Modulation of Aqueous Extracted *Angelicae sinensis Radix* on Nitric Oxide Production and Pro-inflammatory Cytokine Gene Expressions in RAW 264.7 Macrophage Cells

Young Sun Lee, Ok Kyung Han, Sang Woo Shin[1], Jong Hyun Park[1], Young Kyu Kwon[1]*

Efficacy and Safety Research Center for Traditional Oriental Medicines, Daegu Haany University, Kyungbuk Technopark, 1: College of Oriental Medicine, Daegu Haany University

Angelica sinensis radix, Danggui, is a traditional oriental medication, which has been used to modulate immune response. We report here that aqueous extract of Angelica sinensis radix (ASR) can induces NO production, and inhibit LPS-induced NO production in dose-dependent manner in RAW 264.7 macrophage cells. ASR also induces iNOS mRNA and iNOS protein expression, and exhibit inhibitory effect on iNOS mRNA and protein expression in a dose-dependent manner in LPS-stimulated RAW 264.7 macrophage cells. Cytokines involved in the regulation of inflammatory reaction and immune response may play a role in the pathogenesis. ASR induces pro-inflammatory cytokine gene expression (IL-1 α, IL-1 β and IL-6 gene) in a dose-dependent manner, and inhibits the expressions of these cytokines in LPS-stimulated RAW 264.7 macrophage cells. These data indicate that (1) ASR may be a potential therapeutic modulator of NO synthesis in various pathological conditions, and (2) the immunomodulatory effects of ASR may be, in part, associated with the inducing or suppression of pro-inflammatory cytokine gene expressions.

Key words : Angelica sinensis radix, NO, iNOS, cytokine, RAW 264.7 cells

Introduction

Nitric oxide (NO), highly unstable gas, plays an important role in diverse physiological processes, including inflammatory, immune responses and neurotransmission[1]. In mammalian cells, NO is generated by NO synthases (NOSs) and two major classes of NOSs have been identified on the basis of physical and biochemical characteristics of the purified enzymes: constitutive and inducible. Between them, the inducible nitric oxide synthase (iNOS), expressed in various cell types including macrophages, hepatocytes and astrocytes, is induced in response to various factors including lipopolysaccharide (LPS), Interferon (IFN), Tumor necrosis factor α (TNF α) and pro-inflammatory cytokines[2,3]. NO has a dual biological role[4,5], as a pro-inflammatory or anti- inflammatory. However, the large amounts of NO release have been implicated in a wide range of inflammatory disease, such as sepsis, arthritis, multiple sclerosis and systemic lupus erythematosus[6]. Therefore, effective inhibition of NO accumu- lation by inflammatory stimuli is a potential therapeutic approach for the treatment of these inflammatory diseases.

The root of Angelica sinensis is known as Danggui in traditional oriental medicines, and is used to treat blood deficiency syndrome, menstrual irregularities, rheumatic arthralgia and constipation[7]. These applications of Danggui are based on experimental knowledge of the effects of tonifying in the blood, promoting blood circulation, relieving pain and moistening the bowels[8].

In this study, using murine macrophage cell line RAW 264.7 cells an experimental model, we have investigated the effects of modulation of NO production by ASR. In order to investigate the modulation mechanism of ASR action, iNOS mRNA and iNOS enzyme protein expressions were examined. Furthermore, we have investigated the effects of pro-inflammatory cytokine gene expression by ASR in RAW 264.7 cells.

Materials and methods

1. Preparation of ASR

Angelica sinensis radix used in this study was supplied

* To whom correspondence should be addressed at : Young Kyu Kwon, Department of Physiology, College of Oriental Medicine, Daegu Haany University, Suseong-gu, Daegu, Korea.

· E-mail : ykkwon@dhu.ac.kr, · Tel : 053-770-2242

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and identified by Dr. Sang-Woo Shin, College of Oriental Medicine, Daegu Haany University. The voucher specimen (No, KOM 0208-04) was deposited in the herbarium College of Oriental Medicine, Daegu Haany University. The dried root (40 g) were boiled gently with 1,500 ㎖ of water for 2 hr 30 min in a glass flask. The extracts were concentrated and the yield of concentration was 47.5% from the original weight.

2. Cell culture

Murine macrophage RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC; TIB71, Maryland, USA). The cells were maintained in complete DMEM supplemented with 10% heat-inactivated FBS, 1% antibiotics/antimycotics (100 U/㎖ of penicillin, 25 ㎍/㎖ of amphotericin D and 100 ㎍/㎖ of streptomycin) and 1.5% sodium bicarbonate at 37℃ in a humidified 5% CO_2 atmosphere. Cells were plated at a density of 1×10^6 cells/well in a 60 mm dish, and allowed to attach for 2 hr. For stimulation, the medium was replaced with 0.1% FBS contained DMEM, and the cells were then stimulated with 1 ㎍/㎖ of LPS in the presence or absence of various concentrations of ASR for 48 hr.

3. Cell proliferation assay

Murine macrophage RAW 264.7 cells were suspended at the concentration of 1×10^6 cells/㎖ in DMEM supplemented with 10% fetal bovine serum (FBS). The 100 ㎕ of RAW 264.7 cells were added to 96-well flat-bottomed microplates and were incubated in triplicate in a humidified atmosphere (5% CO_2/95% air) at 37℃ and allowed to attach for 2 hr. For stimulation, the medium was replaced with 0.1% FBS contained DMEM, and the cells were then stimulated with various concentration of ASR. After 48 hr of incubation, 20 ㎕ of CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) was added in the cells, and plate re-incubated at 37℃ for 1 hr 30 min. The plates were read on a micro ELISA reader (Molecular Devices Corp. Sunnyvale, CA) using a test wavelength of 490 nm. Mean background values were obtained by scanning blank wells. Results are expressed as mean optical density (O.D.) absorbance minus background O.D. absorbance.

4. Measurement of nitrite concentration

NO synthesis in cell culture was measured by a Griess reagent. Briefly, 100 ㎕/well of sample was incubated with an equal volume of the Griess solution (1% sulfanilamide in 5% phosphoric acid + 1% α-naphthylamide in distilled water) at room temperature for 10 min. The absorbance was measured with a micro ELISA reader at 540 nm. Nitrite was determined by using sodium nitrite as the standard. The level of nitrite reflects NO synthesis.

5. Total RNA isolation and RT-PCR

Murine macrophage RAW 264.7 cells in the TRIzol reagent were well homogenized and vortexed after a 1/10 volume of chloroform was added. After incubating the mixture on ice for 15 min, the samples were centrifuged at 12,000 rpm, for 15 min at 4℃. The aqueous phase was transferred to a new 1.5 ㎖ micro centrifuge tube. RNA from the aqueous phase was precipitated by mixing it with the same volume of isopropyl alcohol, and was centrifuged at 12,000 rpm for 15 min at 4℃, following 30 min of incubation on ice. Precipitated RNA pellets were washed once with 70% ethyl alcohol, and were redissolved in DEPC-treated water (Quality Biological Inc., Gaithersburg, MD, USA).

RT reaction of 4 ㎍ of RNA was performed in a 20 ㎕ RT reaction mixture containing 0.5 ㎕ of MMLV reverse transcriptase (200 U/㎕, Promega), 4.0 ㎕ of 5 MMLV RT buffer (Promega), 2.0 ㎕ of dNTP mixture (10 mM, BM), 0.5 ㎕ of RNasin (RNase inhibitor, 40 U/㎕, Promega), and 2.0 ㎕ of oligo dT (50 mM) in DEPC-treated water. The reaction was performed under the conditions of 42℃ for 1 hr and 95℃ for 5 min. PCR was carried out with the use of 1.0 ㎕ of RT products as templates : 94℃ for 3 min followed by 35 cycles of denaturation at 94℃ for 45 sec, annealing at 57℃ for 45 sec, and elongation at 72℃ for 45 sec. The last cycle was followed by a 10 min extension step at 72℃. The amplified products were analyzed by ethidium bromide-stained agarose gel electrophoresis. The PCR primer sequences are shown in Table 1.

Table 1. Primer sequence used for detection of pro-inflammatory cytokine gene expression

	Oligonucleotide sequence
G3PDH	5'-CCA CCC AGA AGA CTG TGG ATG GC-3' 5'-CAT GTA GGC CAT GAG GTC CAC CAC-3'
IL-1a	5'-CAC TAT CTC AGC ACC ACT TG-3' 5'-CTG GAA GTC TGT CAT AGA GG-3'
IL-1β	5'-CCG TGG ACC TTC CAG GAT GA-3' 5'-GAT CCA CAC TCT CCA GCT GC-3'
IL-6	5'-AGA GGA GAC TTC ACA GAG GA-3' 5'-ATC TCT CTG AAG GAC TCT GG-3'
iNOS	5'-GAC AAG CTG CAT GTG ACA TC-3' 5'-GCT GGT AGG TTC CTG TTG TT-3'

6. Western blotting

Cellular lysate were prepared by suspending 1×10^6 cells in 100 ㎕ of lysis buffer (50 mM Tis-Cl, 25 mM EDTA, 650 mM NaCl, 5% Triton X-100, 100× PMSF, 100× protease inhibitor cocktail, 5× lysis buffer). Proteins in the cell lysates were then separated by a 12% SDS-polyacrylamide gel

electrophoresis and transferred to a Protran nitrocellulose membrane (S&S, GmbH, Germany). The membrane was then blocked with 5% skim milk in PBS-Tween-20 for 1 hr at room temperature and then incubated with an anti-iNOS antibody (Transduction lab, Lexington, KY). After washing in PBS-Tween-20 three times, the blot was incubated with a secondary antibody. Detection of specific proteins was carried out with an ECL Western blotting kit (Amersham Pharmacia Biotech, USA) according to the manufactures instructions.

7. Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate the statistical significance of changes in all indices as a function of dose and treatment, followed by Duncans new multiple range test using $p<0.05$ as the level of significance.

Results

1. Modulation of NO production by ASR in RAW 264.7 macrophage cells

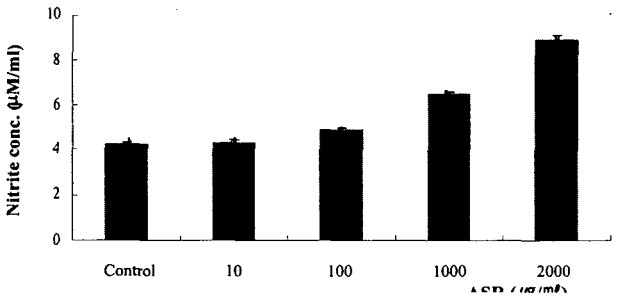

Fig. 1. Dose response of ASR on NO synthesis in RAW 264.7 cells. RAW 264.7 cells were incubated with 10 ㎍/㎖ to 2000 ㎍/㎖ ASR for 24 hr. NO synthesis was determined by measuring accumulation of nitrite in the incubation medium. Data are mean±S.D. of three independent experiments. Different letter on the top of the line indicates significant difference ($p<0.05$).

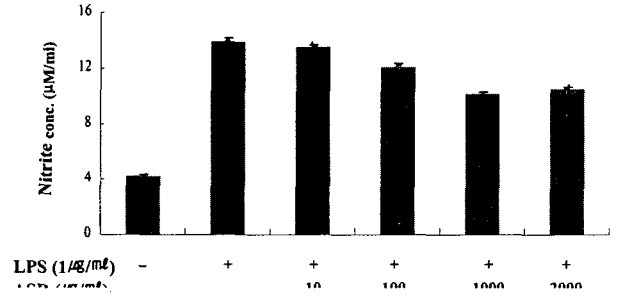

Fig. 2. Effect of ASR on NO production by LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were incubated with or without lipopolysaccharide (LPS: 1 ㎍/㎖) for 24 hr in the presence or absence of ASR at indicated doses. The amount of NO released by cells was measured by the method of Griess. Data are mean±S.D. of three independent experiments. Different letter on the top of the line indicates significant difference ($p<0.05$).

To evaluate whether ASR could modulate the production of NO by ASR, we examined the effect of ASR on NO production in RAW 264.7 cells with or without LPS (1 ㎍/㎖). ASR alone induced NO production in a dose-dependent manner, and LPS markedly induced NO production in RAW 264.7 cells. However, ASR significantly inhibited NO production by LPS-activated RAW 264.7 cells in a dose-dependent manner (Fig. 1, 2). The observed effect was not due to a potential cytotoxicity of ASR, since ASR showed no alteration of cell viability.

2. Time kinetics of NO production by ASR

To examine the time course of NO production by ASR, various time periods (0, 2, 4, 6, 8, 10, 12 and 24 hr) were used. ASR alone did not significantly affect basal NO production up to 12 hr, but NO production slightly induced at 24 hr, and LPS induced an effectively on NO production at 24 hr in RAW 264.7 cells. However, LPS plus ASR markedly inhibited NO production in contrast to LPS only stimulated RAW 264.7 cells (Fig. 3)

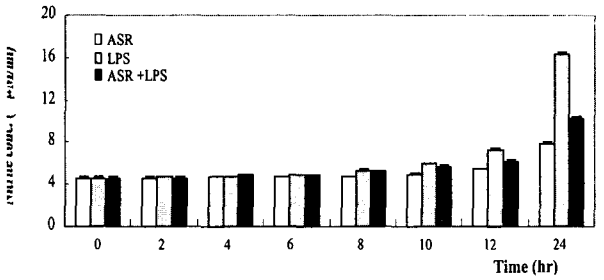

Fig. 3. Time kinetics of inhibition of NO production by ASR. RAW 264.7 cells were incubated with 1000 ㎍/㎖ ASR for various time points (0, 2, 4, 6, 8, 10, 12, 24 hr) in the presence or absence of 1 ㎍/㎖ LPS. NO synthesis was determined by measuring accumulation of nitrite in the incubation medium. The data present the means for triplicate, and the bars represent the standard deviation.

3. Effect of ASR on iNOS mRNA and iNOS protein expression in RAW 264.7 cells

To evaluate a possible mechanism by which the ASR could induce or inhibit NO production, we examined iNOS mRNA and iNOS enzyme protein contents. On the basis of RT-PCR data, ASR induced the expression of iNOS mRNA in a high dose. However, when the cells were activated with LPS, iNOS mRNA expressions decreased a dose-dependent manner of ASR (Fig. 4).

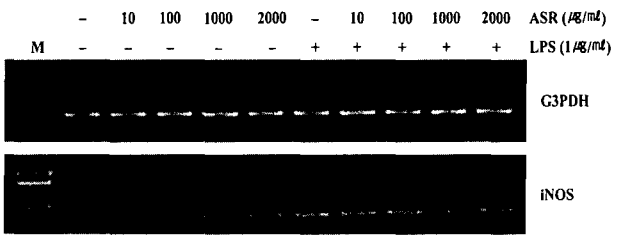

Fig. 4. Inhibition of iNOS mRNA expression by ASR in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were stimulated with 1 ㎍/㎖ of LPS in the presence or absence of various concentrations of ASR for 24 hr. Total RNA was prepared, and iNOS mRNA was analyzed by RT-PCR. G3PDH was used as control genes.

The contents of iNOS enzyme protein were also analyzed by Western blotting. ASR induced the expression of iNOS enzyme protein in a dose-dependent manner. However, in the case of LPS-stimulated cells, the expression of iNOS enzyme protein decreased a dose-dependent manner. On the basis of Western blotting, expressed iNOS enzyme protein levels were correlated with iNOS mRNA levels. The normal expression of HSP70 suggests that inhibition by ASR was not due to nonspecific or cytotoxic effects (Fig. 5).

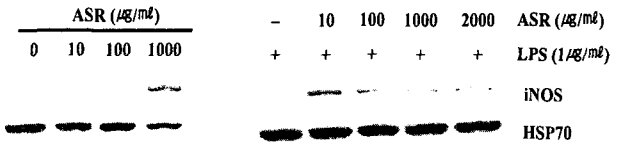

Fig. 5. Inhibition of iNOS protein expression by ASR in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were stimulated with 1 ㎍/㎖ LPS in the presence or absence of various concentration of ASR for 24 hr. The protein extracts were prepared, and then the samples analyzed for iNOS expression by Western blotting as described in the method. HSP70 was used as control protein

4. Effect of ASR on the pro-inflammatory cytokine gene expression of RAW 264.7 cells

To investigate the effect of ASR on the expression of pro-inflammatory cytokine mRNA in RAW 264.7 cells, the cells were treated with various concentrations of ASR for 24 hr. In RT-PCR, ASR induced the expression of IL-1α, IL-1β and IL-6 mRNA in a dose-dependent manner (Fig. 6).

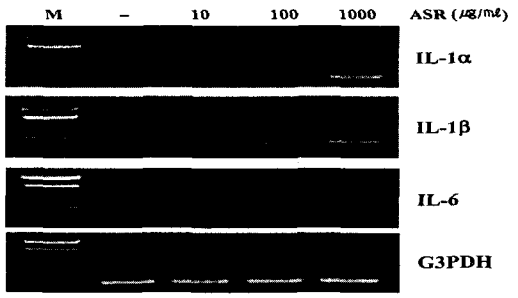

Fig. 6. Pro-inflammatory cytokine gene expression in RAW 264.7 cells by ASR. RAW 264.7 cells were stimulated with various amount of ASR for 24 hr. Total RNA was prepared from each sample and RT-PCR were performed as described in materials and methods. G3PDH was used as control genes.

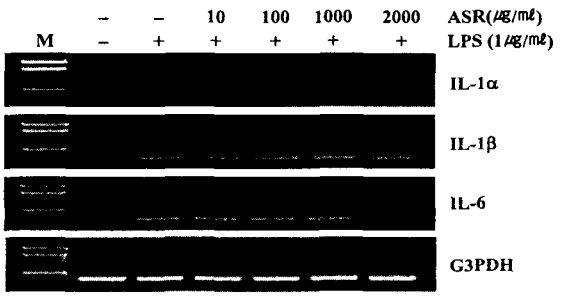

Fig. 7. Pro-inflammatory cytokine gene expression in LPS-stimulated RAW 264.7 cells by ASR. RAW 264.7 cells were stimulated with various amount of ASR with LPS (1 ㎍/㎖) 24 hr. Total RNA was prepared from each sample and RT-PCR were performed as described in materials and methods. G3PDH was used as control genes.

However, when the cells were activated with LPS, these cytokine expressions decreased a high dose of ASR. The G3PDH was also amplified from each RNA preparation to enable comparisons of the PCR products in different samples (Fig. 7).

Discussions and Conclusion

Despite its beneficial role in host defense, excessive NO production has been implicated in various diseases. Several researchers reported that NO has a dual biological role[4,5]. Low level of NO play a role as neurotransmitter, and high level of NO induces host cell death and inflammatory tissue damage[1,9]. Therefore, a number of plant materials have been investigated as modulator of NO production, and the development of effective modulators for the NO production in inflammatory cells are eagerly expected for the treatment of diseases mediated by NO[10,11]. In this study, we have assessed whether the extract of ASR could modulate NO production. Our results demonstrate that ASR could induce NO production, and inhibit NO production in LPS-stimulated RAW 264.7 cells. After than, to determine whether the modulation of NO in RAW 264.7 cells by ASR is correlated with iNOS mRNA and iNOS enzyme protein expression, we examined the expression of iNOS mRNA and iNOS enzyme protein using RT-PCR and Western blotting. LPS-induced iNOS mRNA and iNOS enzyme protein expression decreased ASR concentration dependently. Data obtained from these results demonstrated that interference with the expression of iNOS mRNA and iNOS enzyme protein may be the factor contributing to the modulator effect of ASR on NO production in RAW 264.7 cells, and the inhibition of iNOS expression occurred at the RNA level, although we did not determine whether the reduction in iNOS mRNA level results from a decrease in de novo transcriptional activity or mRNA stability. From these results, we conclude that the ASR modulate the NO production through modulate of iNOS mRNA expression, which leads to a modulation in iNOS protein expression.

Macrophages have the secretion capacity for a various kind of mediators such as the IL-1, IL-6, granulocyte macrophage colony stimulating factor (GM-CSF) and TNFα, which lead to secondary immune response such as proliferation of T and B cells, activation of macrophages for phagocytosis, and killing of microorganisms. Among theses mediators, pro-inflammatory cytokines such as IL-1α, IL-1β and IL-6 can be generated in response to immunological reaction inflammation, and microbial invasion[12-14]. Recently, several studies have shown that the various herbs can alter the cytokine expression in macrophages[15-17]. Therefore, we have

examined the effect of ASR on the expression of pro-inflammatory cytokine mRNA in RAW 264.7 cells. ASR induced the expressions of pro-inflammatory cytokine gene (IL-1α, IL-1β and IL-6 mRNA) in a dose-dependent manner, and in the high dose of ASR decreased these cytokine expressions in LPS stimulated RAW 264.7 cells. Therefore these results demonstrate that ASR is involved in the modulation of pro-inflammatory cytokine gene expressions in RAW 264.7 cells.

In conclusion, our data suggest that ASR can induce NO production and pro-inflammatory cytokines mRNA expressions. However, in a process independent of cytotoxicity, ASR inhibited NO production in LPS-stimulated RAW 264.7 cells. The inhibition of NO production may be associated with an inhibition of iNOS mRNA and iNOS enzyme protein expression. These results further provide a rational basis for therapeutic usages of ASR in various NO-associated inflammatory disease.

Acknowledgements

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