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In-vitro and in-vivo anti-inflammatory and antinociceptive effects of the methanol extract of the roots of *Morinda officinalis*

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Abstract

The anti-inflammatory effects of the methanol extract of the roots of *Morinda officinalis* (MEMO) (Rubiaceae) were evaluated in-vitro and in-vivo. The effects of MEMO on lipopolysaccharide (LPS)-induced responses in the murine macrophage cell line RAW 264.7 were examined. MEMO potently inhibited the production of nitric oxide (NO), prostaglandin E_2 and tumour necrosis factor-α (TNF-α) in LPS-stimulated RAW 264.7 macrophages. Consistent with these results, the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at the protein level, and of iNOS, COX-2 and TNF-α at the mRNA level, was also inhibited by MEMO in a concentration-dependent manner. Furthermore, MEMO inhibited the nuclear factor kappa B (NF-κB) activation induced by LPS, and this was associated with the prevention of degradation of the inhibitor κB (IκB), and subsequently with attenuated p65 protein in the nucleus. The anti-inflammatory effect of MEMO was examined in rats using the carrageenan-induced oedema model. The antinociceptive effects of MEMO were assessed in mice using the acetic acid-induced abdominal constriction test and the hot-plate test. MEMO (100, 200 mg kg^{-1} per day, p.o.) exhibited anti-inflammatory and antinociceptive effects in these animal models. Taken together, the data demonstrate that MEMO has anti-inflammatory and antinociceptive activity, inhibiting iNOS, COX-2 and TNF-α expression by down-regulating NF-κB binding activity.

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Introduction

Chronic inflammations and infections lead to the up-regulation of a series of enzymes and signalling proteins in affected tissues and cells. Among these pro-inflammatory enzymes, the inducible forms of nitric oxide synthase (NOS) and cyclooxygenase (COX), which are responsible for increasing the levels of nitric oxide (NO) and prostaglandins (PGs), respectively, are known to be involved in the pathogenesis of many chronic diseases, including multiple sclerosis, Parkinson's and Alzheimer's diseases, and colon cancer (Heiss et al 2001). NO is generated enzymatically by NOS, which oxidizes L-arginine to L-citrulline (Michel & Feron 1997; Ignarro 2002). There are three isoforms of NOS: NOS-1 or neuronal NOS, NOS-2 or inducible NOS (iNOS), and NOS-3 or epithelial NOS (Michel & Feron 1997; West et al 2001; Ignarro 2002). The three isoforms have similar molecular structures and all require multiple cofactors. Neuronal NOS and epithelial NOS produce NO within seconds, and their activities are direct and short acting, whereas iNOS produces large toxic amounts of NO in a sustained manner (Salvemini et al 2003).

COX is the enzyme that converts arachidonic acid to PGs. COX exists in two isoforms: COX-1 and COX-2 (Funk et al 1991). COX-1 is expressed constitutively in most tissues and appears to be responsible for maintaining normal physiological functions. In contrast, COX-2 is detectable in only certain types of tissues and is induced transiently by growth factors, pro-inflammatory cytokines, tumour promoters and bacterial toxins (Prescott & Ritzpatrick 2000; Hinz & Brune 2002). Moreover, elevated levels of COX-2 expression have been detected in various tumour types, and may account for the excessive production of the inflammatory mediator PGs (Subbaranaiah & Dannenberg 2003).

Nuclear factor kappa B (NF-κB) is a ubiquitous transcription factor that regulates the expression of genes involved in cellular proliferation, inflammatory response and cell adhesion. The activation of NF-κB has been reported to induce the gene transcription of multiple pro-inflammatory mediators, including iNOS, COX-2, tumour necrosis factor-α (TNF-α), interleukin-6 and interleukin-8 (Surh et al 2001; Lappas et al 2002). Functionally active NF-κB exists mainly as a heterodimer consisting of subunits of the Rel family p50 and p65, which is normally sequestered in the cytosol as an inactive complex with inhibitors of κB (IκB) in unstimulated cells (Baeuerle 1998). The activation of NF-κB involves the phosphorylation of IκB in two critical serine residues (Ser^{32}, Ser^{36}) by the IκB kinase signalosome complex (Brown et al 1995; DiDonato et al 1996; O'Conell et al 1998). The resulting free NF-κB is translocated to the nucleus, where it binds to κB binding sites in the promoter regions of target genes, and then induces the transcriptions of pro-inflammatory mediators (Baeuerle & Baltimore 1996; Barnes & Karin 1997).

As a part of our on-going screening programme to evaluate the anti-inflammatory potential of natural compounds, we investigated the in-vitro and in-vivo anti-inflammatory activity of the methanol extract of the roots of *Morinda officinalis* (MEMO). Among Morinda species, *Morinda citrifolia* (Rubiaceae), commonly known as noni, is a plant typically found in the Hawaiian and Tahitian islands. From this plant, a number of constituents have already been isolated, including anthraquinones, flavonoids, iridoids and oligosaccharides (Sang et al 2001). Iridoids isolated from *Morinda moindoides* have been reported to have complement inhibitory activity (Cimanga et al 2003). The roots of *M. officinalis* have been used to treat impotence, menstrual disorders, and inflammatory diseases such as rheumatoid arthritis and dermatitis in the Orient (Evans 2002). However, there is no report on their anti-inflammatory activity or mode of action. We therefore investigated the effects of MEMO on lipopolysaccharide (LPS)-induced NO, prostaglandin E_2 (PGE_2) and TNF-α production in the RAW 264.7 macrophage cell line, and subsequently evaluated its anti-inflammatory and antinociceptive effects in-vivo.

Materials and Methods

Reagents and animals

Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, USA). COX-2, iNOS, p65 and IκB-α monoclonal antibodies and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The enzyme immunoassay (EIA) kits for PGE_2 and TNF-α were obtained from R&D Systems (Minneapolis, MN, USA). NS-398, a COX-2 enzyme inhibitor, was from Calbiochem (San Diego, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyl tertazolium bromide (MTT), aprotinin, leupeptin, phenylmethylsulfonylfluoride, dithiothreitol, caffeic acid, L-N^6-(1-iminoethyl) lysine, *Escherichia coli* LPS, acetylsalicylic acid (Aspirin), carrageenan and all other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

ICR male mice, 20–25 g, and Sprague-Dawley male rats, 100–120 g, were purchased from Daehan Biolink (Eumsung-Gun, Chungbuk, Korea) and maintained under constant conditions (temperature 20°C; humidity 40–60%; 12-h light/dark cycle) for 2 weeks or more. At 24 h before the experiment, only water was offered to the animals. Considering the variation of enzyme activity during one day, the animals were killed at a fixed time (10:00–12:00 hours). The experiments were approved by the University of Kyung-Sung Animal Care and Use Committee. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health.

Extraction, fractionation and isolation of iridoid glycosides

The roots of *M. officinalis* were purchased from the Chun-Il Oriental Herbal Store in Wonju, Korea, and the plant origin was identified by S. Y. Yun (Department of Botanical Resources, Sangji University, Korea). A voucher specimen (NATCHEM-29) was deposited in the Laboratory of Natural Products Analysis, Division of Applied Plant Sciences, Sangji University, Korea. The plant material (2.5 kg) was extracted three times with MeOH under reflux. The extract was filtered and evaporated on a rotary evaporator under reduced pressure. The concentrated extract was again dried using a freeze dryer to give a solid MeOH extract (185 g), which was then partitioned between distilled water and $CHCl_3$, and then the residual water layer was successively extracted with EtOAc and BuOH. The $CHCl_3$, EtOAc and BuOH layers were evaporated under reduced pressure to give $CHCl_3$ extract, EtOAc extract and BuOH extract. The BuOH extract was chromatographed over silica gel using the solvent $CHCl_3$/MeOH/H_2O (65:35:10, lower layer) and then collected (120 mL). Purification of the fractions with the iridoid glycoside afforded compounds such as geniposidic acid (Lee et al 2004), deacetylasperulosidic acid (Ling et al 2002) and monotropein (Boros & Stermitz 1990), confirmed by comparison with literature physicochemical and spectroscopic data.

Cell culture and sample treatment

The RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37°C in DMEM medium supplemented with 10% FBS, penicillin (100 units mL^{-1}) and streptomycin sulfate (100 $\mu g\,mL^{-1}$) in a humidified atmosphere of 5% CO_2. Cells were incubated with MEMO at different concentrations (40, 60 and 80 $\mu g\,mL^{-1}$) or positive chemical and stimulated with 1 $\mu g\,mL^{-1}$ LPS.

Nitrite determination

The nitrite accumulated in culture medium was measured as an indicator of NO production based on the Griess reaction. Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine-HCl), incubated at room temperature for 10 min, and then the absorbance at 550 nm was measured using a microplate reader. Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was measured with the sodium nitrite serial dilution standard curve and nitrite production was measured.

MTT assay for cell viability

Cell viability studies were performed in 96-well plates. RAW 264.7 cells were mechanically scraped and plated at 5×10^5 cells/well in 96-well plates containing 100 μL of DMEM with 10% FBS and incubated overnight. MEMO was dissolved in DMSO; the same volume of DMSO was added to all plates. After overnight incubation, the test material was added, and the plates were incubated for 24 h. Cells were washed once before adding 50 μL of FBS-free medium containing 5 mg mL^{-1} MTT. After 4 h of incubation at 37°C, the medium was discarded and the formazan blue that formed in the cells was dissolved in 100 μL DMSO. The optical density was measured at 540 nm.

PGE_2 and TNF-α assay

PGE_2 and TNF-α levels in macrophage culture medium were quantified using EIA kits according to the manufacturer's instructions.

Western blot analysis

Cellular proteins were extracted from control and MEMO-treated RAW 264.7 cells. The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM Na fluoride, 0.5 mM Na orthovanadate) containing 5 μg mL^{-1} each of leupeptin and aprotinin and incubated for 15 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacture's instructions. Cellular protein (40 mg) from treated and untreated cell extracts was electroblotted onto a nitrocellulose membrane following separation by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4°C, followed by incubation for 4 h with a 1:500 dilution of monoclonal anti-iNOS, 1:1000 dilution of anti-COX-2 antibody, 1:1000 dilution of anti-IκB-α antibody and 1:500 dilution of anti-p65 antibody (Santa Cruz Biotechnology Inc.). Blots were washed twice with Tween 20/Tris-buffered saline and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Blots were again washed three times with Tween 20/Tris-buffered saline and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, USA).

RNA preparation and polymerase chain reaction (PCR)

Total cellular RNA was isolated using Easy Blue kits (Intron Biotechnology, Sungnam, Kyungki-Do, Korea) according to the manufacturer's instructions. From each sample, 1 μg of RNA was reverse-transcribed using MuLV reverse transcriptase, 1 mM dNTP and 0.5 μg μL^{-1} oligo (dT_{12-18}). PCR analyses were then performed on aliquots of the cDNA preparations to detect iNOS, COX-2, TNF-α and β-actin (as an internal standard) gene expression using a thermal cycler (Perkin Elmer Cetus, Foster City, CA, USA). The reactions were carried out in a volume of 25 μL containing (final concentration) 1 unit of Taq DNA polymerase, 0.2 mM dNTP, $\times$10 reaction buffer, and 100 pmol of 5′ and 3′ primers. After initial denaturation for 2 min at 95°C, 30 amplification cycles were performed for iNOS (1 min of 95°C denaturation, 1 min of 60°C annealing, and 1.5 min 72°C extension), COX-2 (1 min of 94°C denaturation, 1 min of 60°C annealing, and 1 min 72°C extension) and TNF-α (1 min of 95°C denaturation, 1 min of 55°C annealing, and 1 min 72°C extension). The PCR primers used in this study are listed below and were purchased from Bioneer (Seoul, Korea): sense strand iNOS, 5′-AATGGCAACATCAG GTCGGCCATCACT-3′, anti-sense strand iNOS, 5′-GCTGTGT-GTCACAGAAGTCTCG-AACTC-3′; sense strand COX-2, 5′-GGAGAGACTATCAAGATAGT-3′, anti-sense strand COX-2, 5′-ATGGTCAGTAGACTTTT ACA-3′; sense strand TNF-α, 5′-ATGAGCACAGAAA GCATGATC-3′, anti-sense strand TNF-α, 5′-TACAGG CTT-GTCACTCGAATT-3′; sense strand β-actin, 5′-TC ATGAAGTGTGACGTTGACATCCGT-3′, anti-sense strand β-actin, 5′-CCTAGAAGCATTTGCGGTGCACG ATG-3′. After amplification, portions of the PCR reactions were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

Nuclear extraction and electrophoretic mobility shift assay

RAW 264.7 macrophages were plated in 100-mm dishes (1×10^6 cells). The cells were treated with various concentrations of MEMO (40, 60 and 80 μg mL^{-1}), stimulated with LPS for 1 h, washed once with phosphate-buffered saline, scraped into 1 mL of cold phosphate-buffered saline, and pelleted by centrifugation at 500 *g*. Nuclear extracts were prepared as described previously with slight modifications (Kim et al 2003). The cell pellet was resuspended in hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonylfluoride, 0.5 mM dithiothritol, 10 μg mL^{-1} aprotinin) and

incubated on ice for 15 min. The cells then were lysed by the addition of 0.1% Nonidet P-40 and vigorous vortexing for 10 s. The nuclei were pelleted by centrifugation at 12 000 *g* for 1 min at 4°C and resuspended in high-salt buffer (20 mM HEPES pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM Na fluoride, 1 mM sodium vanadate). A total of 10 μg of nuclear extract was mixed with the double-stranded NF-κB oligonucleotide: 5′-AGTTGAGGGGACTTTCCCAGGC-3′ end-labelled by [γ-^{32}P]dATP (the underlined section indicates a κB consensus sequence or a binding site for NFκB/cRel homodimeric and heterodimeric complex). Binding reactions were performed at 37°C for 30 min in 30 μL of reaction buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% glycerol, 1 μg of poly(dI-dC) and 1 mM dithiothreitol. The specificity of binding was examined by competition with the 80-fold unlabelled oligonuclotide. DNA–protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 100 V in 0.5 × TBE buffer. The gels were vacuum-dried for 1 h at 80°C and exposed to X-ray film at −70°C for 24 h.

Carrageenan-induced hind-paw oedema in rats

The initial hind paw volume of Sprague-Dawley rats was determined volumetrically. A 1% solution of carrageenan in saline (0.1 mL per rat) was injected subcutaneously into the right hind paw 1 h after the test substances had been administered orally. The control group received the vehicle. Paw volumes were measured for up to 5 h at intervals of 1 h, and the volume of the oedema was measured with a plethysmometer. Ibuprofen was used as a positive control (Yang et al 1996).

Abdominal constriction test in mice

The acetic acid-induced abdominal constriction test was performed as described by Hayashi & Takemori (1971). Vehicle, aspirin (100 mg kg^{-1}) and test solution (100 and 200 mg kg^{-1}) were orally administered 30 min before the experiment, and 0.1 mL/10 g of 0.7% acetic acid-saline was then injected intraperitoneally. At 10 min after the injection, the frequency of abdominal constriction in mice was counted for the next 20 min.

Hot-plate test

The hot-plate test was used to measure the response latencies according to the method described previously by Eddy & Leimback (1953), with minor modifications. In these experiments, a transparent plastic cylinder (14 cm diameter, 20 cm height) was used to confine the mouse on the heated (56 ± 1°C) surface on the hot plate (model-DS 37; Ugo Basile, Comerio, VA, Italy). The reaction time was noted by observing either the licking of the hind paws or the jumping movements 1 h before and 1 h after the administration of vehicle and test solution (100 and 200 mg kg^{-1}). Five groups of 10 mice per group were used. The cut-off time was 20 s. Morphine sulfate (10 mg kg^{-1}, i.p.; Kuju Pharmaceutical Co., Seoul, Korea) was used as reference drug (Yang et al 1996).

Statistical analysis

All data are expressed as mean ± s.d. of n animals in the in-vivo experiments. Statistical analysis was performed by analysis of variance for multiple comparisons followed by Dunnett's test. In-vitro experiments were performed three times and analysed using a non-parametric multiple comparisons test (Kruskal–Wallis test) followed by Dunn's test. Statistical significance was set at $P < 0.05$.

Results

Effect of MEMO on LPS-induced NO and PGE_2 production

To determine the effect of MEMO on LPS-induced NO production in RAW 264.7 cells, cells were treated with or without MEMO for 1 h before being treated with LPS (1 μg mL^{-1}) for 24 h. LPS and MEMO were not added to the control group. Cell culture medium was then harvested, and NO levels were determined using the Griess reaction. LPS induced 100-fold more NO compared with the control group, and MEMO inhibited this NO production in a concentration-dependent manner over the concentration range 40–80 μg mL^{-1} with an IC50 of 61.83 μg mL^{-1} (Figure 1A). L-N^6-(1-iminoethyl) lysine (IC50 1.8 μg mL^{-1}) was used as a positive inhibitor of NO production. To determine if MEMO inhibits PGE_2 production, RAW 264.7 cells were pre-incubated with MEMO (40, 60 or 80 μg mL^{-1}) for 1 h, and then stimulated with 1 μg mL^{-1} LPS for 24 h. As shown in Figure 1B, PGE_2 production was significantly inhibited by MEMO in a concentration-dependent manner. Cytotoxicity of MEMO was evaluated in the presence or absence of LPS by using the MTT assay. MEMO did not affect the cell viability of RAW 264.7 cells in either the presence or absence of LPS even at a dose of 200 μg mL^{-1} after a period of 24 h (data not shown).

Inhibition of LPS-induced iNOS and COX-2 protein and mRNA expression by MEMO

Western blot analysis and reverse transcription PCR were performed to determine if the inhibitory effects of the pro-inflammatory mediators NO and PGE_2 are related to the expressional modulations of iNOS and COX-2. Neither iNOS or COX-2 protein nor mRNA were detectable in unstimulated RAW 264.7 cells. In response to LPS, iNOS expression was markedly augmented and pre-treatment for 1 h with MEMO significantly inhibited iNOS protein induction in a concentration-dependent manner (Figure 2A). Pre-incubation with 60 μM MEMO resulted in a marked reduction of LPS-induced iNOS expression (Figure 2A). Moreover, reverse transcription PCR analysis showed that the amount of iNOS mRNA was correlated with its protein level (Figure 2B). A similar pattern was observed when we

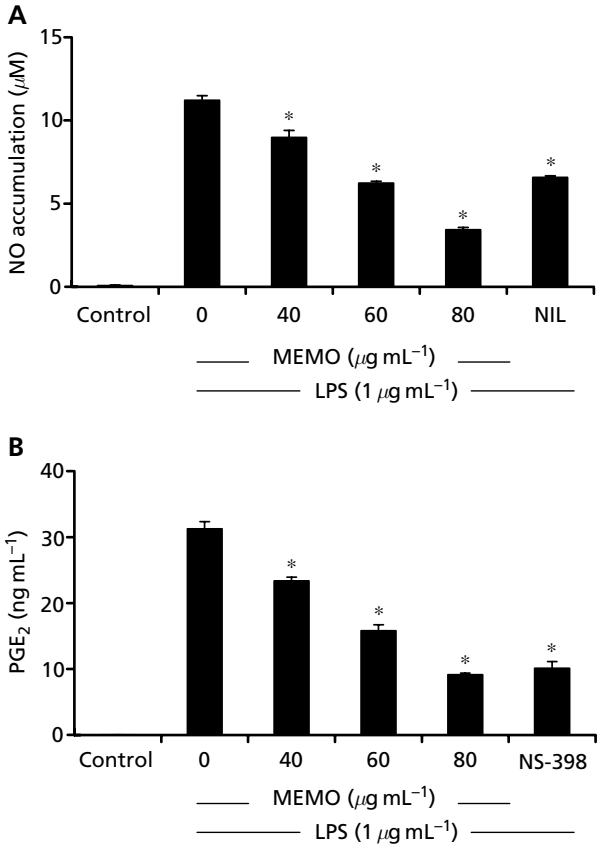

Figure 1 Effects of the methanol extract of the roots of *Morinda officinalis* (MEMO) on (A) nitric oxide (NO) and (B) prostaglandin E_2 (PGE_2) production in lipopolysaccharide (LPS)-induced RAW 264.7 cells. Cells were pre-treated for 1 h with different concentrations (40, 60 or 80 $\mu g\,mL^{-1}$) of MEMO, and then LPS (1 $\mu g\,mL^{-1}$) was added and the cells were incubated for 24 h. Control values were obtained in the absence of LPS and MEMO. L-N^6-(1-iminoethyl) lysine (10 μM; NIL) or NS-398 (10 μM) was used in the assay as a positive control. The values shown are the means ± s.d. of three independent experiments. $^*P < 0.05$ compared with LPS alone.

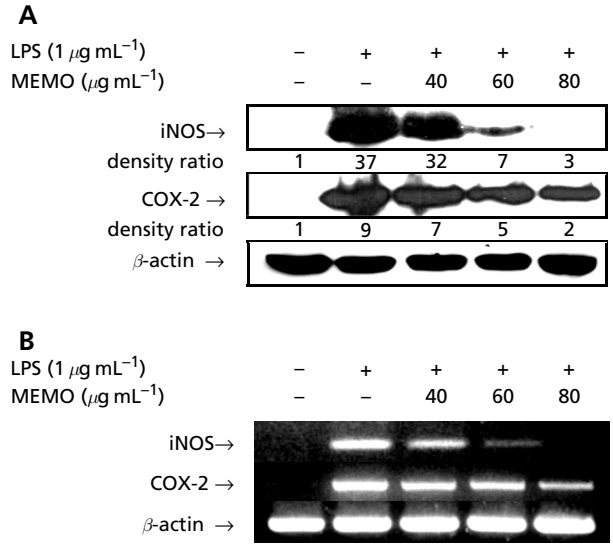

Figure 2 Effects of the methanol extract of the roots of *Morinda officinalis* (MEMO) on lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein, and mRNA expression in RAW 264.7 cells. A. Lysates were prepared from control or LPS-stimulated cells (1 $\mu g\,mL^{-1}$ LPS for 24 h) alone or after pre-treatment with MEMO (40, 60 or 80 $\mu g\,mL^{-1}$) for 1 h. All lanes contained 40 μg of total protein. A representative immunoblot from three separate experiments is shown. B. Cells were lysed and total RNA was isolated for reverse transcription polymerase chain reaction (PCR) analysis of gene expression. The cDNA (10 ng) of RAW 264.7 macrophages pre-treated with various concentrations of MEMO (40, 60 or 80 $\mu g\,mL^{-1}$) for 1 h and subsequently treated or not treated with LPS (1 $\mu g\,mL^{-1}$) for 4 h were subjected to reverse transcription PCR and the mRNA levels of iNOS and COX-2 were compared. PCR of β-actin was performed to confirm that the initial cDNA content of the samples was similar. The experiment was repeated twice and similar results were obtained.

examined the effect of MEMO on LPS-induced COX-2 expression; densitometric analysis demonstrated that COX-2 protein expression induced by LPS was inhibited by 55.6% in cells treated with 60 μM MEMO (Figure 2A). Under the same conditions, COX-2 mRNA levels were also significantly decreased in a similar way (Figure 2B). MEMO did not affect the expression of the housekeeping gene β-actin. In general, these results are consistent with the observed inhibitory effects of MEMO on NO and PGE_2 release (Figure 1).

Effects of MEMO on LPS-induced TNF-α production and mRNA expression

To determine the effect of MEMO on LPS-induced TNF-α release, we investigated its effect on TNF-α using EIA and reverse transcription PCR. Pre-treating RAW 264.7 cells with MEMO reduced TNF-α production and mRNA expression in a concentration-dependent manner (Figure 3A, B).

Effects of MEMO on LPS-induced NF-κB DNA binding activity, IκB-α degradation and p65 nuclear translocation

To further investigate the mechanism of MEMO-mediated inhibition of iNOS, COX-2 and TNF-α transcription, we focused on NF-κB, which is known to transactivate iNOS, COX-2, TNF-α and other genes (Baeuerle 1998). Electrophoretic mobility shift assay demonstrated that LPS-induced NF-κB DNA binding activity in RAW 264.7 macrophages is significantly reduced by MEMO treatment in a concentration-dependent manner (Figure 4A). Moreover, an excess of unlabelled NF-κB oligonucleotide completely inhibited in-vitro NF-κB-DNA binding activity. To determine if MEMO inhibits NF-κB via IκB-α degradation in RAW 264.7 cells, cytosolic extracts were prepared and assayed for IκB-α degradation by Western blotting. As shown in Figure 4B, MEMO inhibited LPS-induced IκB-α degradation in a concentration-dependent manner. In

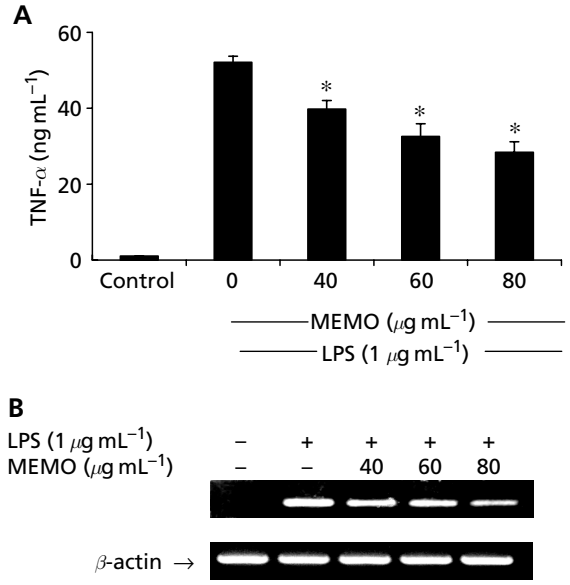

Figure 3 Effect of the methanol extract of the roots of *Morinda officinalis* (MEMO) on lipopolysaccharide (LPS)-induced TNF-α release in RAW 264.7 cells. A. Cells were pre-treated for 1 h with MEMO (40, 60 or 80 μg mL^{-1}) and then LPS (1 μg mL^{-1}) was added and the cells were incubated for 24 h. Control values were obtained in the absence of LPS and MEMO. The values are the means $\pm$ s.d. from three independent experiments. $^*P < 0.05$ compared with LPS alone. B. Total RNA was prepared as described in Figure 2B. TNF-α specific sequences (351 bp) were detected by agarose gel electrophoresis. Polymerase chain reaction of β-actin was performed to confirm that the initial cDNA content of the samples was similar. The experiment was repeated three times and similar results were obtained.

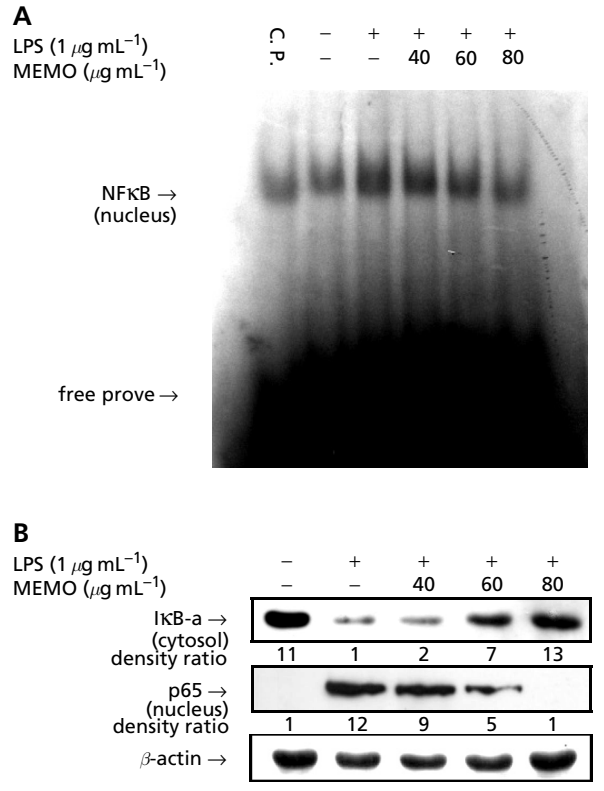

Figure 4 Inhibition of nuclear factor kappa B (NF-κB) DNA binding activity, inhibitor kappa B-α (IκB-α) degradation, and the nuclear translocation of p65 by the methanol extract of the roots of *Morinda officinalis* (MEMO). A. Nuclear extracts prepared from control or 24 h LPS (1 μg mL^{-1})-stimulated cells alone or after pre-treatment with MEMO (40, 60 or 80 μg mL^{-1}) for 1 h were prepared and analysed for NF-κB binding by electrophoretic mobility shift assay. The specificity of binding was examined by competition with an 80-fold cold probe (C.P.). B. RAW 264.7 macrophages were pre-treated with MEMO (40, 60 and 80 μg mL^{-1}) and then LPS (1 μg mL^{-1}) was added and incubated for 24 h. For each condition, the protein level of IκB-α in cytosolic fractions and the p65 level in the nuclear protein fractions were determined by Western blotting. The experiment was repeated three times and similar results were obtained.

addition, we investigated if MEMO prevented the translocation of the subunit of NF-κB, p65 from the cytosol to the nucleus after its release from IκB. In the case of the treatment with MEMO, a decrease in the level of p65 in the nuclear fraction was detected in a concentration-dependent manner by Western blot analyses (Figure 4B).

In-vivo anti-inflammatory and antinociceptive activity of MEMO

We examined the anti-inflammatory effect of MEMO using the carrageenan-induced oedema model, and observed maximal oedema inhibition 3 h after oedema induction. In particular, treatment with MEMO (100 and 200 mg kg^{-1}, p.o.) reduced the oedema rate by 24.8% and 34.4%, respectively, at 3 h; ibuprofen (100 mg kg^{-1}, p.o.) treatment decreased the oedema rate by 52.0% at 3 h (Table 1). This reduction remained statistically significant for 2–4 h after oedema induction.

The antinociceptive effects of the test samples were determined in mice using the acetic acid-induced abdominal constriction test and the hot-plate test. In the case of the acetic acid-induced abdominal constriction test, MEMO showed antinociceptive activity after oral administration (100 and 200 mg kg^{-1}). Aspirin (100 mg kg^{-1}) also exerted a significant protective effect (Table 2). The results of the hot-plate test in mice (Table 2) showed that MEMO at doses of 100 and 200 mg kg^{-1} significantly increased the latency of the jumping response but did not affect the animal's ability to detect pain. Morphine (10 mg kg^{-1}, i.p) was used as a positive control in the hot-plate test.

Discussion

In the present study, we evaluated the effects of MEMO on LPS-induced pro-inflammatory molecules, including NO, PGE_2 and TNF-α. We found that LPS-induced NO, PGE_2 and TNF-α production is inhibited by MEMO in a concentration-dependent manner. To further explore the possible mechanism of this inhibition by

Table 1 Inhibitory effect of the methanol extract of the roots of *Morinda officinalis* (MEMO) on carrageenan-induced oedema in the hind paws of rats

Group	Dose (mg kg^{-1})	Swelling volume (mL)				
		1 h	2 h	3 h	4 h	5 h
Control		1.32 ± 0.08	2.38 ± 0.06	2.94 ± 0.05	2.38 ± 0.03	1.90 ± 0.05
MEMO	100	1.28 ± 0.04	1.96 ± 0.07*	2.21 ± 0.06*	1.83 ± 0.04*	1.87 ± 0.06
	200	1.23 ± 0.06	1.53 ± 0.03*	1.93 ± 0.03*	1.64 ± 0.06*	1.58 ± 0.07*
Ibuprofen	100	0.78 ± 0.04*	1.16 ± 0.06*	1.41 ± 0.05*	1.22 ± 0.08*	0.93 ± 0.04*

Values are expressed as mean ± s.d. The number of rats in each group was 10. *$P < 0.05$ compared with control.

Table 2 Antinoceptive effect of the methanol extract of the roots of *Morinda officinalis* (MEMO) on stretching episodes in the acetic acid-induced abdominal constriction test and action time in the hot-plate test in mice

Group	Dose (mg kg^{-1}, p.o.)	Stretching episodes (counts/20 min)	Action time (s)
Control		60.6 ± 2.0	8.7 ± 1.3
MEMO	100	46.8 ± 2.4*	13.8 ± 1.3*
	200	37.2 ± 1.5*	15.3 ± 1.3*
Aspirin	100	19.0 ± 1.6*	–
Morphine	10	–	22.8 ± 2.0*

Values are expressed as mean ± s.d. The number of mice in each group was 10. *$P < 0.05$ compared with control.

MEMO, the expression levels of iNOS and COX-2 protein, and iNOS, COX-2 and TNF-α mRNA were examined. The inhibition of the LPS-stimulated expression of these molecules by MEMO in RAW 264.7 cells was not attributed to cytotoxicity, as assessed by MTT assay and β-actin housekeeping gene expression. The inhibition of iNOS and COX-2 gene expression was evidenced by reductions in their mRNA levels, and these occurred simultaneously in a concentration-dependent manner. Thus, the inhibition of NO and PGE_2 release may be attributed to the suppression of iNOS and COX-2 mRNA transcription followed by protein expression.

NF-κB is known to play a critical role in the regulation of genes involved in cell survival, and to coordinate the expression of pro-inflammatory enzymes such as iNOS, COX-2 and TNF-α (Xie et al 1993; Xie et al 1994; Roshak et al 1996; Schmedtje et al 1997). Therefore, we examined the DNA binding activity of NF-κB to confirm that the inhibition of iNOS, COX-2 and TNF-α was influenced by the NF-κB signalling pathway. Our results indicate that the DNA binding activity of NF-κB is inhibited in a concentration-dependent manner by MEMO (Figure 4), and this was similar for the inhibition of iNOS, COX-2 and TNF-α expression. NF-κB is associated with an inhibitory subunit called IκB, which is present in the cytoplasm in an inactive form; moreover, NF-κB is tightly controlled by IκB. However, IκB is phosphorylated and its subsequent proteolysis allows NF-κB translocation to the nucleus, where it activates the transcription of NF-κB-responsible genes (Henkel et al 1993). We also examined the effect of MEMO on IκB-α degradation, and found that MEMO exerted an inhibitory effect on the LPS-induced degradation of IκB-α in a concentration-dependent manner (Figure 4B). Corresponding with this result, the translocation of the p65 subunit in the nucleus was blocked in a concentration-dependent manner (Figure 4B).

While investigating the anti-inflammatory and antinociceptive effects of MEMO in-vivo, we found that MEMO slightly decreased the oedema induced by carrageenan, in which the peak in oedema is characterized by the presence of PGs (Yang et al 1996).

The antinociceptive effects of MEMO were assayed using the acetic acid-induced abdominal constriction test and the hot-plate test in mice. MEMO was found to significantly inhibit the acetic acid-induced abdominal constriction response at doses of 100 and 200 mg kg^{-1}. A significant response in the abdominal constriction model is also related to the sensitization of nociceptive receptors to PGs (Koppert et al 2004). Therefore, it is possible that MEMO exerts a peripheral analgesic effect, probably by inhibiting the synthesis or the action of PGs. In order to distinguish between the peripheral and central analgesic action, the hot-plate test was used to examine the antinociceptive effect in mice, since it is known that centrally acting analgesic drugs elevate the pain threshold of mice towards heat. It was found that 100 and 200 mg kg^{-1} MEMO significantly increased the hot-plate reaction time in mice. The antinociceptive effects of MEMO in these models indicate that MEMO may possess peripherally and centrally mediated antinociceptive properties.

MEMO exhibited anti-inflammatory and antinoceptive effects, as demonstrated by the carrageenan-induced oedema model, and by the abdominal constriction and hot-plate tests. The extract was further fractionated into $CHCl_3$, EtOAc or BuOH extracts for activity-guided fractionation; the chloroform fraction of *M. officinalis* was found to be the most active in the inhibition of NO and PGE_2 production (data not shown). The activity-guided fractionation led to the isolation of three iridoid glycosides: geniposidic acid, deacetylasperulosidic acid and monotropein. The structures of these compounds were fully elucidated on the basis of spectroscopic methods and confirmed by comparison of their spectroscopic data with those reported in the literature (Boros & Stermitz 1990; Ling et al 2002; Lee et al 2004). Since *Gardeniae Fructus*, one of the important Chinese medicines that contains iridoid glycosides such as gardenoside, shanzhiside and methyl deacetylasperuloside, has been used for the treatment of rheumatoid arthritis (Han 2001), further pharmacological examination on the iridoid glycosides isolated from *M. officinalis* will provide clearer information on the active components.

Conclusion

We found that MEMO is a potent inhibitor of LPS-induced NO, PGE_2 and TNF-α production at the gene expression level, and the inhibition was found to be caused by the blocking of NF-κB activation in RAW 264.7 macrophages. In addition, our results demonstrate that MEMO has anti-inflammatory and antinociceptive effects in animals. We conclude that MEMO appears to have potential as an analgesic for the treatment for inflammatory disease.

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