JPP Journal of Pharmacy and Pharmacology

ROYAL PHARMACEUTICAL SOCIETY

JPP Journal of Pharmacy and Pharmacology

JPP 2011, 63: 1211–1218

Received October 13, 2010
Accepted June 15, 2011
DOI
10.1111/j.2042-7158.2011.01329.x
ISSN 0022-3573

Research Paper

Myrrh mediates haem oxygenase-1 expression to suppress the lipopolysaccharide-induced inflammatory response in RAW264.7 macrophages

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Abstract

Objectives To elucidate a novel anti-inflammatory mechanism of myrrh against lipopolysaccharide (LPS)-induced inflammation.

Methods RAW264.7 macrophages were cultured in DMEM and then cells were treated with LPS or LPS plus a myrrh methanol extract (MME) for 24 h. The culture medium was collected for determination of nitric oxide (NO), prostaglandin (PG)E_2, interleukin (IL)-1β, and tumour necrosis factor (TNF)-α, and cells were harvested by lysis buffer for Western blot analysis.

Key findings Our data showed that treatment with the MME (1~100 μg/ml) did not cause cytotoxicity or activate haem oxygenase-1 (HO-1) protein synthesis in RAW264.7 macrophages. Furthermore, the MME inhibited LPS-stimulated NO, PGE_2, IL-1β and TNF-α release and inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 protein expression. Zn(II) protoporphyrin IX, a specific inhibitor of HO-1, blocked the inhibition of iNOS and COX-2 expression by the MME.

Conclusions These results suggest that among mechanisms of the anti-inflammatory response, the MME inhibited the production of NO, PGE_2, IL-1β and TNF-α by downregulating iNOS and COX-2 gene expression in macrophages and worked through the action of HO-1.

Keywords cyclooxygenase-2; haem oxygenase-1; inducible nitric oxide synthase; lipopolysaccharide; myrrh

Introduction

Myrrh (*Commiphora mukul*, called *mo yao* in Chinese), is a reddish-brown resinous exudate obtained from the Burseraceae family and has been used for a long time in the traditional medicines of China, India, Rome, Greece, etc.[1,2] For over 2000 years, myrrh has been used as a natural medicine to treat conditions such as inflammation, skin infection, pain, diarrhoea and periodontal diseases.[3] Myrrh is widely used today in Chinese traditional medicine as it activates the blood circulation, transforms blood stasis, alleviates pain, reduces swelling and aids regeneration of damaged tissues.[2] As to the chemical component analysis of myrrh, it consists of approximately 3~8% volatile oils, 30~60% water-soluble gum and 20~40% alcohol-soluble resin.[4,5] The volatile oils contain steroids, sterols and terpenes, and furanosesquiterpenes provide the main characteristic odour of myrrh.[6] Myrrh was demonstrated to possess efficacious biological actions, such as antibacterial,[7] antifungal,[7] anti-ulcer,[8] antioxidant,[9] anti-inflammatory,[10,11] anti-tumour,[12] anti-atherosclerotic,[13] anti-hypercholesterolaemic,[14–16] anti-hypertensive,[17] anti-obesity[18] and antidiabetic properties.[19]

Overall, myrrh has a broad-spectrum of activity as indicated above, some of which possibly act through an anti-inflammatory mechanism. The anti-inflammatory activity of myrrh and its components, such as myrrhanol A, myrrhanone A and guggulsterone, have been reported,[10,11,19–25] and an understanding of the molecular mechanisms is gradually emerging.

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Mostly, such studies showed that myrrh and its components can suppress inflammation by inhibiting lipopolysaccharide (LPS)-induced inducible nitric oxide (NO) synthase (iNOS) expression in macrophages. In inflammation-related diseases, the suppression of the iNOS/NO pathway is believed to ameliorate inflammatory responses.[26] Prostaglandin (PG)E_2 is another important mediator produced in the inflammatory response that is catalysed by cyclooxygenase (COX)-2 from arachidonic acid metabolites.[27] Expression of the iNOS and COX-2 genes in macrophages is under the control of various transcription factors, such as nuclear factor (NF)-κB.[28] NF-κB is sequestered in the cytoplasm as a heterotrimeric complex by binding to IκB-α. The phosphorylation, ubiquitination and further degradation of IκB-α unmask the nuclear localization signal motif of the NF-κB (p50 and p65) dimer, thereby inducing translocation of NF-κB into the nucleus with subsequent binding to κB-sites on the iNOS and COX-2 genes, consequently initiating gene expression.[29]

Haem oxygenase (HO)-1, a stress-inducible enzyme, is believed to have an important function in reducing inflammatory stimulus-induced tissue injury.[30] It is well known that HO-1 provides cytoprotection against oxidative damage through haem metabolites, biliverdin/bilirubin and carbon monoxide.[31,32] Recent reports demonstrated anti-inflammatory properties for HO-1; notably, upregulation of HO-1 was shown to protect against LPS-induced iNOS expression in RAW264.7 macrophages.[33,34] Some studies indicated that both humans and mice deficient in HO-1 expression display a phenotype of an increased inflammatory state *in vivo*.[35–37]

To date, limited scientific research has indicated that myrrh has anti-inflammatory activity to mediate inhibition of NF-κB-regulated gene production and iNOS expression. However, the precise mechanisms responsible for the inhibition of iNOS by myrrh remain unclear. In this study, we confirmed the anti-inflammatory role of myrrh and demonstrated another mechanism for this anti-inflammatory effect. The goal of this study was to examine the effects of myrrh on LPS-induced NO, PGE_2 and cytokine (IL-1β and TNF-α) production and iNOS and COX-2 expression using RAW264.7 macrophages. In this regard, our data indicate a novel pathway of HO-1 induction by the methanol myrrh extract (MME) and thus the attenuation of LPS-induced inflammation.

Materials and Methods

Plant material

Commercially available myrrh was purchased from a traditional Chinese medicine drug store, and its botanical source was the Burseraceae imported from China. The authenticity of the myrrh was confirmed by Prof. Hui-Wen Cheng of the School of Pharmacy, Taipei Medical University. A voucher of the source was kept at the traditional Chinese medicine drug store, and a voucher specimen was deposited at our laboratory. Myrrh (400 g) was extracted with 1000 ml of methanol for 72 h, and then the extract was filtered and centrifuged at 12 500 rpm for 15 min. The supernatant of the methanol extract was passed through a 0.22-μm sterile filter (Millipore, Billerica, USA) and first concentrated using a Yamato vacuum rotary evaporator (Tokyo, Japan) at 45°C.

Chromatographic analysis of methanol myrrh extract

The high-performance liquid chromatography (HPLC) system consisted of a Shimadzu (Kyoto, Japan) LC-10ATvp liquid chromatograph equipped with a DGU-14A degasser, an FCV-10ALvp low-pressure gradient flow control valve, an SIL-10ADvp auto injector, an SPD-M10Avp diode array detector and an SCL-10Avp system controller. Peak areas were calculated with Shimadzu Class-VP software (version 6.12 sp5). The mobile phase was composed of 0.05% trifluoroacetic acid–acetonitrile (v/v) with gradient elution (0 min, 100 : 0; 10 min, 100 : 0; 40 min, 0 : 100 and 50 min, 0 : 100). A Purospher STAR RP-18e reversed-phase column (250 × 4 mm i.d.) and a Purospher STAR RP-18e guard column (4 × 4 mm i.d.) (Merck, Darmstadt, Germany) were used. The flow-rate was 1.0 ml/min with UV absorbance detection at 280 nm. The analysis involved 10 μl of 2 mg/ml MME solution. The operation was carried out at room temperature (25°C).

Results are expressed in (*Z*)-guggulsterone equivalents per amount of the MME. A calibration curve was created with (*Z*)-guggulsterone (1.25~80 μg/ml, $y = 1.16x + 1.021$, $r^2 = 0.991$). According to the analytical conditions, the MME was quantified to contain 3.21 ± 0.15% *Z*-guggulsterone (w/w). Currently, a 2.5% guggulsterone content is the minimum standard for quality gugulipid preparations. Our determination of the MME was the same as that of a previous report.[3]

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Life Technologies (Gibco, Grand Island, USA). Monoclonal antibodies and a peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). The enzyme immunoassay kits for PGE_2, IL-1β and TNF-α were purchased from R&D System (Minneapolis, USA). Other agents were obtained from Sigma Chemical (St Louis, USA).

Cell culture

RAW264.7 macrophages were purchased from the American Type Culture Collection (Rockville, USA). Cells were cultured in DMEM with 10% FBS, penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml) in a 95% air and 5% CO_2 humidified atmosphere at 37°C.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay for cell viability

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan. Cells (10^4 cells/well) in 96-well plates were incubated with various indicated concentrations of myrrh at 37°C in 5% CO_2 for 24 h. The MTT assay was performed as described previously.[34]

Lactate dehydrogenase release assay for cytotoxicity

MME-induced cytotoxicity leading to plasma membrane damage was measured using a Cytotoxicity Detection Kit

(LDH) (Boehringer Mannheim, Mannheim, Germany). The lactate dehydrogenase (LDH) release assay is widely used in cytotoxicity studies.[38] To determine the maximum LDH release (high control), some cells were solubilized with 1% (w/v) Triton X-100. The spontaneous LDH release (low control) was determined by incubating cells with DMEM. The detailed assay was performed as described previously.[39]

Determination of nitrite production

As an indicator of NO production, the nitrite concentration in the culture medium was determined by Griess reagent performed as described previously.[34]

Measurements of prostaglandin E_2, interleukin-1β and tumour necrosis factor-α

Levels of PGE_2, IL-1β and TNF-α in the RAW264.7 macrophage culture media were measured by enzyme immunoassay (EIA) kits according to the manufacturer's instructions.

Bilirubin determination in culture medium

RAW264.7 macrophages (8×10^5 cells/well) in 6-well plates were treated with or without various concentrations of the MME (100 µg/ml) for 24 h. The supernatant (0.5 ml) of the culture medium was then collected and barium chloride (250 mg) was added. After vortexing, benzene (0.75 ml) was added and mixed again. The benzene phase containing the extracted bilirubin was separated from the aqueous phase by centrifugation at 13 000*g* for 30 min. Bilirubin was determined spectrophotometrically using an absorbance of 450 nm ($\varepsilon 450 = 27.3\ mM^{-1}\,cm^{-1}$).[40]

RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol purchased from Invitrogen (Carlsbad, USA), following the manufacturer's instructions. In total, 5 µg RNA was used as the template for complementary (c)DNA synthesis. Reverse transcription was performed with 4 U/µl M-MLV reverse transcriptase, 1 mM dNTP, 5 mM $MgCl_2$, 0.02 µg/µl oligo(dT) primers, 10 mM DTT, and 1.4 U/µl RNasin Plus RNase inhibitor at 37°C for 1 h. The reaction was stopped at 94°C for 3 min, and the cDNA products were stored at −20°C. The PCR was performed in a thermal cycler (Gene Amp PCR System 9700; Applied Biosystems, Foster City, USA) with 1 mM specific primers, 0.2 mM dNTP, 1 mM $MgCl_2$, and 0.05 U/µl DNA polymerase. Amplification products (HO-1, 364 bp; GAPDH, 600 bp) were resolved by 2.0% agarose gel electrophoresis, stained with 0.5 µg/ml ethidium bromide, and photographed with the BioDoc-ItTM imaging system (Ultraviolet Products, Upland, USA). Forward and reverse primers were as follows: mouse HO-1, forward 5′-TTACCTTCCCGAACATCGAC-3′ and reverse 5′-GCATAAATTCCCACTGCCAC-3′; and mouse GAPDH, forward 5′-TGAAGGTCGGTGTGAACGCATTTG GC-3′ and reverse 5′-TTCTGGGTGGCAGTGATGGC-3′.

Electrophoretic mobility shift assay

NF-κB activation was analysed by electrophoretic mobility shift assay (EMSA) gel shift kit (Panomics, Fremont, USA) according to the manufacturer's instructions. In brief, nuclear extracts (10 µg) were incubated with 2 µl of 5× binding buffer and 1 µg of poly d(I-C) at room temperature for 5 min. Then 1 µl of the biotin-labelled transcription factor probe was added to each sample at 15°C for 30 min. The DNA protein complexes were separated by 6% nondenaturing polyacrylamide gel electrophoresis (PAGE). The transblotted membrane was blocked by incubating it at room temperature with 1× blocking buffer for 15 min. After the blocking buffer was removed, the membrane was incubated with streptavidin-horseradish peroxidase (HRP) at room temperature for another 15 min. The membrane was washed in wash buffer and developed for visualization using Hyperfilm enhanced chemiluminescence (ECL; Amersham, Arlington Heights, USA).

Haem oxygenase activity measurement

HO-1 activity was determined as previously described.[34] Briefly, harvested cells after various treatments were sonicated and centrifuged (18 000*g* for 10 min at 4°C). After removing the supernatant buffer, the pellet was resuspended in potassium phosphate buffer (100 mM) at 4°C. Suspended proteins (1 mg) were incubated with 0.2 ml of the reaction mixture (1 mg protein of mouse liver cytosol, 50 µM haemin, 1 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase, and 100 mM potassium phosphate; pH 7.4) at 37°C for 1 h in the dark. Using 1 ml of chloroform to extract bilirubin, the difference in the absorbances at 464 and 530 nm (extinction coefficient, $40\ mM^{-1}\,cm^{-1}$) was measured. HO-1 activity was expressed as the concentration (nmol) of bilirubin formed per milligram protein per hour.

Preparation of total cell lysates and nuclear and cytosolic extracts

RAW264.7 macrophages (5×10^5 cells/well) were incubated in 6-well plates with or without various concentrations of the MME and the MME plus LPS (100 ng/ml) for 1 or 24 h. Total cell lysates were determined as described previously.[34] The nuclear extract was prepared according to Choy's method.[41] The protein content of cell lysates was determined using a Bio-Rad Protein Assay system.

Western blot analysis

A Western blot analysis was performed as described previously.[34] The intensity of each band was quantified using density analysis software (MetaMorph Imaging System, Meta Imaging Series 4.5, West Chester, USA), and the density ratio represented the relative intensity of each band against controls in each experiment.

Data and statistical analysis

Results of all experiments are expressed as the mean ± standard error (SE) obtained from the number of replicate treatments. Data were analysed by analysis of variance followed by Dunn's post-hoc test for comparisons; $P < 0.05$ was considered statistically significant.

Results

Cell viability of RAW264.7 macrophages with MME treatment was measured by an MTT assay. The cytotoxicity of the

Table 1 Effect of the methanol myrrh extract (MME) on lipopolysaccharide (LPS)-induced inflammatory mediators produced in RAW264.7 macrophages

Treatment	Inflammatory mediators			
	Nitric oxide (μM)	PGE_2 (ng/ml)	IL-1β (pg/ml)	TNF-α (ng/ml)
Control	0.26 ± 0.01	0.35 ± 0.09	19.4 ± 7.8	5.02 ± 1.14
LPS	15.14 ± 1.72	6.56 ± 0.66	225.3 ± 20.6	33.18 ± 4.27
MME 100	0.35 ± 0.04	0.49 ± 0.05	23.0 ± 9.8	6.80 ± 0.63
MME 10/LPS	9.88 ± 1.03*	6.10 ± 0.60	210.0 ± 24.8	30.51 ± 1.46
MME 20/LPS	6.46 ± 1.64**	4.57 ± 0.26*	138.5 ± 15.0*	22.01 ± 1.91
MME 50/LPS	2.75 ± 0.16**	2.88 ± 0.25**	76.8 ± 8.3**	14.67 ± 1.19*
MME 100/LPS	0.69 ± 0.17**	0.79 ± 0.18**	44.1 ± 15.5**	6.94 ± 0.85**

Cells were treated with 10, 20, 50 and 100 μg/ml of the MME for 2 h, followed by 24 h of culture in the presence or absence of 100 ng/ml LPS. The nitrite concentration in the culture medium was determined by Griess reagent. Prostaglandin (PG)E_2, interleukin (IL)-1β and tumour necrosis factor (TNF)-α production was determined by EIA kits, as described in 'Materials and Methods'. Data are expressed as the mean ± standard error from three independent experiments. *$P < 0.05$, **$P < 0.01$ compared with LPS alone.

MME was determined by an LHD release assay. Cells were not significantly injured by treatment with the MME at up to 100 μg/ml, and microscopic examination of cell cultures showed a reversal of LPS-induced alteration in cell morphology after treatment with different concentrations of the MME (10~200 μg/ml) (data not shown). These results suggest that concentrations of <100 μg/ml of the MME were not toxic to RAW264.7 macrophages; however, cells were injured with 200 μg/ml of the MME. Thus, cells were treated with the MME in the concentration range of 10~100 μg/ml for the following experiments.

To assess the effects of the MME on LPS-induced NO, PGE_2, IL-1β and TNF-α production in RAW264.7 macrophages, PGE_2, IL-1β and TNF-α concentrations were determined by EIA kits and nitrite production was assessed by the Griess reagent. Our results showed that LPS stimulation caused a significant accumulation of NO in the culture medium; however, pretreatment with 10, 20, 50 and 100 μg/ml of the MME significantly ($P < 0.01$) attenuated LPS-induced NO production in a dose-dependent manner. PGE_2, IL-1β and TNF-α are inflammatory mediators, and effects of the MME on PGE_2, IL-1β and TNF-α production in LPS-stimulated RAW264.7 macrophages were measured in the culture medium. Similar to NO accumulation, the release of PGE_2, IL-1β and TNF-α significantly decreased in a dose-dependent manner with an increasing concentration (10, 20, 50 and 100 μg/ml) of the MME before LPS treatment of cell cultures Table 1).

Western immunoblot analyses were performed to determine whether the inhibitory effects of the MME on pro-inflammatory mediators, such as NO, PGE_2, IL-1β and TNF-α, were related to changes in the protein levels of iNOS and COX-2 expression. LPS significantly induced the protein expression of iNOS and COX-2, and the MME markedly inhibited expression of these proteins in a dose-dependent manner (Figure 1). For quantitative analysis, β-actin levels were used as an internal standard with results that showed comparable densities among the cell samples. The density ratio of iNOS and COX-2 was significantly ($P < 0.01$) suppressed by the MME in LPS-induced RAW264.7 macrophages. To elucidate the pathway underlying the inhibition of iNOS protein expression by the MME, cytoplasmic IκB-α, phospho-NF-κB, and nuclear NF-κB levels were determined by Western blotting. As shown in Figure 1, IκB-α was degraded after treatment with LPS in RAW 264.7 cells for 1 h, and this degradation was markedly inhibited by pretreatment with the MME (10, 20, 50 and 100 μg/ml) for 2 h. Consistently, the MME had significant inhibitory effects on LPS-induced cytoplasmic phospho-NF-κB and nuclear NF-κB expression in a dose-dependent manner. In the EMSA, LPS markedly enhanced the binding activity of NF-κB in the nucleus, and NF-κB activation was suppressed by pretreatment with the MME, as shown in Figure 2. Collectively, these results suggest that the MME inhibited NF-κB phosphorylation by preventing IκB-α degradation.

Interestingly, as shown in Figure 3a, the MME significantly increased HO-1 protein expression in a concentration-dependent manner. However, the MME did not induce nuclei Nrf2 activation. Moreover, the bilirubin production of the cell-culture medium insignificantly increased in response to the MME (Figure 3b). To determine whether HO-1 expression induced by the MME was due to changes in the mRNA level or protein synthesis, either actinomycin D (ActD), a transcription inhibitor, or cyclohexamide (CHX), a protein synthesis inhibitor, was administered before treatment with the MME. As shown in Figure 3c, 0.3 μg/ml CHX blocked MME-induced HO-1 protein expression whereas 50 ng/ml ActD did not. Similarly, the HO-1 mRNA expression level did not increase after treatment with 10, 20, 30, 50 and 100 μg/ml MME for 3 h (Figure 3d). These findings indicate that the MME increased HO-1 expression at the level of protein synthesis. Taken together, the MME increased HO-1 protein expression in unstimulated RAW264.7 macrophages. In addition, as shown in Figure 4a, the MME induced HO-1 expression and markedly suppressed the expression of iNOS, COX-2 and phospho-NF-κB in macrophages. Therefore, we assumed that this effect was well correlated with HO-1 induction. To elucidate this effect, an inhibitor of HO-1 activity, ZnPP, was used as co-treatment with the MME in LPS-stimulated RAW264.7 cells. The data showed that the reduction in iNOS, COX-2 and phospho-NF-κB protein expression by the MME was reversed by treatment with ZnPP. In contrast, ZnPP did not suppress MME-induced HO-1 protein expression; however, ZnPP reduced MME-induced HO-1

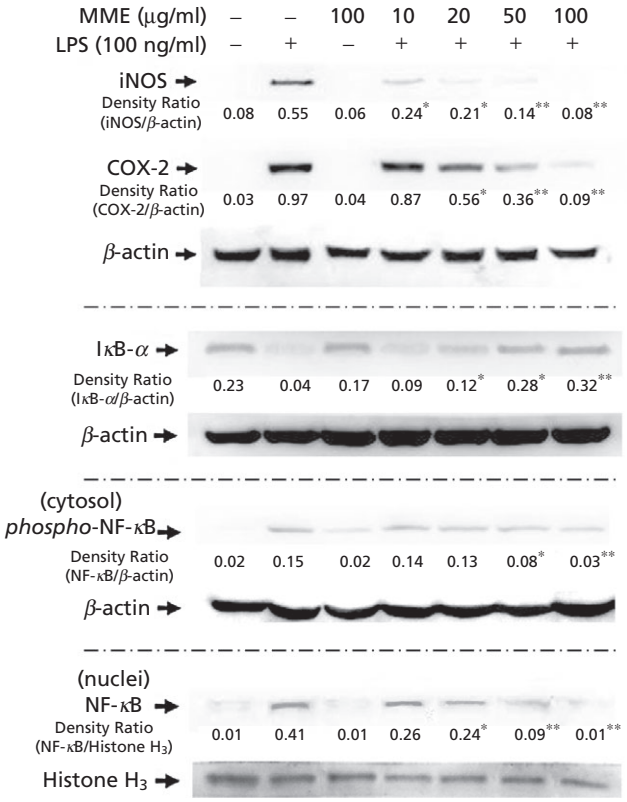

Figure 1 Effect of the methanol myrrh extract (MME) on lipopolysaccharide (LPS)-induced iNOS, COX-2 and IκB-α degradation and NF-κB activation of protein expression. Cells (5×10^5 cells/well) were pretreated with the indicated concentrations of the MME for 2 h before incubation with 100 ng/ml LPS for 1 or 24 h. Total cell lysates (25 μg) were analysed on 10% SDS-PAGE for anti-iNOS, COX-2 and β-actin. Cytosolic lysates (30 μg) were analysed on 10% SDS-PAGE for anti-IκB-α, phospho-NF-κB and β-actin. Nuclear extracts (20 μg) were analysed on 10% SDS-PAGE for anti-NF-κB and histone H_3. iNOS, COX-2 and phospho-NF-κB expression, IκB-α degradation and nuclear NF-κB expression were quantified by a densitometric analysis, and the relative density was calculated as the ratio of iNOS, COX-2 and phospho-NF-κB expression and IκB-α degradation, or nuclear NF-κB expression to β-action or histone H_3 expression. Data are expressed as the mean ± SE from five independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, compared with LPS treatment alone.

activity, as shown in Figure 4b. Taken together, these results powerfully support the hypothesis that activation of HO-1 mediates the inhibitory effects of the MME in LPS-induced inflammation.

Discussion

Our results suggest that the MME certainly has some effects on LPS-stimulated inflammation in RAW264.7 macrophages. In response to LPS or cytokines, the induction of iNOS in macrophages subsequently led to a substantial increase in NO production, which may play an important role in promoting inflammatory conditions.[42] The production of PGE_2 and some cytokines, such as TNF-α, IL-1β, and IFN-γ, plays a crucial role in modulating immune responses resulting from

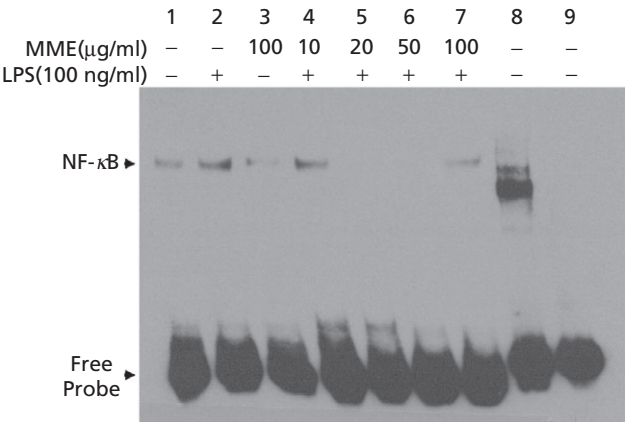

Figure 2 Electrophoretic mobility shift assay (EMSA) for the binding of NF-κB in nuclear extracts isolated from RAW264.7 macrophages. Cells were treated with the indicated concentrations of the methanol myrrh extract (MME) for 2 h followed by stimulation with lipopolysaccharide (LPS) for 1 h. Lanes 1–7, labelled EMSA probe with treated samples (10 μg). Lane 8, labelled EMSA probe with positive control sample of 4 μg (nuclear extract prepared from the HeLa cell line). Lane 9, cold and labelled EMSA probe with the positive control.

inflammatory stimuli. For example, excessive production of these mediators is detected in septic and haemorrhagic shock, rheumatoid arthritis, chronic hepatitis, etc. Through future drug development, targeting the iNOS gene, blocking LPS-binding receptors, or decreasing activities of other enzymes involved in inflammatory processes may have potential therapeutic effects in treating septic shock and many other inflammatory and infectious disorders.[43]

According to our results, the MME suppressed the production of NO, PGE_2, IL-1β and TNF-α, and decreased iNOS and COX-2 protein expression levels in LPS-induced RAW264.7 macrophages. In addition, it is well known that LPS induces inflammatory responses through NF-κB activation, which in turn regulates the expression of iNOS and COX-2 in macrophages.[44] Under normal conditions, NF-κB cannot be translocated into the nucleus due to inactivation by IκB-α.[45] However, with LPS stimulation, IκB-α is phosphorylated and then targeted for ubiquitination and degradation, so that the activated NF-κB will then be able to be translocated into the nucleus.[46] Our data showed that MME-induced HO-1 expression was correlated with suppression of the LPS-induced inflammatory response. ZnPP, an HO-1 activity inhibitor, reversed MME-induced HO-1 activation and the anti-inflammatory action. In principle, HO-1 is understood to play a vital role in cytoprotection and anti-inflammatory responses in a variety of immune-mediated inflammatory diseases.[32] Over the past several decades, the beneficial role of HO-1 has been highlighted. More recently, it was reported that the anti-inflammatory effects of some polyphenols (such as curcumin, caffeic acid phenethyl ester and rosolic acid), sulforaphanes, statins and antineoplastic compounds were attributable to upregulation of HO-1.[47] Although some signalling pathways (such as MAP kinase, activating transcription factor 2 and the transcription factor, NF-E2-related factor 2 (Nrf2), etc.) are thought to lead to HO-1 induction, we found that MME-induced HO-1 expression was eliminated by treatment with

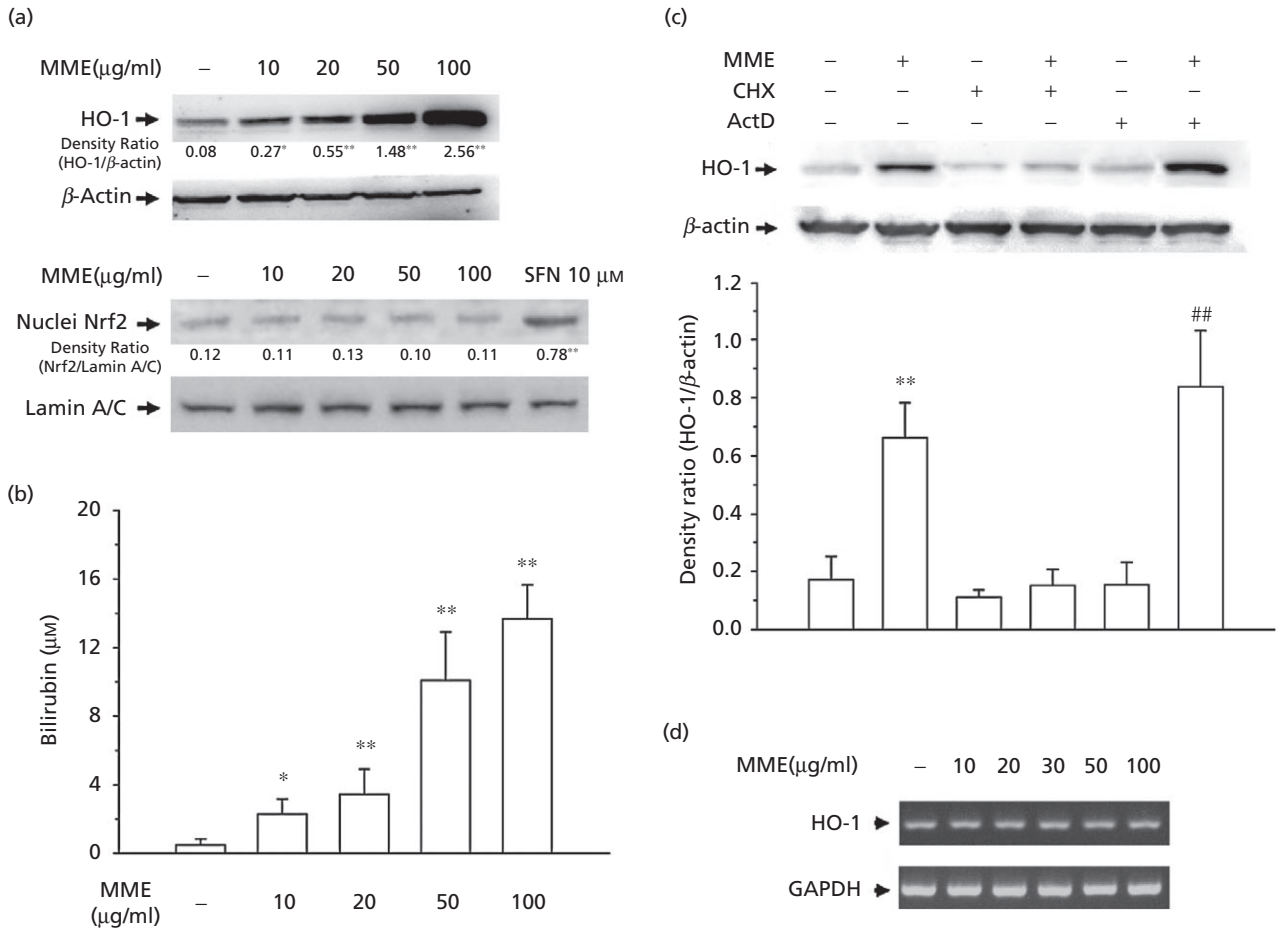

Figure 3 Effect of the methanol myrrh extract (MME) on RAW264.7 macrophages. (a) The MME induced HO-1 and nuclear Nrf2 expressions in unstimulated RAW264.7 macrophages. β-Actin was used as a loading control. The relative density was calculated as the ratio of HO-1 and nuclear Nrf2 expression to β-actin and lamin A/C expression. Sulforaphane (SFN) (10 μM) was used as a positive control. $*P < 0.05$, $**P < 0.01$ compared with control. (b) The MME induced bilirubin production in cell-culture medium for 24 h. $*P < 0.05$, $**P < 0.01$ compared with control. (c) Effect of CHX or ActD on MME-induced HO-1 protein expression. $**P < 0.01$ compared with control; $\#\#P < 0.01$ compared with ActD alone. (d) The MME induced HO-1 mRNA expression in unstimulated RAW264.7 macrophages.

CHX (an inhibitor of protein synthesis) but not ActD (an inhibitor of transcription). These results suggest that MME-induced HO-1 protein expression occurs on a protein synthesis stage. Which components of the MME induce HO-1 expression in RAW264.7 cells? As described in the 'Introduction', myrrh is an oleo-gum resin containing 2~8% volatile oils and also contains many constituents, such as terpenoids and steroids. In this study the MME was examined by HLPC, which confirmed that it contained 3.21% (*Z*)-guggulsterone. We also found that (*Z*)-guggulsterone can induce HO-1 expression and inhibit LPS-induced iNOS and COX-2 expression (data not shown). Therefore, we can say at least that (*Z*)-guggulsterone is involved in MME-induced HO-1 expression.

HO-1 catalyses the rate-limiting step in the degradation of haem and produces CO, free iron and biliverdin (which is rapidly converted to bilirubin). Increasing numbers of reports have found that CO and biliverdin/bilirubin are beneficial compounds that may play a major role in counteracting anti-inflammatory effects.[48] Since the MME induced the expression of HO-1 in RAW264.7 macrophages, the anti-inflammatory actions of the MME might be mediated by the products of HO-1, such as CO or biliverdin/bilirubin. Based on these results, we theorized that MME-induced HO-1 expression possibly inhibited LPS-stimulated expression of the iNOS and COX-2 genes through inactivation of NF-κB. However, we cannot exclude the possibility that the anti-inflammatory effects of MME may involve the inhibition of other transcription factors or may occur through different pathways.

Conclusions

In this study, we demonstrated an important inhibitory effect of the MME via HO-1 induction in LPS-stimulated macrophages for the first time. Although the molecular basis for the anti-inflammatory activity of myrrh's constituents has not been entirely elucidated, inhibition of iNOS and COX-2 expression by MME-induced HO-1 expression may, in part, delineate a potential anti-inflammation pathway. Therefore,

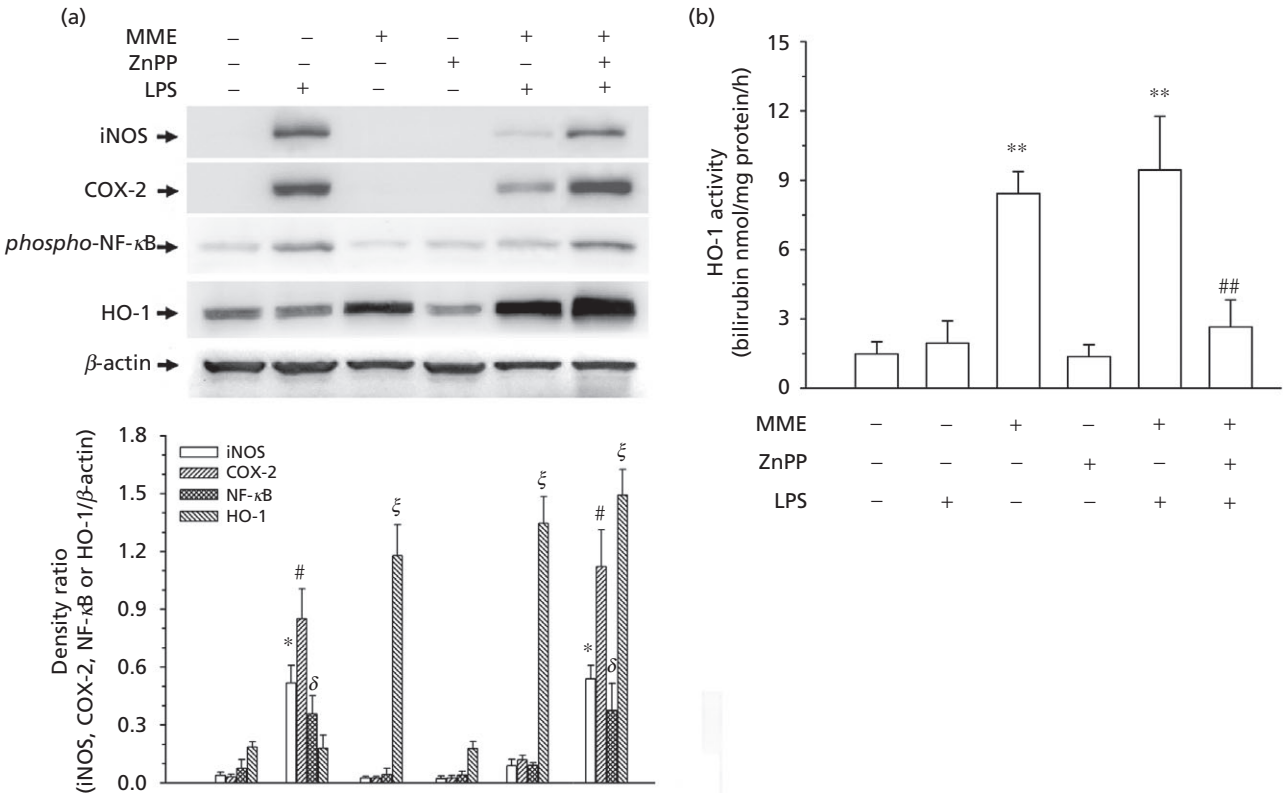

Figure 4 Induction of HO-1 protein mediates the suppression of iNOS, COX-2 and phospho-NF-κB expression in RAW264.7 macrophages following prolonged treatment with the methanol myrrh extract (MME). (a) Effect of ZnPP on MME-induced HO-1 protein expression. $*P < 0.01$, $\#P < 0.01$, $^{\delta}P < 0.01$ and $^{\xi}P < 0.01$ compared with control. (b) Microsomal HO-1 activity was measured by the production of bilirubin from the added haemin upon addition of mouse liver cytosol as described in 'Materials and Methods'. $**P < 0.01$ compared with control cells; $\#\#P < 0.01$ compared with MME plus ZnPP.

myrrh might be a potential therapeutic drug for clinical use in inflammatory diseases.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This work was supported by the Taipei Medical University Hospital, Taiwan, R.O.C. (98TMU-TMUH-09).

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