyme, catalyze of PGE2 from arachidonic acid[7]. Several reports demonstrated that overproduction of PGE2 by COX-2 are in close connection with NO generation[8]. Thus, COX-2 mediated production of PGE2 take important part in the process of inflammation[9].

Quercus salicina Blume (Fagaceae) which is distributed through the southern parts of the Korean Peninsula and Japan, is an oriental medicine, used as diuretic, anti-inflammatory, antiedemic agent[10,11]. Previous phytochemical studies of this plant have shown the presence of stenophyllanin A, B, C, salidroside gallate, scyllo quercitol gallate, quinic acid gallate, stenophynin A, B, grandinin, acuttissimin A, B, guaiacyl glycerol[12-15]. Previous pharmacological studies on *Q. salicina* revealed its antioxidant properties[16] and therapeutic effect in the rat urolithiasis model[17]. However, the anti-inflammatory activity of MeOH extracts of Q. salicina (QSM) in IFN-γ and LPS-stimulated mouse peritoneal macrophages is still unclear.

In the present study, the inhibition effect of QSM on NO production in the IFN-γ/LPS-stimulated macrophages was investigated. For clear the evident mechanism of NO suppression, the author also assessed the expression level of iNOS, COX-2 as well as NF-κB activation.

Materials and Methods

1. Animals

Male C57BL/6 mice were purchased from Damul Science

Co. (Daejon, South Korea). All animals were housed at 22±1℃ with a 12 h light/dark cycle and fed a standard pellet diet with tap water ad libitum.

2. Preparation of sample

The plant materials were purchased from Wansanyakupsa (Jeonju, South Korea) in March 2010. A voucher specimen (WME072) has been deposited at the Department of Oriental Pharmacy, College of Pharmacy, Woosuk University. An extract was obtained twice from the dried sample (300 g) with 6,000 mL of MeOH under ultrasonification for 2 h. The solutions were filtered and the solvents were evaporated in the rotary vacuum evaporator. The evaporated samples were lyophilized and then stored at －20℃ until use.

3. Peritoneal macrophage culture

Thioglycollate-elicited macrophages were harvested 3～4 days after i.p. injection of 2.5 ml TG to the mice and isolated. Using 8 ml of cold HBSS containing 10 U/ml heparin, peritoneal lavage was performed. Then, the cells were distributed in DMEM, which was supplemented with 10% heat-inactivated FBS, in 24-well tissue culture plates (3×10^5 cells/well) incubated for 3 h at 37℃ in an atmosphere of 5% CO_2, washed three times with HBSS to remove non-adherent cells and equilibrated with DMEM that contained 10% FBS before treatment.

4. MTT assay

Cell respiration, an indicator of cell viability, was performed by the mitochondrial dependent reduction of 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan, as described by Mosmann[18]. Peritoneal macrophages (3×10^5 cells/well) were cultured with various concentrations of QSM. After 24 h of incubation, The extent of the reduction of MTT to formazan within cells was quantified by measuring the optical density (OD) at 540 nm using an automated microplate reader (GENios, Tecan, Austria).

5. Assay of nitrite concentration

Peritoneal macrophages (3×10^5 cells/well) were cultured with various concentrations of QSM. The cells were then stimulated with rIFN- γ (20 U/ml). After 6 h, the cells were finally treated with LPS (10 μg/ml). After 48 h, NO synthesis in cell cultures was measured by a microplate assay method. To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent at room temperature for 10 min. The absorbance at 540 nm was determined by an automatic microplate reader. NO_2- was determined by using sodium nitrite as a standard.

6. Preparation of nuclear extracts

Nuclear extracts were prepared essentially according to described previously[19]. Briefly, dishes were washed with ice-cold PBS. The dishes were then scraped and transferred to microtubes. Cells were allowed to swell by adding lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol and 0.5 mM phenylmethyl-sulfonylfluoride). The tubes were vortexed to disrupt cell membranes. The samples were incubated for 10 min on ice and centrifuged for 5 min at 4℃. Pellets containing crude nuclei were resuspended in extraction buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride) and incubated for 30 min on ice. The samples were centrifuged at 12,000 rpm for 10 min to obtain the supernatant containing nuclear extracts. Extracts were stored at -70℃ until use.

7. Western blot analysis

Whole cell lysates were made by boiling peritoneal macrophages in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then blocked with 5% skim milk for 2 h at room temperature and then incubated with anti-iNOS, anti-COX-2 and anti-p65. After washing in with phosphate buffered saline (PBS) containing 0.05% tween 20 three times, the blot was incubated with secondary antibody (anti-rabbit, anti-mouse) for 1 h and the antibody specific proteins were visualized by the enhanced chemiluminesence detection system according to the recommended procedure (Amersham Corp. Newark, NJ, Germany).

8. Densitometric and statistical analysis

All measurement are expressed as the mean ± S.D. of independent experiments. Data between groups were analyzed by a paired Student's t-test and p-values less than 0.01 were considered significant. Intensity of the bands obtained from Western blotting were estimated with ImageQuantTL (GE Healthcare, Sweden) and the values were expressed as mean ± standard error.

Results

1. Effects of QSM on cell viability

To determine the effects of QSM on viability of mouse peritoneal macrophages, we carried out MTT assay. IFN-γ (20 U/ml) and LPS (10 μg/ml) treated macrophages were exposed to various concentrations of QSM and it did not showed significant cytotoxicity on activated macrophages(Fig. 1).

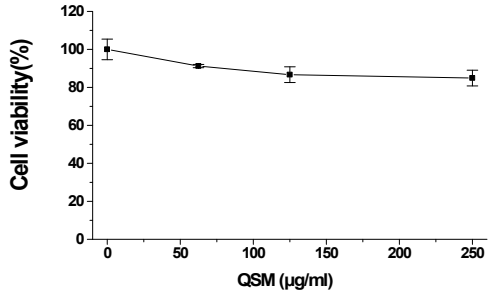

Fig. 1. Effect of QSM on the viability in rIFN-γ/LPS-treated peritoneal macrophages. Peritoneal macrophages (3×10^5 cells/well) were incubated with various concentrations of QSM for 24h. Cell viability was evaluated by MTT colorimetric assay as described in the method. The results are expressed as means ± S.D. of three independent experiments duplicate in each run.

2. Inhibitory effects of QSM on NO Production

To determine the effect of QSM on the production of NO in mouse peritoneal macrophages, we pretreated the cells with various concentration QSM (62.5, 125, 250 μg/ml). And then we stimulated them with IFN-γ (20 U/ml) and LPS (10 μg/ml). The resultant NO production was determined by detecting nitrite concentrations in the cell supernatants after 48 h treatment. As shown in Fig. 2, IFN-γ and LPS stimulation caused an increase of NO production about 12 folds and QSM showed suppression of NO production in a dose dependent manner(Fig. 2).

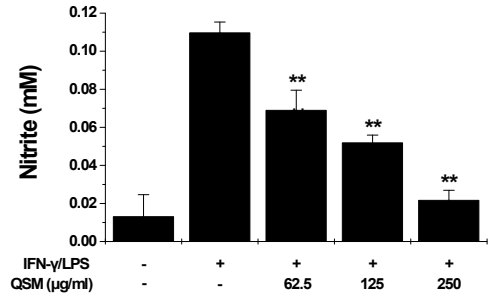

Fig. 2. Dose-dependent inhibition effects of QSM on NO production in rIFN-γ/LPS-treated peritoneal macrophages. Peritoenal macrophages (3×10^5 cells/well) were cultured with various concentration of QSM. The peritoneal macrophages were then stimulated with rIFN-γ (20 U/ml) and LPS (10 μg/ml). After 48 h of culture, NO release was measured by the Griess method (nitrite). NO (nitrite) released into the medium is presented as the mean ± S.D. of three independent experiments duplicate in each run; $^{**}p < 0.001$ compared to rIFN-γ + LPS.

3. Effects of QSM on expression of iNOS and COX-2

In order to investigate the mechanism of action of QSM (100 μg/ml) on the inhibition of NO production, we investigate the effect of the QSM on inflammatory mediators at protein level by western blotting. As shown in Fig. 3, the expression of iNOS and COX-2 was increased after IFN-γ (20 U/ml) and LPS (10 μg/ml) stimulation for 24 h. This enhanced expression of iNOS and COX-2 was significantly reduced by QSM(Fig. 3).

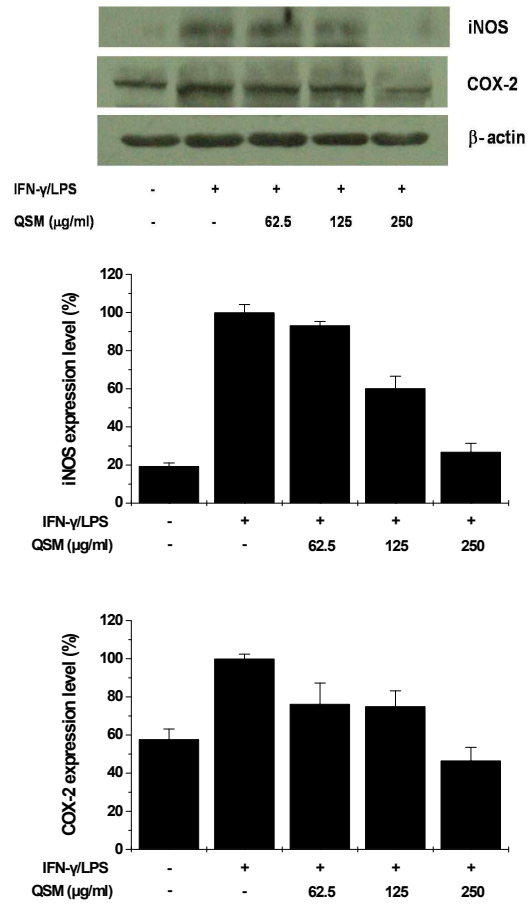

Fig. 3. Effects of QSM on the expression of iNOS and COX-2 by rIFN-γ/LPS-treated peritoneal macrophages. Peritoneal macrophages (5×10^6 cells/well) were pretreated with QSM and then stimulated for 6 h with rIFN-γ (20 U/ml). The peritoneal macrophages were then stimulated with LPS (10 μg/ml) for 24 h. The protein extracts were prepared and samples were analyzed for iNOS and COX-2 expression by Western blotting as described in the Method section. The expression of iNOS and COX-2 was quantified by densitometric analysis. The expression levels of the rIFN-γ/LPS treated control cells were considered to be 100% for the percentage calculations.

4. Effects of QSM on activation of NF-κB

NF-κB is activated in cells stimulated with LPS and other inflammatory insults, a process that is related to the transcriptional activation of responsive genes. As shown in Fig 4, co-incubation with LPS plus QSM decreased the p65 level in the nucleus and increased in the cytosol, respectively. These findings strongly suggest that QSM inhibited the LPS-stimulated transcriptional activity of NF-κB by

suppressing phosphorylation-dependent proteolysis of IkB-α in the cells.

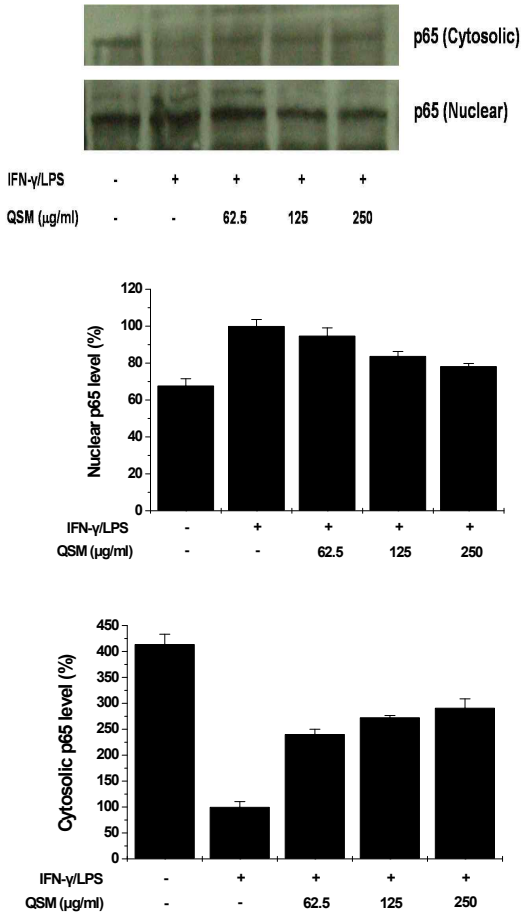

Fig. 4. Effects of QSM on the activation of NF-κB by LPS-stimulated peritoneal macrophages. Peritoneal macrophages (5×10^6 cells/well) were pretreated with QSM for 30 min and then stimulated with rIFN-γ (20 U/ml) for 2 h. After 1 h stimulation with LPS (10 μg/ml), the nuclear extracts were prepared and samples were analyzed by western blotting as described in the Method section and quantified by densitometry. The expression levels of the rIFN-γ/LPS treated control cells were considered to be 100% for the percentage calculations.

Discussion

NO is a free radical produced from L-arginine by NOS and has diverse physiological roles and also contributes to the immune defense against viruses, bacteria and other parasites[7]. However, over-production of NO is recognized to play a crucial role in the pathogenesis of inflammation and result in septic shock, neurologic disorders, rheumatoid arthritis and autoimmune diseases[20]. Therefore, to avoid excessive NO production, the use of exogenous modulators become necessary.

At this point of view, it was evaluated that the effect of methanol extract of QSM on NO production in IFN-γ and LPS-stimulated mouse peritoneal macrophages. Nitrite assay by Griess reaction revealed that the markedly increased production of nitrite by activated mouse peritoneal macrophages was dose-dependently inhibited by QSM (Fig. 2) and this effect was shown without notable cytotoxic activity(Fig. 1).

iNOS is up-regulated in response to inflammatory and pro-inflammatory mediators and their products can influence many aspects of the inflammatory cascade. nNOS and eNOS were critical to normal physiology and thus, inhibition of these enzymes caused damage. In the contrary, the level of iNOS is associated with excess production of NO in activated macrophages. Therefore, suppression of NO production via inhibition of iNOS expression might be an attractive therapeutic target for the treatment of numerous pathological conditions, including inflammation. Thus the possibility that QSM might inhibit iNOS expression was examined. The results demonstrated that QSM suppressed the expression of iNOS significantly in IFN-γ and LPS-stimulated mouse peritoneal macrophages(Fig. 3).

COX, another key enzyme in inflammation, is the rate-limiting enzyme that catalyzes the formation of prostaglandins (PGs) from arachidonic acid. Levels of PGs increase early in the course of the inflammation[21]. Like NOS, COX also exists in both constitutive (COX-1) and inducible (COX-2) forms. It is well known that the COX-1 is a housekeeping protein in most tissues and it catalyzes the synthesis of PGs for normal physiological functions. In constant, inducible isoform, COX-2, is rapidly stimulated by tumor promoters, growth factors, cytokines and pro-inflammatory molecules[22] and responsible for the production of the high levels of PGs in several pathological conditions such as inflammation. Since, COX-2 is induced by stimulation in inflammatory cells, inhibitors of COX-2 induction might be candidates for the new type of nonsteroidal anti-inflammatory drugs (NSAIDs). The present study showed that methanol extract of QSM inhibited IFN-γ and LPS-stimulated expression of COX-2 in mouse peritoneal macrophages(Fig. 3). Thus, it seems quite reasonable to speculate that QSM may inhibits PGE2 production. However, further studies are required to determine whether QSM is selective inhibitor of COX-2.

The iNOS and COX-2 expression is regulated mainly at the transcriptional level and its major transcriptional regulator is the NF-κB, which is also key regulators of a variety of genes involved in immune and inflammatory responese[23]. In unstimulated cells, inactive NF-κB is sequestered in the cytoplasm and is linked to the inhibitory IκB protein. However, in active state, following the induction of NF-κB by

appropriate extracellular stimulation such as LPS, TNF-α or tissue plasminogen activator, it translocate to the nucleus with phosphorylation, ubiquitation and degradation of IκBα and also acts upon the iNOS gene promoter to activate transcription. In this study, we investigated that the effect of QSM on the translocation of NF-κB using Western blotting. The result showed that translocation of NF-κB to nucleus was attenuated strongly by QSM(Fig. 4). These results indicate that the inhibitory action of QSM on the expression of iNOS and COX-2 was might be due to blocking of NF-κB activation.

In summary, the production of NO, iNOS and COX-2 takes an important part in the immune response to many inflammatory stimulti. Excessive production of these mediators is implicated in acute and chronic inflammatory diseases including septic shock, hemorrhagic shock, multiple selerosis, rheumatoid arthritis, ulcerative colitis and atherosclerosis[24]. The present results demonstrate that QSM inhibits over-production of NO and this inhibitory effect was consistent with its down-regulation effect on the expression of iNOS in mouse peritoneal macrophages. QSM also suppressed expression of COX-2 in a concentration-dependent manner via down-regulation of NF-κB activation.

These findings in the present study provide scientific supporting evidence of the use of clinical practice of QSM for the treatment of inflammation. In conclusion, these results establish that QSM has potent anti-inflammatory effects and may hold great promise for use in macrophage-mediated inflammatory diseases as an effective immunomodulatory mediators.

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