Journal of Biochemistry and Molecular Biology, Vol. 40, No. 5, September 2007, pp. 636-643

JOURNAL OF Biochemistry and Molecular Biology

Macrophage Activation by an Acidic Polysaccharide Isolated from *Angelica Sinensis* (Oliv.) Diels

Xingbin Yang[1,*], Yan Zhao[2], Haifang Wang[2] and Qibing Mei[2,*]

[1]*Key Laboratory of Ministry of Education for Medicinal Plant Resource and Natural Pharmaceutical Chemistry, College of Life Sciences, Shaanxi Normal University, Xi'an 710062, China*

[2]*Faculty of Pharmaceutical Sciences, Fourth Military Medical University, Xi'an 710032, China*

Received 13 February 2007, Accepted 29 March 2007

This study was designed to identify and characterize the mechanism of macrophage activation by AAP, an acidic polysaccharide fraction isolated from the roots of *Angelica sinensis* (Oliv.) Diels. As a result, AAP significantly enhanced nitric oxide (NO) production and cellular lysosomal enzyme activity in murine peritoneal macrophages *in vitro* and *in vivo*. Furthermore, L-NAME, a specific inhibitor of inducible nitric oxide synthase (iNOS), effectively suppressed AAP-induced NO generation in macrophages, indicating that AAP stimulated macrophages to produce NO through the induction of iNOS gene expression and the result was further confirmed by the experiment of the increase of AAP-induced iNOS transcription in a dose-dependent manner. To further investigate, AAP was shown to strongly augment toll-like receptor 4 (TLR4) mRNA expression and the pretreatment of macrophages with anti-TLR4 antibody significantly blocked AAP-induced NO release and the increase of iNOS activity, and tumor necrosis factor-α (TNF-α) secretion.

Keywords: A*ngelica* polysaccharide, Nitric oxide, Peritoneal macrophage, Toll-like receptor 4, Lipopolysaccharide

Introduction

Polysaccharides isolated from various traditional medicinal plants have been shown to profoundly affect the immune system both *in vivo* and *in vitro*, and therefore have the potential as a immuno-modulator with a wide application (Jeon *et al*., 1999; Tzianabos, 2000; Lee and Jeon, 2003). It is well known that macrophages play a significant role in the host defense mechanism and many polysaccharides activated immune responses primarily by activation of macrophages, although direct activation of B cells and other immune cells also were implicated (Lee and Jeon, 2005). Activated macrophages released many inflammatory cytokines to exert their biological effects. Nitric oxide (NO) and tumor necrosis factor-α (TNF-α) as the main effector molecules play a critical role in the non-specific immune defense against tumor, bacterial infection and lethal endotoxicosis (Rojas *et al*., 1993; MacMicking *et al*., 1997).

Toll-like receptors (TLRs) are of key importance in the recognition and response to infectious microbes by a variety of cells of the innate immune system. Toll-like receptor 4 (TLR4) as a member of the TLRs family has been identified as the main pattern recognition receptor expressed by macrophages and mediates macrophage activation by transmitting a variety of extracellular signals, resulting in the secretion of cytokines such as NO, TNF-α (Werling and Jungi, 2003; Franchini *et al*., 2006). TLR4 is also known as primary bacteria lipopolysaccharide (LPS) receptor and plays a crucial role in host immune responses against infection (Beutler *et al*., 2003). Recently, the enhancement of host immune responses of polysaccharides from herbs has been recognized as a possible means for inhibiting microorganism invasion and tumor growth without harming the host, and extensive studies on antibacterial, antifungal and antiviral properties have been undertaken to find effectively immuno-stimulatory polysaccharides from a variety of sources. More recently, several polysaccharides isolated from bacterium were shown to have high immunostimulatory activity and the action could be associated with the activation of TLR4 in macrophages, T cells and B cells, suggesting that the innate immune response to some polysaccharides was probably mediated via TLR4 triggering (Ando *et al*., 2002; Han *et al*., 2003a, b).

*To whom correspondence should be addressed.
Tel: 86-29-85310266; Fax: 86-29-85303736
E-mail: xbyang@snnu.edu.cn. (Xingbin Yang);
qbmei@fmmu.edu.cn (Qibing Mei)

It is also noticed that natural polysaccharides cannot penetrate the cellular membrane due to their structurally diverse class of large molecular mass. However, various polysaccharide administrations such as ig, ip and iv have confirmed its extensive bioactivities although polysaccharides cannot penetrate the cellular membrane due to that large molecular mass (Jeon *et al*., 1999; Tzianabos, 2000; Lee and Jeon, 2003). The evidence indicates that the responsive cells such as lymphocytes and macrophages may be activated by the surface binding of polysaccharides to cell-specific surface molecules (or receptors). For this reason, polysaccharides have recently become attractive as health beneficial medicines for the treatment of digestive tract diseases and TLR4 has become a new target for combating infection. Therefore, many attempts have been made to develop new polysaccharide biological response modifier for the treatment of infectious diseases (Komatsu *et al*., 1997; Senel and McClure, 2004).

In recent years, the polysaccharides rich in the roots of *Angelica sinensis* (Oliv.) Diels have drawn the attention of researchers and consumers due to their nutritional and health protective value in gastrointestinal protection (Cho *et al*., 2000; Ye *et al*., 2001a, b), anti-ulcer (Ye *et al*., 2003), anti-tumor (Shang *et al*., 2003), anti-hepatic injury (Ye *et al*, 2001c) and radio-protective effects (Mei *et al*., 1988). However, the molecular mechanism responsible for immuno-stimulatory activity of this polysaccharide is not fully understood and it is unclear whether TLR4 is involved in the polysaccharide-induced activation of macrophage. In the present study, we isolated an acidic *Angelica* polysaccharide (AAP) purified from the crude polysaccharide extract of *Angelica sinensis* (Oliv.) Diels and further investigated the mechanism responsible for macrophage activation by AAP.

Materials and Methods

Materials. LPS, polymyxin B and MTT (Thiazolyl blue) were purchased from Sigma Co. Triton X-100 was obtained from Amersco Inc. N^G-nitro-L-arginine methyl ester (L-NAME) was from Calbiochem-Behring. AMV RT, Oligo (dT) 15, Rnase inhibitor, Taq DNA polymerase and DNA marker were obtained from TaKaRa Biotechnology. Trizol reagent was obtained from Invitrogen. Sc-650 NOS2 and sc-16240 TLR-4 antibody were purchased from Santa Cruz Biotechnology Inc. RPMI1640 and fetal bovine serum (FBS) were the product of Gibco (Grand Island). All other chemicals and solvents were of analytical grade.

Preparation and characterization of AAP. The acidic Angelica polysaccharide (AAP) was isolated from the roots of Angelica sinensis (Oliv.) Diels using ethanol precipitation followed by a further purification with Sephacryl S-400 gel filtration chromatography (Yang *et al*., 2005). The obtained AAP is a white powder readily soluble in water and the average molecular weight was estimated to be approximately 52 kDa determined by high-performance size-exclusion chromatography combined with phenol-sulfuric acid method. AAP is the main fraction in Angelica polysaccharides and consisted of mannose, rhamnose, galacturonic acid, glucose, galactose and arabinose with the average molar ratio of 0.44 : 1.00 : 10.52 : 7.52 : 8.19 : 14.43, where the molar percentage amount of galacturonic acid was 25.0% (Yang *et al*., 2005).

Animals. Female specific pathogen-free BALB/c mice (6-8 weeks old, 17-20 g body weight) were obtained from the Experimental Animal Center of Fourth Military Medical University. Mice were housed in plastic cages with free access to water and food at 20-25 °C under a 12 h light/dark cycle at least 6 days before experiments. The guidelines for the care of the animals were strictly followed throughout the studies.

Cell isolation and culture. Macrophages were prepared from BALB/c mice as described previously (Kim *et al*., 2004). Briefly, peritoneal macrophages were harvested from 2 to 3 BALB/c mice, which had been injected intraperitoneally with 3 ml of thioglycollate three days before sterile peritoneal lavage with 10 ml of Hank's balanced salt solution. The collected cells were seeded and cultured in RPMI1640 containing 10% heat-inactivated FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin at a density 2×10^6 cells/well. The cells were allowed to adhere for 3 h to a 96-well culture plate at 37°C in a 5% CO_2 incubator. Then the cultures were washed twice with RPMI1640 to remove nonadherent cells prior to the addition of 1ml of fresh RPMI1640 containing 10% FBS. The purity of the adherent macrophages was assessed by Giemsa staining (>95%).

Measurement of NO release and iNOS activity. NO production was determined indirectly by assaying the culture supernatant for accumulated nitrite, the stable end product of NO reacted with molecular oxygen as previously described (Green *et al*., 1982). Briefly, murine peritoneal exudate was plated into 96-well plate at 2×10^6 cells/well and adhered macrophages were cultured with various concentrations of AAP, LPS or H_2O_2 at 37°C for 36 h. After treatment, 100 μl of isolated supernatants were allowed to react with Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylethyl-enediamine dihydrochloride and 2.5% phosphoric acid) at room temperature for 10 min. Nitrite products were determined by measuring absorbance at 550 nm versus a $NaNO_2$ standard curve. In addition, inducible NO synthase (iNOS) activity was determined using a Diagnostic Reagent Kit from Jiancheng BioEngineering and assay procedure was based on the direction of the kit and optical density was measured at 530 nm (wavelength). One activity unit of iNOS was defined as the production of 1 nmol NO per 1×10^6 cells per min and expressed as U/ml.

TNF-α bioassay. Adhered macrophages at 2×10^6 cells/well were incubated with either various concentrations of AAP or LPS for at 37°C 48 h. After incubation, conditioned supernatants were collected for assaying TNF-α release by determining cytotoxicity using a bioassay of TNF-sensitive L929 cells (Ferrari *et al*., 1990; Ando *et al*., 2002). L929 cells (3×10^5 cells, 100 μl) were cultured with serially diluted supernatants (100 μl) in the presence of actinomycin D (1 μg/ml) for 18 h in 96-well microtiter plates. The cells were washed once with PBS and stained with 0.5% crystal violet in methanol for 15 min. The plates were extensively washed with water, and the dye was extracted with methanol. The

percentage of cell survival was calculated from the absorbance (A) values at 570 nm as follow: $A_{\text{treated}}/A_{\text{untreated control}} \times 100\%$.

Determination of cellular lysosomal enzyme activity. Cellular lysosomal enzyme activity was measured according to the procedure of Suzuki *et al* (1990). Macrophage monolayers in 96-well culture plates (2×10^6 cells/well) were solubilized by adding 25 ml of 0.1% Triton X-100 and incubated for 30 min at room temperature. Then 150 µl of 10 mM *p*-nitrophenyl phosphate was added to per well as a substrate for acid phosphatase, followed by the addition of 0.1 M citrate buffer (50 µl, pH 5.0). After the incubation for 1 h at 37°C, 0.2 M borate buffer (50 µl, pH 9.8) was added to the mixture to stop the reaction. Optical densities were measured at 405 nm by spectrophotometer. The lysosomal enzyme activity was calculated as the percentage of the absorbance of a sample to a control (saline).

RT-PCR analysis. Total RNA was isolated from macrophages using TriZol Reagent. Total RNA was reverse-transcribed into cDNA and then PCR amplification of the cDNA was performed. The forward and reverse primer sequences of PCR primers were as follows: murine TLR4: 5'-TGT CCC AGG GAC TCT GCG CTG CCA C-3' and 5'-GTT CTC CTC AGG TCC AAG TTG CCG TTT C-3'; murine iNOS: 5'-CTG CAG CAC TTG GAT CAG GAA CCT G-3' and 5'-GGG AGT AGC CTG TGT GCA CCT GGA A-3'; murine β-actin: 5'-ATG AAG ATC CTG ACC GAG CG-3' and 5'-TAC TTG CGC TCA GGA GGA GC-3. RT-PCR reaction: first strand cDNA was synthesized in a 20 µl reverse transcription reaction system as follows: 5 × RT buffer 4 ml, 2.5 mmol β-actin/ 1.28 ml dNTP 3 µl, RNA template 1 µl (1.0 µg), oligo (dT) 15 primer 1 µl, RNase inhibitor 1 µl. 5 U/µl AMV RTase 1 µl was added to each reaction tube, and then all tubes were kept at 30°C for 8 min, 42°C for 1 h and 99°C for 5 min. PCR reaction was carried out in a 50 µl reaction system as follows: 10 × PCR buffer 5 µl, 2.5 mM $MgCl_2$ 5 µl, 2.5 mmol/1.28 ml dNTP 4 ml, 0.1 µM primer 0.75 µl and cDNA template 3 µl. 5 U/µl Taq DNA polymerase 0.5 µl was added to each reaction tube. The PCR reaction was run for 28-30 cycles of 95°C for 1 min, 57°C for 1 min and 72°C for 1 min, followed by an additional extension step at 72°C for 7 min. The PCR products were electrophoresed on a 2% agarose gel and photographed after staining with ethidium bromide.

Statistic analysis. Values are expressed as mean ± SD. Statistical differences between the treatment and untreatment groups were assessed by the unpaired Student *t*-test. A value of $p < 0.05$ was considered statistically significant.

Results

Induction of AAP on lysosomal enzyme activity and NO production in macrophages. Phagocytosis is the first step in the response of macrophages to invading microorganisms. Lysosomal enzyme and phagocytic activities are crucial aspects of macrophage functional assessments (Jeong *et al*., 2006). Here, we examined the effects of an acidic *Angelica* polysaccharide (AAP) fraction isolated from the roots of

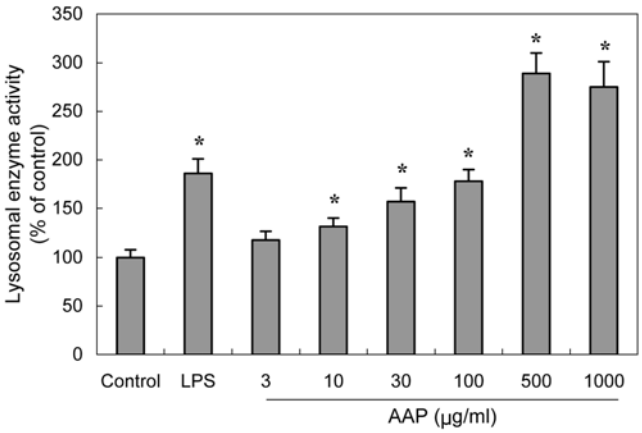

Fig. 1. Cellular lysosomal enzyme activities of murine peritoneal macrophages treated with various concentration of AAP or 3 µg/ml of LPS for 24 h. Saline was used in the negative control and LPS was used as positive group. The macrophage concentration was 2×10^6 cells/well and the ysosomal enzyme activity was calculated as the percentage of the absorbance of a sample to a saline control at 405 nm. Values are the means of three replicates. *$p < 0.05$ as compared with control group.

Table 1. *In vivo* effect of AAP on NO production and lysosomal enzyme activity in murine peritoneal macrophages

Stimulus (mg/kg)	Lysosomal enzyme activity (% of control)[a]	Nitrite (nmol/10^6 cells)[b]
Control (saline)	100.0 ± 8.3	3.8 ± 0.3
AAP 30	166.6 ± 10.8*	24.4 ± 2.1*
100	188.9 ± 11.2*	30.7 ± 2.3*
300	258.3 ± 15.9*	35.6 ± 3.2*
500	326.3 ± 19.7*	51.5 ± 4.7*

[a]Peritoneal macrophages were isolated 8 h after intraperitoneal administration of BALB/c mice with the indicated amount of AAP and the adhered cells were cultured for 24 h. The culture supernatants were subsequently isolated and determined as the percent amount (% of control) of the relative activity of cellular lysosomal enzyme as described in Fig. 1.
[b]Nitrite production was determined from the culture supernatants using Griess reagent as described in Methods. Each value represents the mean ± S.D. of triplicate determinations and *$p < 0.05$ indicates statistically different from control (saline).

Angelica sinensis (Oliv.) Diels on the lysosomal enzyme activities of murine peritoneal macrophages *in vitro* and the results were shown in Fig. 1. It was found that AAP at concentrations of 10, 30, 50, 100 and 500 µg/ml significantly enhanced the relative enzyme activity in a dose-related manner by 132, 157, 178, 289 and 275%, respectively ($p < 0.05$ versus physiological saline control) and the maximal effect was observed at a concentration of 500 µg/ml. When the concentrations were higher than 500 µg/ml, a decreased trend was observed. However, the activity of AAP (3 µg/ml) was 1.6 times lower than that of the same concentration of

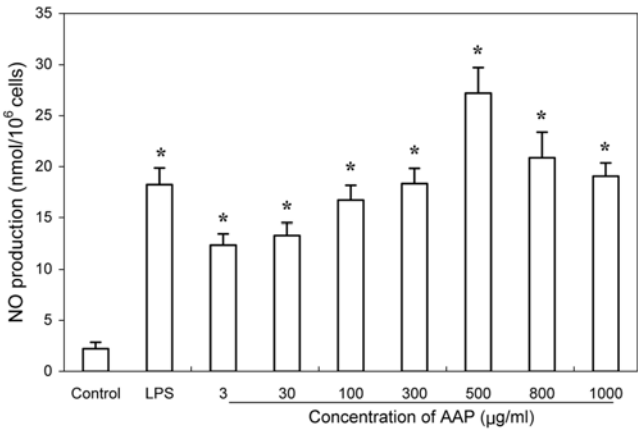

Fig. 2. AAP concentration-dependently induced NO production in murine peritoneal macrophages. The macrophages at 2×10^6 cells/well were incubated with either LPS (3 μg/ml) or serially diluted concentrations of AAP at 3, 30, 100, 300, 500, 800, 1000 μg/ml for 36 h, respectively. LPS was used as a positive reference and control indicated the untreated negative group. NO production was determined by measuring the accumulation of nitrite in the medium. Data are mean ± S.D. of three separate experiments. *$p < 0.05$ compared to control group.

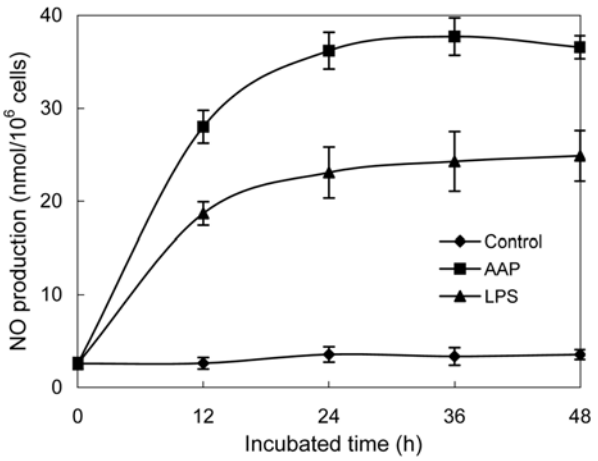

Fig. 3. Time-dependent induction of NO production by AAP in murine peritoneal macrophages. The macrophages (2×10^6 cells/well) were incubated with 300 μg/ml of AAP or 3 μg/ml of LPS for the indicated time and NO production was determined as described in Materials and Methods.

LPS, a potent activator of macrophages. From the results it was concluded that AAP possessed the potential of enhancing innate immune response.

To further confirm, we treated female BABL/c mice with AAP *in vivo* and measured the relative enzyme activity and NO production in isolated peritoneal macrophages. From the data presented in Table 1, it was further confirmed that intraperitoneal administration of AAP at the concentrations of 30, 100, 300, and 500 mg/kg to BABL/c mice not only improved lysosomal enzyme activities but also promoted NO generation in the isolated peritoneal macrophages.

Effects of AAP on NO production and iNOS expression in macrophages. For further estimating the potential of AAP to induce NO production from macrophages, *in vitro* concentration-response experiment of AAP was carried out. Adhered macrophages were incubated with either LPS (3 μg/ml) or various concentrations of AAP at 37°C for 36 h. The culture supernatants were measured for the accumulation of nitrite, the stable end product of NO and compared with untreated control groups. As shown in Fig. 2, the NO release from macrophages was significantly dependent on the employed dosage of AAP. Namely at the concentration range of 0-500 μg/ml, AAP enhanced NO production in a dose-dependent manner and went through a peak at a concentration of 500 μg/ml (12.2-fold versus control), and then decreased. The EC_{50} (50% of maximal AAP induction) value was 250 μg/ml. The treatment of the cells with AAP significantly increased NO production in the medium at the concentrations as low as 3 μg/ml (5.4-fold) whereas potent macrophage activator LPS (3 μg/ml) caused a 8.2-fold increase in NO synthesis as compared to the control group. Furthermore, the time-dependently induced effects of AAP on NO production were also examined and the results were shown in Fig. 3. It was evident that the NO production from AAP-treated macrophages was significantly increased from 12 h (10.7-fold) and peaked at 36 h (14.5-fold), which was similar to LPS.

However, it was unclear whether the stimulatory effect of AAP on NO production was attributable to its influence on the expression of iNOS gene expression. To elucidate the underlying mechanisms of the regulation of NO synthesis by AAP, the effect of L-NAME, an inhibitor of iNOS activity, on AAP-stimulated NO production was evaluated after the cells were incubated with AAP (10, 100 and 500 μg/ml) or LPS (3 μg/ml) as stimulus in the presence and absence of L-NAME. As a result, the enhancing effects of AAP or LPS on NO production were markedly inhibited by L-NAME (Fig. 4A). This inhibitory experiment of iNOS showed that the increase of NO in macrophage was mediated by the expression of the iNOS gene. Furthermore, the iNOS expression level in macrophages was investigated by RT-PCR under the stimulation of AAP at 0-500 μg/ml (10 μg/ml of LPS as positive control). As shown in Fig. 4B, the transcription of iNOS mRNA (311 bp) was concentration-dependently induced by AAP in macrophages. This was further evidenced that AAP exhibited a potential inductive effect on iNOS gene expression in macrophages, resulting in the enhancement of NO release.

TLR4-dependent activation of macrophages by AAP. To further insight into the activation mechanism, we examined whether the NO production was a mediated outcome of TLR4 activation by AAP. Fig. 5A and B show the effects of the treatment of macrophages with anti-TLR4 antibody on AAP- or LPS-induced NO production by macrophages, respectively.

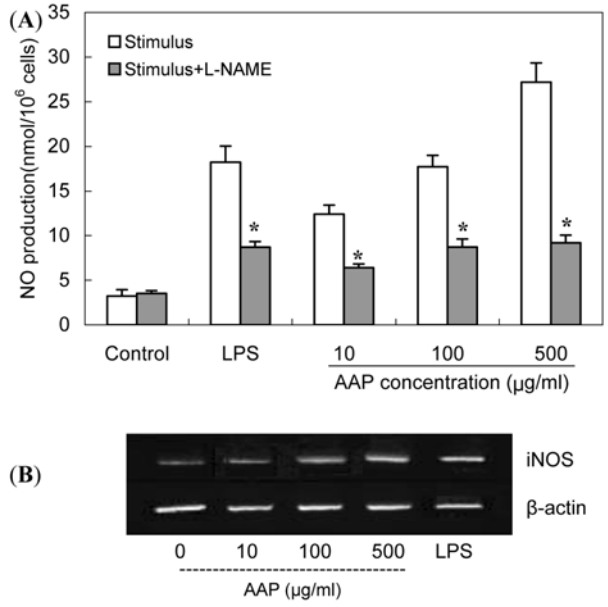

Fig. 4. Effects of AAP on NO production and iNOS expression in mouse peritoneal macrophages. (A) Culture macrophages were treated with stimulus (3 μg/ml LPS or AAP at the concentration of 10, 100 and 500 μg/ml, respectively) for 36 h in the absence (□) or presence (■) of L-NAME (500 mM, iNOS inhibitor). Data are mean ± S.D. of three separate experiments and $*p < 0.05$ indicates significant different versus only stimulus-treated groups. (B) Cells were treated with AAP (0-500 μg/ml) or LPS (10 μg/ml) for 48 h as described in Methods and iNOS mRNA levels were determined by RT-PCR. The experiments were repeated three times and similar results were obtained.

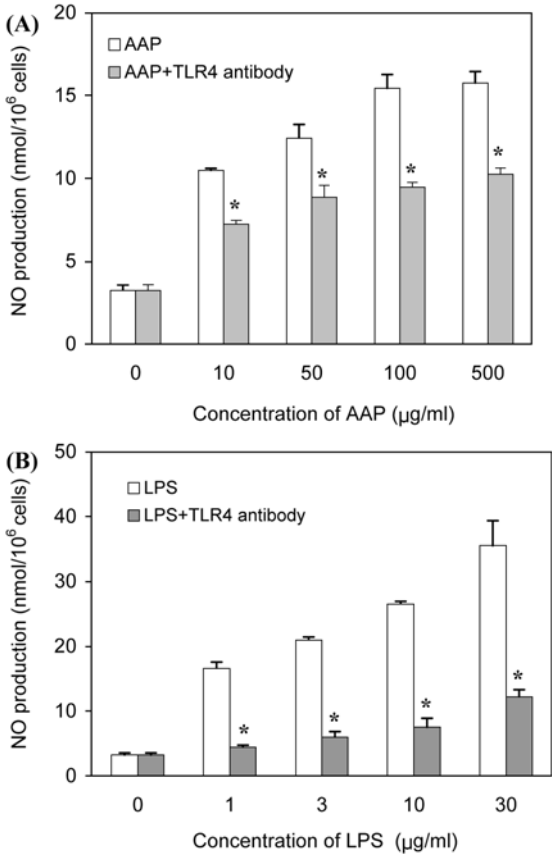

Fig. 5. Involvement of TLR4 in the activation of NO release by AAP. The cells were pre-incubated with 10 μg/ml of anti-TLR4 antibody for 1 h, and then washed out and treated with serially indicated concentrations of AAP (A) or LPS (B) for 36 h. The nitrite levels in supernatants were determined using Griess reagent. Each column shows the mean ± S.D. of triplicate determinations. Significance was determined using the Student's t-test versus the corresponding group without antibody treatment ($*p < 0.05$).

As shown in Fig. 5A, the pretreatment of macrophages with anti-TLR4 antibody obviously blocked AAP-mediated NO production. Similarly, LPS-induced NO production was also significantly suppressed by the pretreatment (Fig. 5B). Unexpectedly, the NO inductive effect of LPS as a potent macrophage activator was restrained to a much lower level than that of AAP by the pretreatment of macrophages with anti-TLR4 antibody. These results suggest that the AAP-induced NO production was associated with TLR4-dependent activation of macrophages.

For confirming the mechanism responsible for TLR4-dependent activation, we investigated the effect of the treatment of macrophages with anti-TLR4 antibody on the change of iNOS activity. As shown in Fig. 6A, it was found that AAP also remarkably increase iNOS activity in macrophages and the pretreatment with anti-TLR4 antibody significantly suppressed AAP-induced iNOS activity in macrophages when compared to the control group treated by AAP alone. To further confirm the consequence of TLR4 triggering, the effect of AAP on the expression of TLR4 mRNA was assessed by RT-PCR. As expected, treatment of macrophages with either AAP or LPS resulted in a drastic increase in the expression of TLR4 mRNA (2500 bp) and the amount of β-actin mRNA, an internal control, was not affected by AAP treatment (Fig. 6B), indicating that the release of NO and increase of iNOS activity in AAP-induced macrophages occurred at the level of transcription of TLR4 although the action of AAP was not so prominent as it was in the action of LPS.

AAP induced TNF-α secretion by TLR4 activation of macrophages. To further confirm the molecular mechanism of AAP-mediated activation of macrophages, we also assessed the effect of anti-TLR4 antibody on AAP-induced TNF-α production by macrophages. Adhered cells were cultivated in the presence or absence of anti-TLR4 antibody and the supernatants were collected at 48 h, and the amounts of TNF-α was measured by bioassay method. As shown in Fig. 7, the treatment of L929 cells with the supernatants from AAP- or LPS-stimulated macrophages resulted in the significant loss of cell viability in response to stimulation with AAP within studied concentration range (10-500 μg/ml), indicating

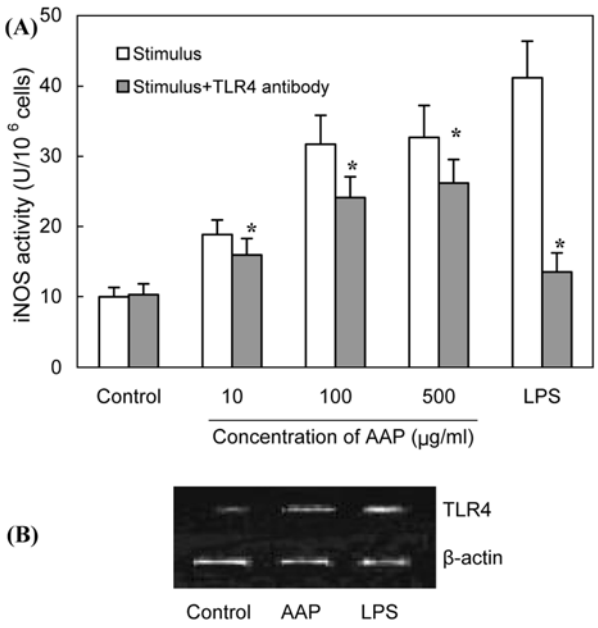

Fig. 6. Effect of the pretreatment of macrophages with anti-TLR4 antibody on iNOS activity in murine peritoneal macrophages by AAP. (A) The macrophages were treated as described in Fig. 5 and iNOS activity was determined as described in Methods. $*P < 0.05$, compared to the values obtained in only stimulus-treated cells. (B) Cells were treated with AAP (300 μg/ml) or LPS (10 μg/ml) for 48h as described in the part of Material and Methods. Total RNA was isolated and analyzed for magnitude of mRNA expression of TLR4 using RT-PCR. The experiments were repeated three times and similar results were obtained.

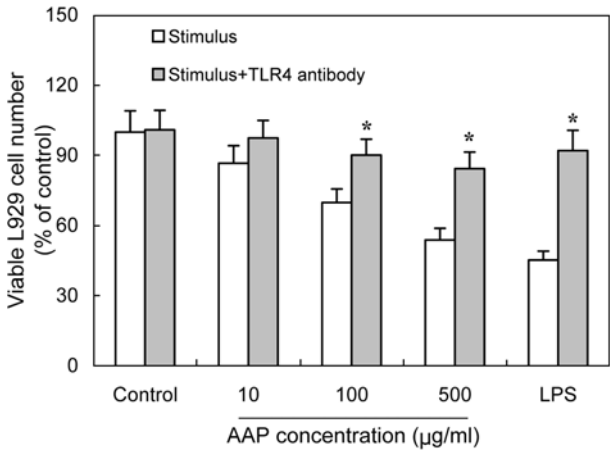

Fig. 7. Effect of the pretreatment of macrophages with anti-TLR4 antibody on AAP- or LPS-induced TNF-α secretion. Macrophages were cultured with various concentration of AAP or 3 μg/ml of LPS as stimulus for 48 h and TNF-α release from macrophages was performed by a bioassay using L929 cells. LPS was used as a positive reference. Viable L929 cell number was expressed and calculated as relative percentage to the control culture incubated without stimulus. Values are the mean ± S.D. of three independent experiments. $*p < 0.05$ vs the values obtained in only stimulus-treated cells.

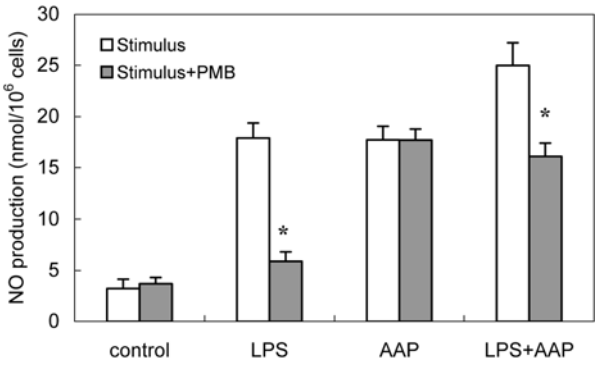

Fig. 8. Effects of PMB treatment on AAP or LPS-induced NO release from macrophages. Stimuli for AAP (100 μg/ml), LPS (3 μg/ml), or AAP (100 μg/ml) combined with LPS (3 μg/ml) were pre-incubated with PMB (1000 U/ml) for 1 h, and then used to activate macrophages for 24 h. Nitrite generation was determined from the culture supernatant using Griess reagent. Results are representative of three separate experiments. Significance was determined using the Student's *t*-test versus the corresponding group without PMB treatment ($*p < 0.05$).

that tested AAP dose-dependently increased TNF-α release from macrophages, resulting in the augment of TNF-α cytotoxicity against L929 cells. Furthermore as shown in Fig. 7, the pretreatment of macrophage with anti-TLR4 antibody remarkably inhibited the TNF-α secretion from cells, as reflected by enhancing relative cell viability as compared to untreated control. From the result it was further confirmed that AAP stimulated macrophages to produce TNF-α via TLR4 although the inhibition of anti-TLR4 antibody on LPS-induced TNF-α production was remarkably more effective than that on the action of AAP, where the pretreatment with anti-TLR4 antibody could increase L929 cell viability from 45.3 to 92.1% and 53.9% to 84.4% in response to stimulation with 3 μg/ml of LPS and a high dose of AAP (500 μg/ml), respectively. This result was in agreement with that of TLR4-mediated NO production of macrophages. Based on the investigation, it was evident that AAP is an activator of macrophages and enhanced the NO and TNF-α production via TLR4.

Analysis for a possible contamination of bacterial LPS in AAP. To ensure that the effects of AAP were not due to endotoxin contamination, we examined the effect of polymyxin B (PMB) on AAP-induced NO production. PMB is known to inhibit LPS activity by binding to the lipid A moiety (Morrison and Jacobs, 1976). In this study, AAP or LPS was mixed with PMB and the immunomodulating activity was examined in macrophages. As shown in Fig. 8, the PMB treatment did not affect AAP-induced NO generation, while it abolished NO production in LPS-induced macrophages and co-stimulated macrophages with LPS and AAP. It was further confirmed that a decrease in the lysosomal enzyme activity was observed in LPS, whereas

there was little change in the activity of AAP (data not shown). These results demonstrate that AAP was not contained with LPS and the activation of macrophages was attributed to the stimulation of AAP.

Discussion

In the present study, we investigated the effect of AAP, an acidic polysaccharide fraction from *Angelica sinensis* (Oliv.) Diels, on the host defense system and was identified as an immunomodulator of macrophages, as reflected by the significant increase in cellular lysosomal enzyme activity, TNF-α secretion and NO production in AAP-stimulated macrophages. It is well known that the activation of macrophages results in the expression of iNOS, which catalyzes the production of a large amount of NO from L-arginine and molecular oxygen (Xie *et al*., 1992; Alderton *et al*., 2001). In our study, the treatment with L-NAME, a specific inhibitor of iNOS, effectively suppressed AAP-induced NO generation in macrophages, indicating that AAP stimulated macrophages to produce NO through the induction of iNOS gene expression and the result was further evidenced by the experiment of AAP-induced iNOS transcription in the cultured macrophages. In addition, in good agreement with the previous reports that natural polysaccharide activated B cells, dendritic cells and macrophages through TLR4 (Ando *et al*., 2002; Han *et al*., 2003a, b), AAP promoted NO production by up-regulating the expression of TLR4 on macrophages and the activation was mediated by activation of the membrane receptor, TLR4 in a similar manner by LPS, indicating that a common signaling pathway mighty be involved.

Although macrophage activation by AAP was quite similar to that by LPS, some discrepancies were also observed between APS and LPS. The blocking of anti-TLR4 antibody on AAP-induced NO production, iNOS activity and TNF-α secretion was less sharper than that on the action of LPS. The present results indicated that TLR4 might be only partially involved in the action of *Angelica* polysaccharides, instead played a pivotal role in LPS action (Ando *et al*., 2002; Han *et al*., 2003a, b). *Angelica* polysaccharides may have a broader receptor profile than LPS. Further study on the activated mechanism of *Angelica* polysaccharides is essential. Moreover, because endotoxin LPS is a strong activator of macrophages and is contaminated in many plant materials, possible contamination of endotoxin is always a matter of concern for the high molecular weight components isolated from plants. In this study, the active component of AAP appeared to be polysaccharides, because the pretreatment of PMB did not affect either nitrite generation or cellular lysosomal enzyme activity in AAP-induced macrophages, while it abolished LPS stimulation of macrophages.

In conclusion, our results demonstrated that AAP stimulated macrophages to produce NO through the induction of iNOS gene expression in a dose-dependent manner. The induction of NO formation by AAP was consistent with increase in iNOS mRNA expression, suggesting that AAP exerted its effect by inhibiting iNOS gene expression. In addition, TLR4 was involved in the release of cytokines such as NO and TNF-α in *Angelica* polysaccharide-activated murine peritoneal macrophages.

Acknowledgments This work was supported by the science and research foundations of Shaanxi Province, China (2002K10-G2) and the Special Foundation of Shaanxi Normal University, China (QDF999133).

References

Alderton, W. K., Cooper, C. E. and Knowles, R. G. (2001) Nitric oxide synthase: structure function and inhibition. *Biochem. J.*, **357**, 593-615.

Ando. I., Tsukumo, Y., Wakabayashi, T., Akashi, S., Miyake, K., Kataoka, T. and Nagai, K. (2002) Safflower polysaccharides activate the transcription factor NF-kappa B via Toll-like receptor 4 and induce cytokine production by macrophages. *Int. Immunopharmacol.* **2**, 1155-1162.

Beutler, B., Hoebe, K., Du, X. and Ulevitch, R. J. (2003) How we detect microbes and respond to them: the Toll-like receptors and their transducers. *J. Leukocyte Biol.* **74**, 479-485.

Cho, C. H., Mei, Q. B., Shang, P., Lee, S. S., So, H. L., Guo, X. and Li, Y. (2000) Study of the gastrointestinal protective effects of polysaccharides from *Angelica sinensis* in rats. *Planta Medica* **66**, 348-351.

Ferrari, M., Fornasiero, M. C. and Isetta, A. M. (1990) MTT colorimetric assay for testing macrophage cytotoxic activity *in vitro*. *J. Immunol. Methods* **131**, 165-172.

Franchini, M., Schweizer, M., Mtzener, P., Magkouras, I., Sauter, K. S., Mirkovitch, J., Peterhans, E. and Jungi, T. W. (2006) Evidence for dissociation of TLR mRNA expression and TLR agonist-mediated functions in bovine macrophages. *Vet. Immunol. Immunopathol.* **110**, 37-49.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S. and Tannenbaum, S. R. (1982) Analysis of nitrate in biological fluids. *Anal. Biochem.* **126**, 131-138.

Han, S. B., Park, S. K., Ahn, H. J., Yoon, Y. D., Kim, Y. H., Lee, J. J., Lee, K. H., Moon, J. S., Kim, H. C. and Kim, H. M. (2003a) Characterization of B cell membrane receptors of polysaccharide isolated from the root of *Acanthopanax koreanum*. *Int. Immunopharmacol.* **3**, 683-691.

Han, S. B., Yoon, Y. D., Ahn, H. J., Lee, H. S., Lee, C. W., Yoon, W. K., Park, S. K. and Kim, H. M. (2003b) Toll-like receptor-mediated activation of B cells and macrophages by polysaccharide isolated from cell culture of *Acanthopanax senticosus*. *Int. Immunopharmacol.* **3**, 1301-1312.

Jeon, Y. J., Han, S. B., Ahn, K. S. and Kim, H. M. (1999) Activation of NFkappaB/Rel in angelan-stimulated macrophages. *Int. Immunopharmacol.* **43**, 1-9.

Jeong, S. C., Jeong, Y. T., Yang, B. K. and Song, C. H. (2006) Chemical characteristics and immuno-stimulating properties of biopolymers extracted from *Acanthopanax* sessiliflorus. *J. Biochem. Mol. Biol.* **39**, 84-90.

Kim, G. Y., Choi, G. S., Lee, S. H. and Park, Y. M. (2004) Acidic

polysaccharide isolated from *Phellinus linteus* enhances through the up-regulation of nitric oxide and tumor necrosis factor-a from peritoneal macrophages. *J. Ethnopharmacol.* **95**, 69-76.

Komatsu, W., Yagasaki, K., Miura, Y. and Funabiki, R. (1997) Stimulation of tumor necrosis factor and interleukin-1 productivity by the oral administration of cabbage juice to rats. *Biosci. Biotechnol. Biochem.* **61**, 1937-1938.

Lee, K. Y. and Jeon, Y. J. (2003) Polysaccharide isolated from *Poria cocos* sclerotium induces NF-kappaB/rel activation and iNOS expression in murine macrophages. *Int. Immunopharmacol.* **3**, 1353-1362.

Lee, K. Y. and Jeon, Y. J. (2005) Macrophage activation by polysaccharide isolated from *Astragalus* membranaceus. *Int. Immunopharmacol.* **5**, 1225-1233.

MacMicking, J., Xie, Q. W. and Nathan, C. (1997) Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **15**, 323-350.

Mei, Q. B., Tao, J. Y., Zhang, H. D., Duan, Z. X. and Chen, Y. Z. (1988) Effects of *Angelica sinensis* polysaccharides on hemopoietic stem cells in irradiated mice. *Acta Pharmacol. Sin.* **9**, 279-282.

Morrison, D. C. and Jacobs, D. M. (1976) Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. *Immunochemistry* **13**, 813-818.

Rojas, A., Padron, J., Caveda, L., Palacios, M. and Moncada, S. (1993) Role of nitric oxide pathway in the protection against lethal endotoxemia by low dose of lipopolysaccharides. *Biochem. Biophys. Res. Commun.* **191**, 441-446.

Senel, S. and McClure, S. J. (2004) Potential applications of chitosan in veterinary medicine. *Adv. Drug Deliv. Rev.* **56**, 1467-1480.

Shang, P., Qian, A. R., Yang, T. H., Jia, M., Mei, Q. B., Cho, C. H., Zhao, W. M. and Chen, Z. N. (2003) Experimental study of anti-tumor effects of polysaccharides from *Angelica sinensis*. *World J. Gastroenterol.* **9**, 1963-1967.

Suzuki, I., Tanaka, H., Kinoshita, A., Oikawa, S., Osawa, M. and Yadomae, T. (1990) Effect of orally administered beta-glucan on macrophage function in mice. *Int. Immunopharmacol.* **12**, 675-684.

Tzianabos, A. O. (2000) Polysaccharide immunomodulators as therapeutic agents: structural aspects and biologic function. *Clin. Microbiol. Rev.* **13**, 523-533.

Werling, D. and Jungi, T. W. (2003) Toll-like receptors linking innate and adaptive immune response. *Vet. Immunol. Immunopathol.* **91**, 1-12.

Xie, Q. W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T. and Nathan, C. (1992) Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* **256**, 225-228.

Yang, X. B., Zhao, Y., Wang, Q. W., Wang, H. F. and Mei, Q. B. (2005) Analysis of the monosaccharide components of *Angelica* polysaccharides by high performance liquid chromatography. *Anal. Sci.* **21**, 1177-1180.

Ye, Y. N., Koo, M. W., Li, Y., Matsui, H. and Cho, C. H. (2001b) *Angelica sinensis* modulates migration and proliferation of gastric epithelial cells. *Life Sci.* **68**, 961-968.

Ye, Y. N., Liu, E. S., Li, Y., So, H. L., Cho, C. C., Sheng, H. P., Lee, S. S. and Cho, C. H. (2001c) Protective effect of polysaccharides-enriched fraction from *Angelica sinensis* on hepatic injury. *Life Sci.* **69**, 637-646.

Ye, Y. N., Liu, E. S., Shin, V. Y., Koo, M. W., Li, Y., Wei, E. Q., Matsui, H. and Cho, C. H. (2001a) A mechanistic study of proliferation induced by *Angelica sinensis* in a normal gastric epithelial cell line. *Biochem. Pharmacol.* **61**, 1439-1448.

Ye, Y. N., So, H. L., Liu, E. S., Shin, V. Y. and Cho, C. H. (2003) Effect of polysaccharides from *Angelica sinensis* on gastric ulcer healing. *Life Sci.* **72**, 925-932.