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Effects of Schisandrin on Transcriptional Factors in Lipopolysaccharide-Pretreated Macrophages

Lian Yu Guo[1], Tran Manh Hung[2], KiHwan Bae[2], Sehyun Jang[1], Eun Myoung Shin[1], Ji Won Chung[1], Sam Sik Kang[1], Hyun Pyo Kim[3], and Yeong Shik Kim[1]

[1]Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul 151-742, Korea, [2]College of Pharmacy, Chungnam National University, Daejon 305-764, Korea, and [3]College of Pharmacy, Kangwon National University, Chunchon 200-701, Korea

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Schisandrin is the main active ingredient isolated from *Schisandra chinensis* Baill. Recent studies have demonstrated that schisandrin exhibits anti-inflammatory effects *in vivo* and *in vitro*. In this study, we examined whether the order of lipopolysaccharide (LPS) treatment affects the mechanism of schisandrin anti-inflammatory activity. We found that the anti-inflammatory mechanisms are not the same depending on whether macrophages were treated with schisandrin before or after LPS. The main difference is that inhibitor kappaBα (IκBα) degradation was not inhibited when macrophages were pretreated by LPS before schisandrin and was weakly inhibited when macrophages were pretreated by schisandrin before LPS.

Key words: Schisandrin, Lipopolysaccharide, c-Jun N-terminal kinase, p38 mitogen-activated protein kinase, RAW 264.7 macrophage

INTRODUCTION

Schisandrin is one of the lignan components of *Schisandra chinensis* Baill., which is widely used to treat hepatitis. Schisandrae Fructus has been reported to protect against CCl_4-induced hepatic damage. Its components can induce production of liver cytochrome P-450 and promote certain anabolic metabolisms such as serum protein biosynthesis and glycogenesis (Liu, 1989). Schisandrae-derived lignans have been reported to induce apoptosis in HL-60 cells and have anti-inflammatory activity (Chen et al., 1999). In a previous study, schisandrin inhibited carrageenan-induced paw edema and acetic acid-induced vascular permeability, protected against LPS-induced sepsis by inhibiting nitric oxide (NO) production, prostaglandin E_2 (PGE_2) release, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression. The latter effects resulted from the inhibition of nuclear factor-kappaB (NF-κB), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) activities in a RAW 264.7 macrophage cell line (Guo et al., 2008).

The most commonly used method of testing compounds for anti-inflammatory effects involves pretreatment of cells with the compound before stimulating the inflammatory response (Rao et al., 1997; Kim and Kim, 2000). However, typically people take medicine after becoming ill. Therefore, we examined whether the order of treatment influences the mechanisms of action of an anti-inflammatory compound (Hong et al., 2006). In this paper, we compared the anti-inflammatory mechanisms induced by schisandrin on macrophages in two treatment models: pretreatment of cells with schisandrin before LPS stimulation (Guo et al., 2008), and pretreatment by LPS followed by treatment with schisandrin.

Correspondence to: Yeong Shik Kim, Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul 151-742, Korea
Tel: 82-2-880-2479, Fax: 82-2-765-4768
E-mail: kims@snu.ac.kr

MATERIALS AND METHODS

Materials

Schisandrin (purity > 96.0%) was isolated according to a previously reported method (Peng et al., 2005). RAW 264.7 cells, a murine macrophage line, were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffer saline (D-PBS), *N-p*-tosyl-l-phenylalanylchloromethyl ketone (TPCK), lipopolysaccharide (*E. coli*, serotype 0127:B8; LPS), 4-methylumbelliferylphosphate (4-MUP), and dimethyl sulfoxide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Geneticin (antibiotic G-418) was purchased from Gibco BRL (Grand Island, NY, U.S.A.). A Cell-Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Tokyo, Japan). Other chemicals and solvents were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All of the samples, solutions and buffers were prepared using deionized water.

Primary antibodies for COX-2, iNOS, IκBα, pIκBα, pp38, p38, pJNK, JNK and secondary antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nitrocellulose membrane (NC membrane) was obtained from Whatman GmbH (Germany). An enhanced chemiluminescence (ECL) detection kit was purchased from LabFrontier (Seoul, Korea).

Cell culture and cell proliferation assay

The macrophage RAW264.7 cell line was incubated in DMEM at 37°C in 5% CO_2 humidified air. The cells were seeded into 96-well plates at the density of 1×10^4 cells/well and allowed to adhere for 24 h. After 24 h treatment with schisandrin (50 and 100 μM), 10 μL of the CCK-8 solution was added to each well, followed by incubation for 2 h at 37°C. The resulting color was assayed at 450 nm using an Emax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) (Liu and Hong, 2005).

Western blot analysis

The RAW264.7 cells (1×10^6 cells/well) were pretreated with LPS (1 μg/mL) for 5 min (IκBα), 1 h (iNOS, COX-2) and then incubated with schisandrin (12.5, 25, 50 and 100 μM) for 20 min (IκBα) or 18 h (iNOS, COX-2). Cells were also co-treated with LPS (1 μg/ml) and schisandrin (25, 50 and 100 μM) for 5 min (pIκBα). The expression of MAPKs (pp38, p38, pJNK, JNK) was detected in a time-course assay. After incubation, cells were lysed to harvest total cytoplasmic extracts as described previously (Zhou et al., 2007). Proteins were separated by SDS-PAGE and transferred to NC membranes. After being blocked by 5% nonfat milk, the membranes were incubated with primary and secondary antibodies in turn. Finally, blots were developed for visualization using the ECL Plus detection kit.

Reporter gene assay

The reporter enzyme activity was measured using a cell-based assay system for monitoring NF-κB activity. Transfected RAW 264.7 cells (1.5×10^5 cells/well) were pretreated with LPS for 1 h, and subsequently with schisandrin in fresh DMEM for 18 h. The fluorescence from the product of the SEAP/4-MUP (secretory alkaline phosphates/4-methylumbelliferylphosphate) reaction, according to excitation at 360 nm and emission at 449 nm, was measured using a 96-well plate Gemini XS fluorometer (Molecular Devices, Sunnyvale, CA, USA) (Moon et al., 2001; Ahn et al., 2003).

Statistical analysis

The results were expressed as means±S.E.M. Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA) followed by a post-hoc Dunnett's test for multiple comparisons and Student's *t*-test. Statistical significance was assessed as $p<0.05$ (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

RESULTS

Protein expression

The effect of schisandrin on iNOS protein expression in macrophages was detected by Western blotting. As shown in Fig. 1, when cells were treated with LPS prior to schisandrin iNOS protein expression was decreased, but not in an obviously dose-dependent manner. Schisandrin treatment significantly inhibited COX-2 protein expression in LPS-pretreated in a dose-dependent manner (Fig. 2). TPCK was positive control, and it had inhibitory effects on LPS-stimulated protein expression.

Activation of NF-κB

An analysis of reporter gene expression in the transfected-RAW 264.7 cells demonstrated that schisandrin inhibited the activation of NF-κB in a dose-dependent manner (Fig. 3).

Effects of schisandrin on phosphorylation and degradation of IκBα

IκBα was phosphorylated 5 min and completely

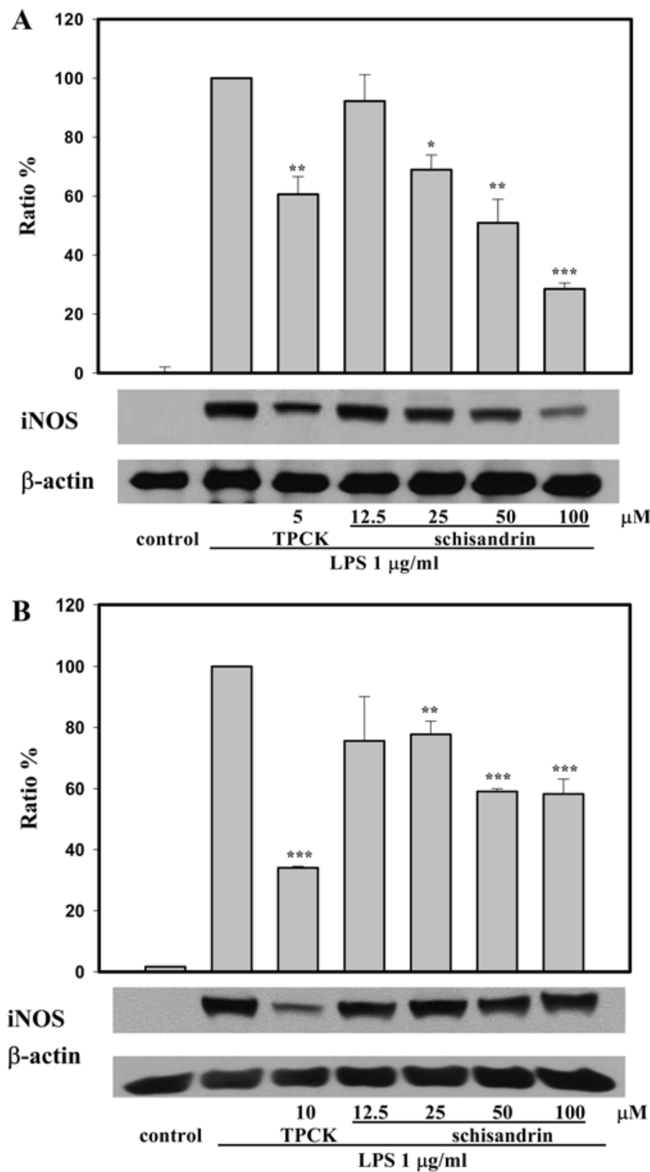

Fig. 1. Effect on iNOS protein expression of macrophages (1×10^6 cells/well) treated with schisandrin (12.5, 25, 50 and 100 μM) or TPCK (positive control: 10 μM) before (A) and after LPS (B), respectively

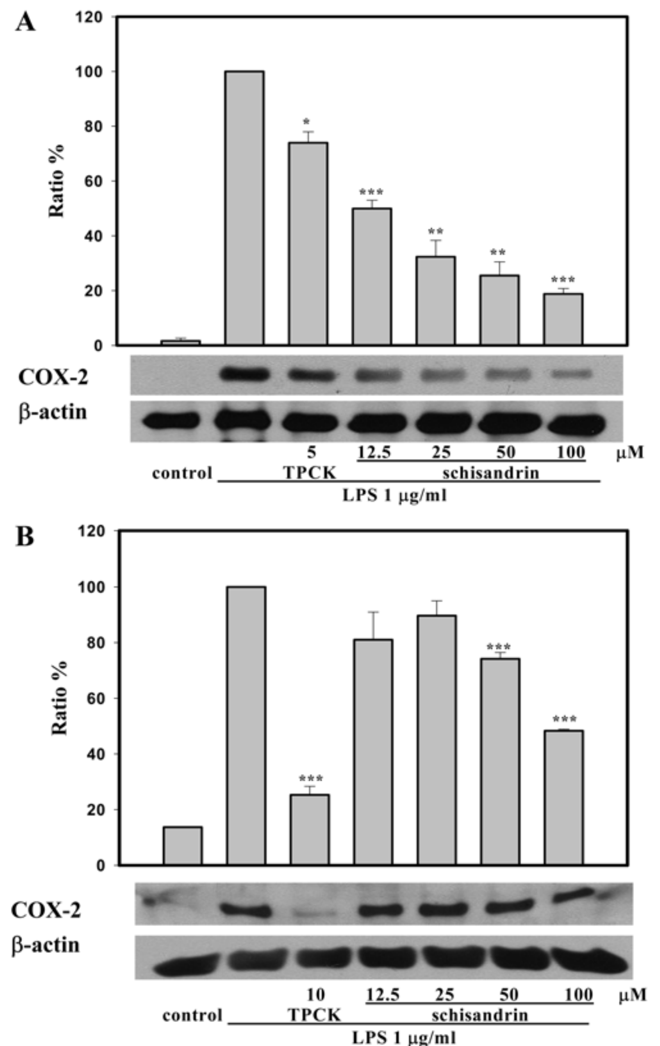

Fig. 2. Effect on COX-2 protein expression of macrophages (1×10^6 cells/well) treated with schisandrin (12.5, 25, 50 and 100 μM) or TPCK (positive control: 10 μM) before (A) and after LPS (B), respectively

degraded 30 min after stimulation with LPS (Fig. 4). Pretreatment with LPS for 5 min followed by incubation with schisandrin for 25 min could not block LPS-induced degradation of IκBα. Furthermore, IκBα phosphorylation was not affected on pretreatment with LPS (Fig. 4).

Activation of JNK and p38 MAPKs

Time-course experiments showed that pretreatment with LPS for 30 min caused a significant increase in the phosphorylation of JNK and p38 MAPK. At 30 min, the phosphorylation of JNK (Fig. 5A) and p38 MAPK (Fig. 5B) was inhibited by schisandrin treatment (50 μM) after LPS stimulation, however, at 5 min there were opposite effects. Treatment with schisandrin after LPS stimulation significantly inhibited the phosphorylation of JNK (Fig. 5C) and p38 MAPKs (Fig. 5D) in a dose-dependent manner.

DISCUSSION

There is no cytotoxicity in 100 μM of schisandrin on macrophages. All experiments had done in the range of non-cytotoxicity concentrations of schisandrin. We compared the results obtained from the two experimental methods. One is that macrophages were pretreated with schisandrin and then

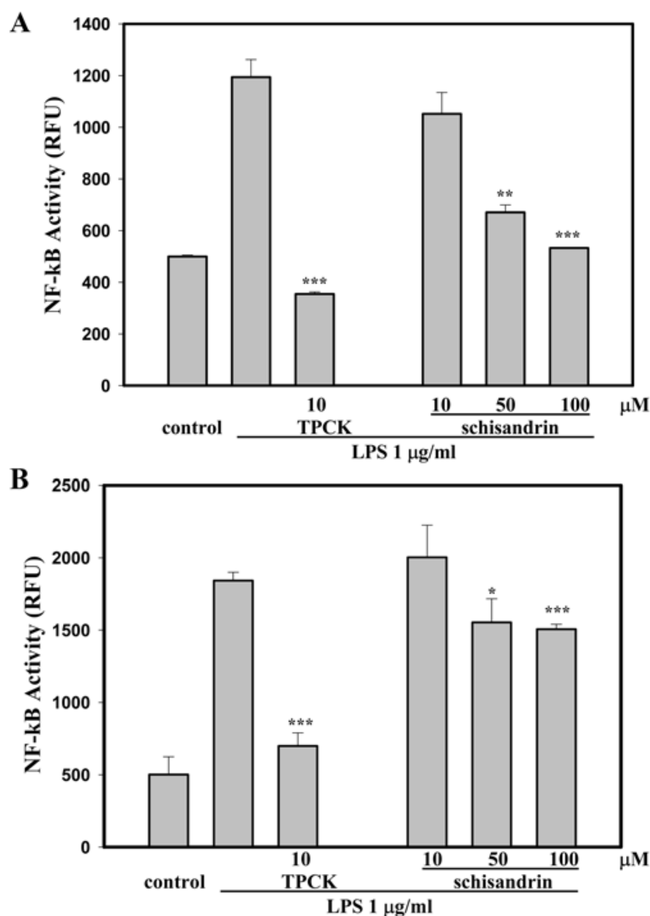

Fig. 3. Effect on NF-κB activity of transfected RAW 264.7 cells (1.5×10^5 cells/well) treated with schisandrin (10, 50 and 100 μM) or TPCK (positive control: 10 μM) before (A) and after LPS (B), respectively

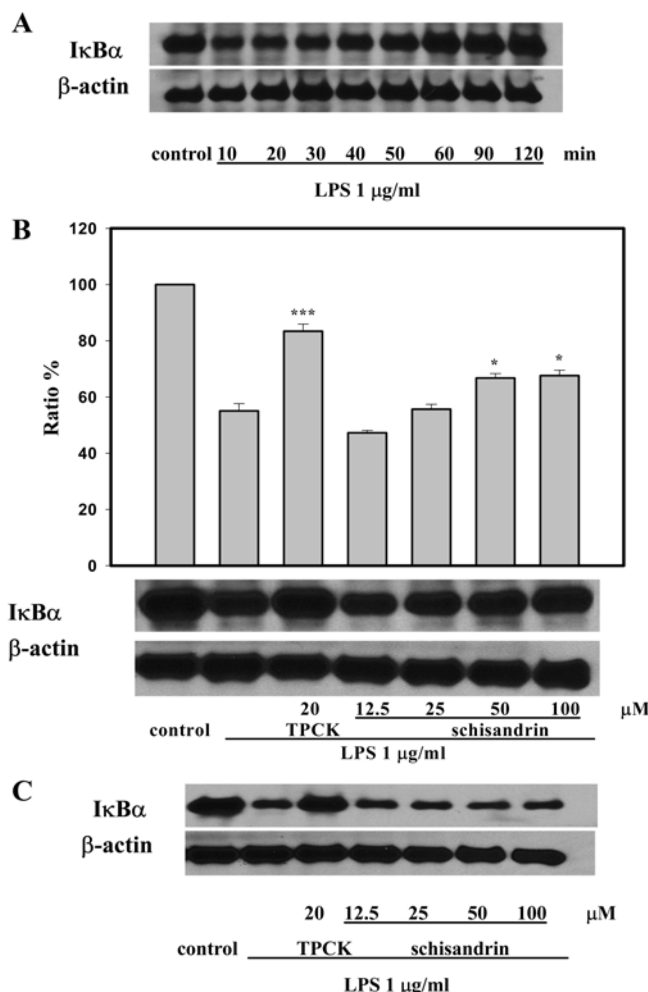

Fig. 4. Time-course assay on IκBα protein expression of macrophages after LPS stimulation (A). Effect on IκBα protein expression of macrophages treated with schisandrin for 2 h before (B) and after (C) LPS.

stimulated by LPS, the other is that macrophages were pretreated by LPS and then treated with schisandrin. The inhibition of iNOS and COX-2 protein expression and NF-κB activation by pretreatment with LPS before schisandrin were weaker than that of pretreatment with schisandrin before LPS (Guo et al., 2008). Pretreatment of cells with LPS had no effect on IκBα degradation. Only when the cells were treated with schisandrin before LPS stimulation was the degradation of IκBα inhibited (Guo et al., 2008). Furthermore, phosphorylation of IκBα was not inhibited even if the cells were pretreated with schisandrin before LPS stimulation.

We also compared the time course of p38 and JNK activation in response to the treatment of cells with schisandrin before and after LPS stimulation. In LPS pretreatment analysis, at 30 min, the phosphorylation of JNK and p38 MAPK was inhibited by schisandrin treatment (50 μM) after LPS stimulation, however, at 5 min, the opposite effects were observed. Schisandrin treatment augmented the phosphorylation of p38 and JNK compared with the parallel assay at 5 min. In some reports, transient activation of JNK induces T-cell proliferation, and persistent activation of JNK causes T-cell apoptosis (Chen et al., 1996). Transient activation of ERK, for 5 min, can induce macrophage proliferation, and prolonged activation of ERK, for 15 min, can induce inflammation (Valledor et al., 2000). These reports indicate that different durations of JNK and ERK induction can have different biological effects. We suggest that the activities of schisandrin in augmenting and inhibiting the activation of JNK and p38 according to transient and persistent stimulation by LPS might be involved in proliferation of cells.

According to the results, it is likely that when

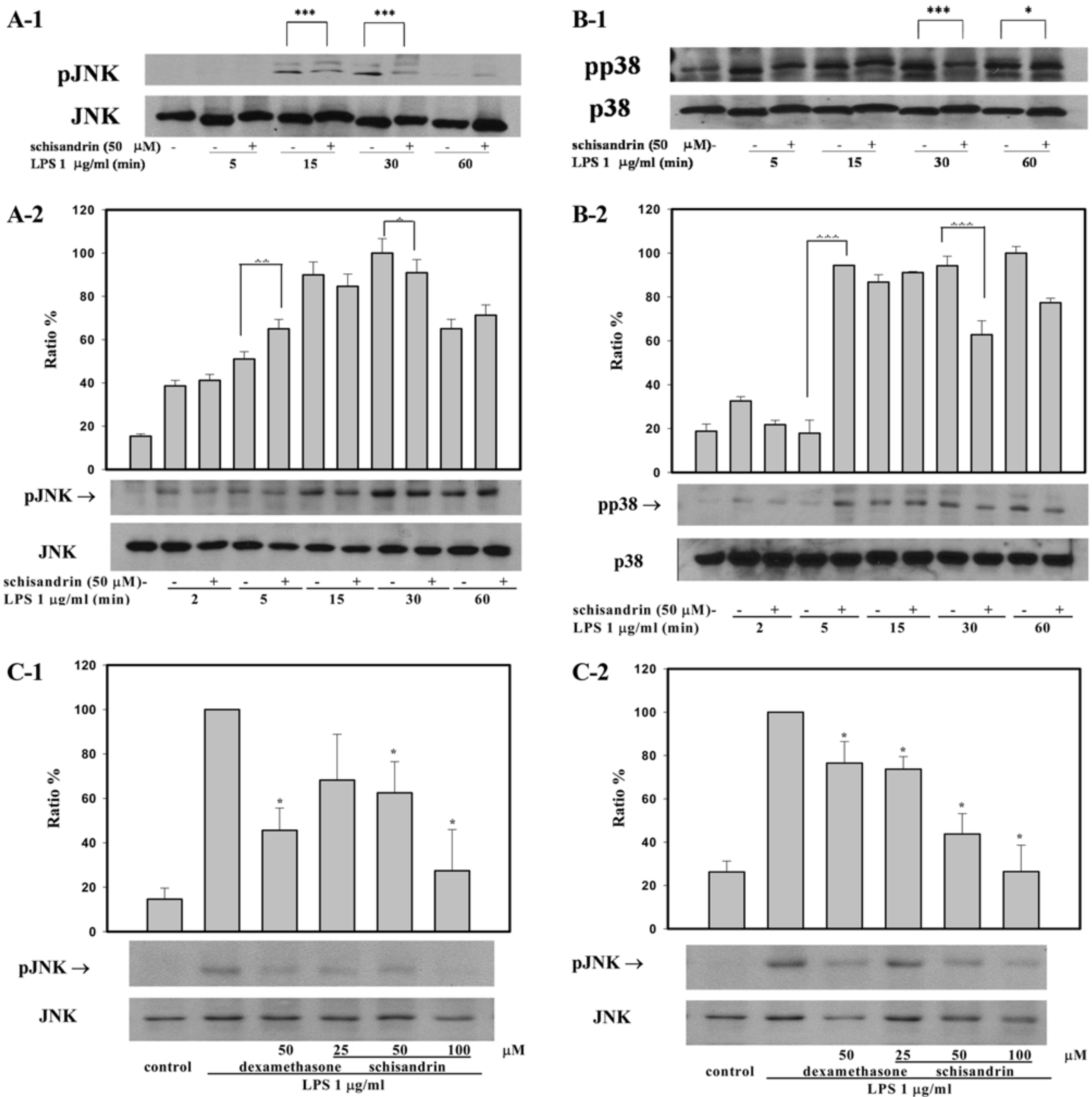

Fig. 5. Effect on JNK protein expression of macrophages treated with schisandrin before (A-1) and after LPS (A-2) in the time-course assay. The effect of schisandrin on p38 protein expression in different sequences of LPS stimulation: B-1 shows pretreatment with schisandrin before LPS stimulation and B-2 shows treatment after LPS stimulation. In the time-course experiment, the cells shown in A-1 and B-1 were pretreated with DMED added to schisandrin or only DMEM for 2 h, then treated with LPS for different periods of time. The cells shown in A-2 and B-2 were pretreated with LPS for different periods of time, then treated with DMEM added to schisandrin or only DMEM for 30 min. In the dose-dependent assay, C-1 shows the effect on JNK protein expression of macrophages treated with schisandrin for 2 h before LPS stimulation for 30 min; C-2 shows the effect of macrophages treated with schisandrin for 30 min after LPS stimulation for 30 min; D-1 shows the effect on p38 protein expression of macrophages treated with schisandrin for 2 h before LPS stimulation for 30 min; D-2 shows the effect of macrophages treated with schisandrin for 30 min after LPS stimulation for 30 min.

cells were pretreated with LPS before schisandrin, the schisandrin leads to a reduction in iNOS and COX-2 expression by the blocking of the p38 and JNK MAPK pathways. With regard to schisandrin pretreatment before LPS, schisandrin inhibits iNOS and COX-2 expression by preventing IκBα degradation, inhibiting NF-κB activation and blocking the p38, JNK MAPK pathways (Guo et al., 2008).

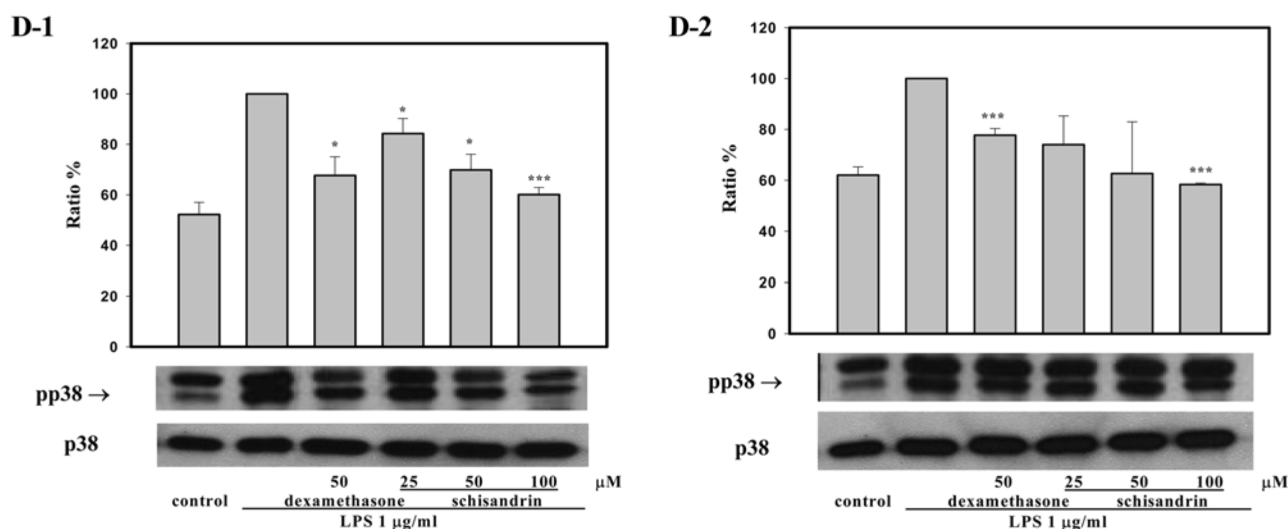

Fig. 5. Continued

In conclusion, the mechanisms of schisandrin-mediated anti-inflammatory action are slightly different depending on the order of LPS and drug treatment. The pharmacological model of LPS pre-treatment accords with the pathological mechanism and clinical use, which is helpful to clarify the mechanisms of effects of anti-inflammatory drugs or compounds. Conversely, some treatments can have a stronger effect when administered before disease by blocking signal transduction or interrupting binding of antigen with receptors (Hong et al., 2006). However, prophylactic treatment is generally not feasible in clinical medicine.

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