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Protopine reduces the inflammatory activity of lipopolysaccharide-stimulated murine macrophages

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Protopine is an isoquinoline alkaloid contained in plants in northeast Asia. In this study, we investigated whether protopine derived from *Hypecoum erectum L* could suppress lipopolysaccharide (LPS)-induced inflammatory responses in murine macrophages (Raw 264.7 cells). Protopine was found to reduce nitric oxide (NO), cyclooxygenase-2 (COX-2), and prostaglandin E_2 (PGE_2) production by LPS-stimulated Raw 264.7 cells, without a cytotoxic effect. Pre-treatment of Raw 264.7 cells with protopine reduced the production of pro-inflammatory cytokines. These inhibitory effects were caused by blocking phosphorylation of mitogen-activated protein kinases (MAP kinases) and also blocking activation of a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). [BMB reports 2012; 45(2): 108-113]

INTRODUCTION

Hypecoum erectum L belongs to the family Hypecoaceae, which is a Mongolian medicinal plant. In the traditional medicine of Tibet and Mongolia, the plant is named "bar ba da" and has been used for many years due to its anti-inflammatory action in disorders involving infection. However, anti-inflammatory compounds derived from *Hypecoum erectum L* have not yet been reported.

Protopine is an isoquinoline alkaloid and is normally found in plants in northeast Asia. Protopine has been shown to exhibit a number of pharmacological activities such as reduction of intracellular calcium flux, and inhibition of arachidonic acid and platelet-activating factor induced platelet aggregation by decreasing cytosolic calcium in the rabbit (1). Protopine inhibits thromboxane A2 synthesis and inflammatory processes via the COX pathway (2). Moreover, it also possesses anti-bacterial (3) as well as anti-histaminic and anti-cholinergic (4) activities. However, there are no reports on the *in vitro* anti-inflammatory effects of protopine. Furthermore, the molecular anti-inflammatory mechanism has not yet been studied.

Inflammation is induced by many factors, including pro-inflammatory enzymes (NO, COX-2, and PGE_2) and cytokines [tumor necrotic factor (TNF)-α, interleukin (IL)-1, and IL-6, *etc.*], which are indicators of inflammatory activity. The role of NO is shown by its elevated levels in the inflammatory response (5, 6). NO is involved in inflammation (7) and autoimmune diseases (8). COX-2, another pro-inflammatory enzyme, is the enzyme which converts arachidonic acid into prostaglandins, and exists as 2 isozymes, COX-1 and COX-2. Of them, COX-2, is induced by pro-inflammatory cytokines or LPS (9-11). Pro-inflammatory cytokines are mainly produced in macrophages activated by LPS produced by Gram negative bacteria (12-14).

NF-κB and MAP kinases are representative cell signaling molecules and are involved in inflammatory responses, including the expression and production of NO, COX-2, and pro-inflammatory cytokines in macrophages (15). NF-κB is constitutively localized in cytosol as a homodimer or heterodimer, which are associated with inhibitor-κB proteins (IκB). The phosphorylation of MAP kinases, including p38 MAPK, ERK, and JNK, is induced by stress and bacterial endotoxin within 10-30 min (16, 17).

In the present study, to clarify the anti-inflammatory activities of protopine, we isolated protopine from *Hypecoum erectum L* and investigated its immune suppressive effects on the production of NO, PGE_2, and pro-inflammatory cytokines, as well as its inhibitory effect on NF-κB and MAP kinase activation in a LPS-stimulated murine macrophage cell line.

RESULTS

Inhibition of NO production by protopine in activated Raw 264.7 cells

Raw 264.7 cells were used to analyze the potential anti-inflammatory activities of protopine because they can produce NO upon stimulation with LPS. The cells were treated with LPS (1 μg/ml) for 24 h after treatment for 1 h with and without protopine. The control (cell-only) group had neither LPS nor protopine. Dexamethasone (10 μM) was used as a positive control for inhibition of NO production. When LPS was added to the Raw 264.7

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cells, NO production increased dramatically in the range of 28.1-35.9 μM, from the basal levels of 0-0.2 μM, and protopine reduced the NO production in a dose-dependent manner (Fig. 1A).

To demonstrate that the inhibitory effect of protopine on NO production did not result from cytotoxicity, the cytotoxicity of protopine was evaluated by a WST-1 assay after the cells were incubated for 24 h in the presence of protopine and LPS. As shown in Fig. 1B, there was no evidence of cytotoxicity by protopine, and all concentrations were tested in the WST-1 assay. Thus, the inhibitory effect of all protopine concentrations on NO production could not be attributed to cytotoxic effects.

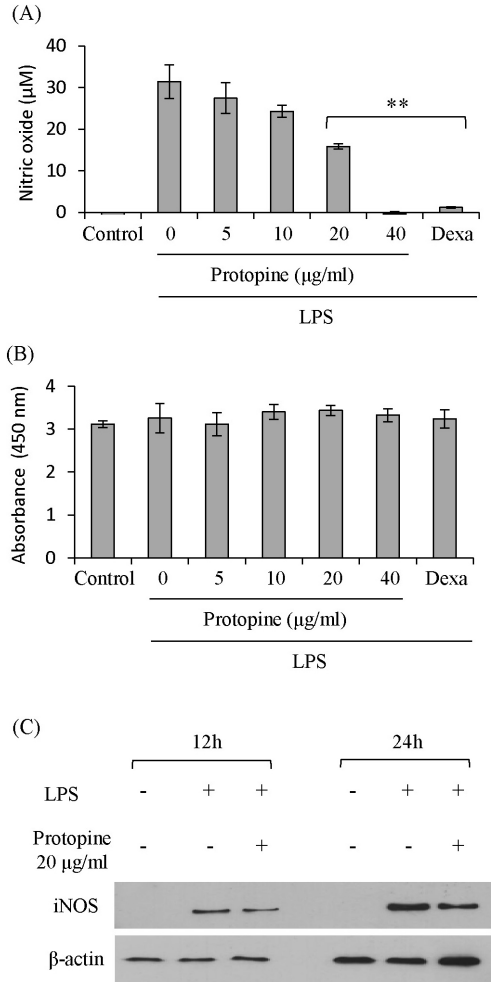

Fig. 1. Effects of protopine on NO production in LPS-stimulated Raw 264.7 cells. Raw 264.7 cells were treated with various concentrations of protopine for 1 h and then the cells were stimulated with 1 μg/ml of LPS. After 24 h, the culture supernatants were subjected to nitrite assay (A). The cell viability was determined by measuring the absorbance at 450 nm after addition of WST-1 reagent (B). LPS (1 μg/ml) was added to Raw 264.7 cells pretreated with protopine and the cells were incubated for 12 h or 24 h. A total protein lysate of each aliquot of Raw 264.7 cells was prepared and the proteins resolved by Western blot were analyzed by an anti-iNOS antibody (C). Anti-β-actin antibody was used as a control. The values shown are the means ± SDs for 3 independent experiments. **P < 0.01 as compared to the cells treated with LPS. Western blot analysis was performed in triplicate, and all analyses showed similar results.

Protopine attenuates inducible nitric oxide synthase (iNOS) protein levels in LPS-stimulated Raw 264.7 cells

To substantiate the inhibitory effects of protopine on LPS-induced nitrite production, the iNOS protein levels were analyzed by Western blotting analysis. Raw 264.7 cells were pretreated with 20 μg/ml protopine for 1 h and stimulated with 1 μg/ml of LPS for 12 and 24 h. The total cell lysates were then probed with iNOS monoclonal antibody. As shown in Fig. 1C, iNOS expression was not detected in unstimulated Raw 264.7 cells (control). Treatment with LPS (1 μg/ml) markedly increased the protein levels, but significantly decreased them in cells pre-treated with protopine at 20 μg/ml.

Protopine inhibits expression of LPS-stimulated COX-2 and PGE_2 production

Because protopine was revealed to be a potent inhibitor of NO,

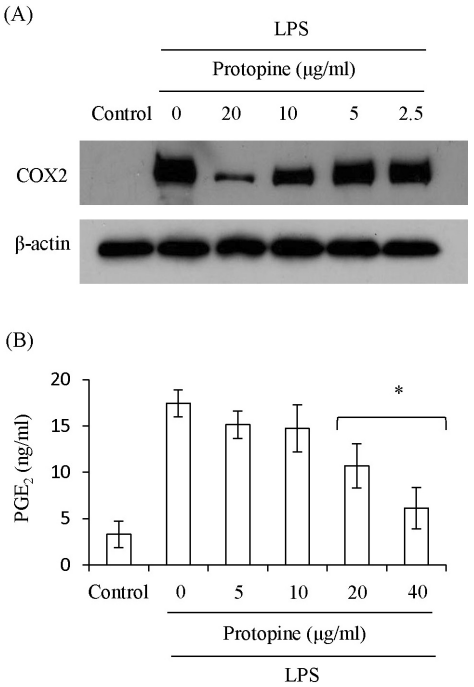

Fig. 2. Effect of protopine on COX-2 and PGE_2 production in Raw 264.7 cells. Raw 264.7 cells were pretreated with different concentrations of protopine for 1 h; LPS (1 μg/ml) was then added. After 6 h, a total protein lysate of each aliquot of Raw 264.7 cells was prepared and the resolved proteins were analyzed by Western blot using an anti-COX-2 antibody (A). After 24 h, the culture supernatant was subjected to PGE_2 assay (B). Western blot analysis using a specific antibody was performed in triplicate and all analyses showed similar results. The values shown are the means ± SD of 3 independent experiments. *P < 0.05 compared to the cells treated with LPS.

we further investigated its effect on the expression levels of COX-2 by Western blot. As shown in Fig. 2A, non-activated cells did not express detectable levels of COX-2, whereas treatment with LPS induced high levels of COX-2. Under these conditions, pre-treatment of Raw 264.7 cells with protopine reduced COX-2 expression in a dose dependent manner. In all experiments the housekeeping protein β-actin was equally expressed irrespective of treatment.

LPS activates macrophages, followed by induction of COX-2, which is a rate-limiting enzyme in PGE_2 synthesis. Therefore, the effect of protopine on production of PGE_2 was investigated in LPS stimulated Raw 264.7 cells. As shown in Fig. 2B, protopine significantly inhibited PGE_2 production in a dose-dependent manner.

Effects of protopine on the protein levels of pro-inflammatory cytokines

Because protopine was found to be a potent inhibitor of the pro-inflammatory mediators (NO, COX-2 and PGE_2), we further investigated its effects on pro-inflammatory cytokine release by ELISA. To examine whether protopine reduced the production of pro-inflammatory cytokines, Raw 264.7 cells were pretreated with protopine for 1 h and stimulated with LPS (1 μg/ml) for 24 h. The culture supernatants were then collected to measure of the amounts of IL-1β, IL-6, and TNF-α. Consistent with results of the NO assay, protopine significantly inhibited LPS-induced IL-1β, IL-6, and TNF-α production (Fig. 3). In particular, protopine strongly reduced IL-6 and IL-1β production to their basal levels, (control) even at 10 μM.

Effects of protopine on the pathways of inflammatory cell signaling

Raw 264.7 cells were pretreated with 20 μg/ml protopine for 1 h and stimulated with 1 μg/ml of LPS. After incubations of 15 min and 30 min the total cell lysates were prepared for assay of MAP kinases (A), and total nuclear protein was prepared after 30 min and 1 h for Western blotting analysis of NF-κB. As shown in Fig. 4A, stimulation with LPS increased the phosphorylation of p38 MAPK, ERK 1/2, and JNK. The protopine treatment reduced the levels of phosphorylated ERK and JNK, while that of p38 was not affected in LPS-stimulated Raw 264.7 cells. The inhibitory effect of protopine on JNK phosphorylation was sustained until 30 min after stimulation. The amounts of non-phosphorylated p38 MAPK, ERK 1/2, and JNK were unaffected by LPS or LPS plus protopine treatment.

NF-κB is known to transactivate various inflammatory enzymes and cytokines. Therefore, we determined if the inhibitory effects of protopine were exerted through the blockade of NF-κB activation in Raw 264.7 cells. As shown in Fig. 4B, LPS enhanced the translocation of p50 and p65 into the nucleus within 30 min, which was inhibited by protopine treatment. Nucleolin was used as an

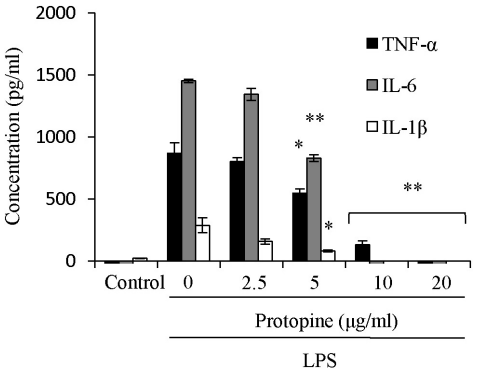

Fig. 3. Effects of protopine on IL-1b, IL-6, and TNF-a production in Raw 264.7 cells. The test samples were treated as described in Fig. 1. The culture supernatant, which was subjected to enzyme immunoassay of cytokines, was harvested. The values shown are means ± S.D. for 3 independent experiments. *P < 0.05; **P < 0.01 as compared to the cells treated with LPS.

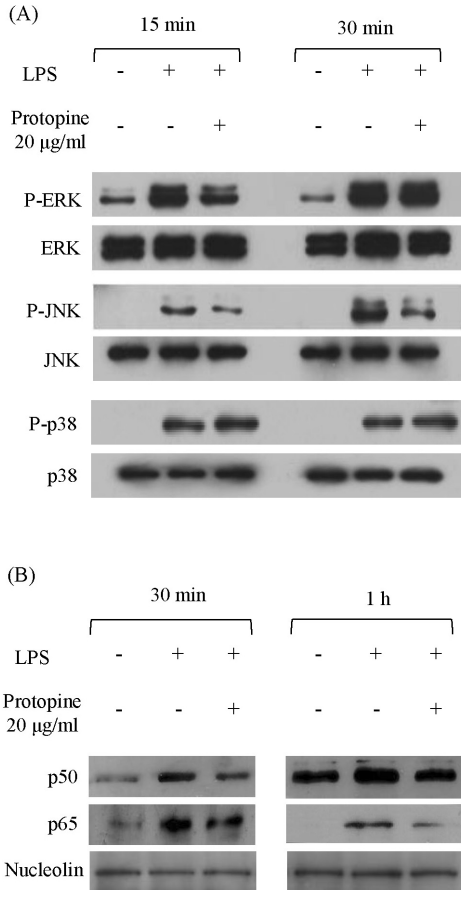

Fig. 4. Effects of protopine on the LPS-induced cell signaling pathway in Raw 264.7 cells. Raw 264.7 cells were pretreated with 20 μg/ml of protopine for 1 h, and then LPS (1 mg/ml) was added. The total cell lysate was prepared after 15 min and 30 min for analysis of MAP kinases (A) and total nuclear protein was prepared after 30 min and 1 h for analysis NF-κB (p50 and p65) (B). Western blot analyses using a specific antibody were performed in triplicate, and all analyses showed similar results.

internal control. These results indicated that protopine reduced the production of NO, iNOS, COX-2, and pro-inflammatory cytokines via the inhibition of phosphorylation of ERK 1/2 and JNK, and inhibition of NF-κB activation in LPS-activated macrophages.

DISCUSSION

The inflammatory response requires cellular factors including pro-inflammatory enzymes and cytokines. NO is one of the pro-inflammatory enzymes (5, 6) which is generated enzymatically by synthases (NOS) and is formed by iNOS in macrophages. NO is released by bacterial infection or inflammatory responses and is toxic to bacteria and other human pathogens. Therefore, bacterial LPS and inflammatory cytokine, IFN-γ, regulate the activity of iNOS in macrophages (18, 19). Although NO is necessary in a defense mechanism for maintaining the dilation of blood vessels (20), the much higher concentrations of NO produced by iNOS in macrophages can cause oxidative damage. iNOS-induced NO is involved in various biological processes, including inflammation (7) and autoimmune diseases (21). Our study clearly showed that pretreatment with protopine inhibited NO production from LPS-activated Raw 264.7 cells.

COX-2, also a pro-inflammatory enzyme, produces PGE_2 by converting arachidonic acid into prostaglandins. COX-2 is generally induced by pro-inflammatory cytokines (TNF-a, IL-1, IL-6, and interferon, etc.) or LPS (9-11). In normal cells, COX-2 is not increased, but it is highly expressed by macrophages during the inflammatory response. COX-2 has been shown to be upregulated in various carcinomas and to have a central role in tumorigenesis. Natural COX-2 inhibitors have been reported in many previous studies. For example, culinary mushrooms and a variety of flavonoids may be able to partially inhibit COX-2 (22, 23). Particularly, Saeed et al. demonstrated that protopine, at a low dose, inhibited arachidonic acid synthesis and showed a marked reduction in carrageenan-induced paw edema (2). These results are in agreement with ours, because our results showed that protopine selectively inhibited the COX pathway and thus reduced the production of PGE_2.

Activated macrophage-derived pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 play critical roles in inflammatory diseases (12-14). TNF-α was recently identified as a main mediator in inflammatory diseases (24), because TNF-α is able to stimulate the production of pro-inflammatory cytokines, such as IL-6, IL-1β, and IFN-γ. Notably, IL-1β has directly opposing activities on the immune system. The first activity is an immunological defense action, because it activates helper T cells and promotes the maturation and clonal expansion of B cells. On the other hand, the second activity is that IL-1β plays an important part in inflammatory diseases, including autoimmune disorders, bacterial infections, noninfectious hepatitis, and graft-versus-host disease (25). IL-6 is also classified as a pro-inflammatory cytokine. It is released from T cells and macrophages in response to TNF-α, IL-1, and LPS. Generally, IL-6 stimulates the immune response to trauma, especially from burns or other tissue damage that leads to inflammation (26). Our results indicate for the first time that protopine significantly inhibits pro-inflammatory cytokine production from activated macrophages, suggesting that protopine could be a potential candidate for treatment of inflammatory disease.

The phosphorylation of MAP kinases, including p38 MAPK, ERK, and JNK, is induced by stress and bacterial endotoxin within 10-30 min, and they are involved in differentiation and proliferation of mammalian cells (16, 17). Several studies have claimed that MAP kinase is involved in the production of LPS-induced inflammatory mediators from macrophages (8). Especially, ERK and JNK are involved in the expression of various inflammatory inducers, including NO, COX, and cytokines. Therefore, these are target molecules in anti-inflammatory medicine. In our experiments, the anti-phosphorylation activity of protopine was seen within 30 minutes. The inhibition of phosphorylated JNK levels by pretreatment with protopine could be affected by an upstream signaling molecule such as NO. Because protopine significantly inhibited NO levels in our results, this inhibition could affect the levels of phosphorylated JNK upon protopine treatment.

Generally, NF-κB, a mammalian transcription factor that regulates various genes, is involved in inflammatory responses, including the expression and production of NO, COX-2, and pro-inflammatory cytokines in macrophages (15, 27). NF-κB is a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors. Homo- and heterodimers of 5 members of the Rel family include NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel (Rel). In this study, we observed that protopine inhibited LPS-stimulated nuclear translocation of p50 and p65, which suggests that protopine may block the NF-κB pathway. This inactivation of NF-κB could be associated with inhibition of phosphorylated ERK levels by protopine. Because activated ERK is known to phosphorylate IκBα, an inhibitor of NF-κB, leading to degradation of IκBα followed by activation of NF-κB, the inhibition of ERK signaling could be a good strategy to pursue s for development of anti-inflammatory agents.

In summary, our results indicate that protopine has anti-inflammatory activities in activated macrophages. That is, we identified that protopine is a potent inhibitor of LPS-induced NO and the production of pro-inflammatory cytokines by gene expression. The inhibitory activities of protopine were found to be caused by blockage of MAP kinase phosphorylation and NF-κB activation in the macrophages. We concluded that protopine has the potential to prevent inflammation.

MATERIALS AND METHODS

Extraction and isolation of protopine (tetrahydro-5-methyl bis-[1,3]benzdioxide[4,5-C:5′,6]-azecin-13(5H)-one)

Hypecoum erectum L was collected in the Bulgan region of Central Mongolia. The plant was identified by Dr. Ch. Dairiimaa (Institute of Botany, Mongolian Academy of Sciences). A voucher specimen (No 588) was deposited in the Herbarium of the Institute of Botany, Mongolian Academy of Sciences, Ulaanbaatar. The air dried and

ground aerial parts (3.5 kg) were extracted with EtOH at room temperature. The combined EtOH extracts were evaporated under reduced pressure, acidified with 10% HCl to pH 1-2, and stored overnight at room temperature. Insoluble non-alkaloid substances were removed by filtration and the filtrate was subjected to n-hexane extraction to eliminate the remaining non-alkaloid compounds. Then, the purified acidic solution was made alkaline with 25% NH_4OH to pH 9-10 and extracted with $CHCL_3$. The combined $CHCL_3$ extracts were evaporated under reduced pressure to give a crude mixture of alkaloids (210 g).

The crude alkaloid mixture (20 g) was separated into fractions by vacuumed liquid chromatography on neutral alumina, and eluting with n-hexane:EtOAc mixtures of increasing polarity (5 : 1, 3 : 1, 1 : 1, and pure EtOAc), and 6 alkaloid fractions (AF1-AF6) were combined. AF2 (3 : 1) was directly subjected to preparative thin layer chromatography with a mobile phase consisting of petroleum : $CHCl_3$: Me_2CO : MeOH (4 : 4 : 1 : 1), and 10.2 g of protopine was isolated.

Reagents and cells

The mouse macrophage cell line, Raw 264.7, was obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone) and penicillin (100 U/ml) / streptomycin (100 μg/ml) (Gibco BRL, Grand Island, NY,USA). LPS, dexamethasone, and Griess reagent were from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal antibodies against p-38, phospho-p-38, ERK 1/2, phospho-ERK 1/2, JNK, phospho-JNK, β-actin, p50, p65, and nucleolin, as well as peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell viability

Cell viability was measured by the WST-1-based colorimetric assay (Takara Bio.,Ohtsu, Japan), which relies on the ability of living cells to reduce a tetrazolium salt to a soluble colored formazan product. Cell suspensions of 5×10^4 cells/well were cultured in a flat- bottom 96-well plate in triplicate for 6 h. Thereafter, 100 μl of medium was replaced with fresh medium containing either LPS (1 μg/ml) alone or LPS with various concentrations of each sample. After 24 h of culture, WST-1 reagent was added to both Raw 264.7 cells and blank samples, and the mixture was incubated at 37°C under 5% CO_2 for 3 h. The level of the dye formed was then measured using a Bio-Rad spectrophotometer at a wavelength of 450 nm. The blank value without cells was subtracted from each experimental value as background.

NO measurement

Production of NO was assayed by measuring the levels of nitrite, as described in ref. 28 (28). Briefly, the test samples were treated as described in the cell viability assay, and the culture supernatant was collected at the end of culture for a nitrite assay, which was used as a measurement of NO production. The culture supernatant (50 μl) was mixed with an equal volume of Griess reagent and the absorbance was measured at 550 nm. Finally, the concentration of nitrite was calculated from a standard curve drawn with known concentrations of sodium nitrite dissolved in DMEM.

Western blot analysis

Raw 264.7 cells were cultured in the presence of LPS or in combination with protopine in a 6-well plate (1×10^6 cells/well). After removal of the supernatants, extracts of Raw 264.7 cells were directly prepared in lysis buffer (0.5% Triton, 50 mM β-glycerophosphate pH 7.2, 0.1 mM sodium vanadate, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl urea, 2 μg/ml of leupeptin, and 4 μg/ml of aprotinin). The lysates were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline (10 mM Tris-Cl pH 7.4) containing 0.5% Tween 20 and 5% nonfat dry milk, incubated with the first specific antibody in blocking solution for 5 h at room temperature, washed, and then incubated with the second developing antibody for 1 h at room temperature. The protein bands were detected by chemiluminescence (Amersham Pharmacia, Biotech, NJ, USA).

Enzyme-linked immunosorbent assay (ELISA)

PGE_2 (PGE_2 immunoassay kit, R&D systems, Minneapolis, MN, USA.), TNF-β, IL-1β, and IL-6 (DuoSet ELISA Development Kit, R&D Systems) in the conditioned supernatants obtained from NO assays were measured by an enzyme-linked immunoassay following the manufacturer's instructions.

Statistics

Analyses of data to determine statistical significance were performed by the Student's *t*-test. Values are given as mean ± S.D.

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