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Inhibition of lipopolysaccharide-induced expression of inducible nitric oxide synthase and tumor necrosis factor-α by 2′-hydroxychalcone derivatives in RAW 264.7 cells

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Abstract

In cultures of the murine macrophage cell line RAW 264.7, effects of four 2′-hydroxychalcone derivatives, 2′-hydroxy-4′-methoxychalcone (compound 1), 2′,4-dihydroxy-4′-methoxychalcone (compound 2), 2′,4-dihydroxy-6′-methoxychalcone (compound 3) and 2′-hydroxy-4,4′-dimethoxychalcone (compound 4), on lipopolysaccharide (LPS)-induced production of nitric oxide (NO) and tumor necrosis factor (TNF)-α were examined. Compounds 1, 2 and 3 at 3–30 μM inhibited the production with almost the same potency. Compound 4 showed no inhibitory activity. Compounds 1, 2 and 3 at 3–30 μM inhibited the LPS-induced expression of inducible nitric oxide synthase (iNOS) and TNF-α mRNA. To clarify the mechanism involved, effects of compounds 1, 2 and 3 on the activation of nuclear factor (NF)-κB and activator protein-1 (AP-1) were examined. Both the LPS-induced activation of NF-κB and AP-1 were blocked by compounds 1, 2 and 3 at 3–30 μM. Moreover, the three compounds at such concentrations inhibited the LPS-induced IκB degradation and the phosphorylation of c-jun N-terminal kinase (JNK) and c-jun. These findings suggest that the inhibition of the LPS-induced production of NO and TNF-α by the 2′-hydroxychalcone derivatives is due to the inhibition of NF-κB and AP-1 activations.

Keywords: 2′-Hydroxychalcone; Nitric oxide; Tumor necrosis factor; NF-κB; AP-1

1. Introduction

Upon inflammatory stimulation, macrophages produce NO, prostanoids and proinflammatory cytokines such as interleukin (IL)-1β and TNF-α [1–4]. NO is generated by NO synthase (NOS) and induces tissue injury at the inflammatory site [5]. To date, three isoforms of NOS have been identified; endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) [6]. Among the three, iNOS is expressed in response to various inflammatory stimuli and causes a large amount of NO to be produced by macrophages during the inflammatory process [7].

TNF-α is one of the most important proinflammatory cytokines and is produced mainly by activated monocytes and macrophages [4]. It induces various biological responses including tissue injury, shock and apoptosis [4,8,9]. TNF-α also induces the secretion of cytokines such as IL-1, IL-6 and IL-10, and activates T cells and other inflammatory cells [10]. Therefore, suppression of the production of NO and TNF-α by activated macrophages using drugs might be useful for the treatment of inflammatory diseases.

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Abbreviations: NO, nitric oxide; TNF, tumor necrosis factor; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; JNK, c-jun N-terminal kinase; AP-1, activator protein-1; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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Chalcone, a flavonoid, is abundantly present in plant kingdom and has various biological activities such as anti-inflammatory, anti-allergic, antioxidant, and antibacterial effects [11–13]. In particular, it has been reported that 2′-hydroxychalcone derivatives showed potent anti-inflammatory activity [14]. For example, 2′,5′-dihydroxychalcone and 2′,3-dihydroxychalcone inhibit polymixin B-induced hind-paw edema in mice [15]. In addition, 2′-hydroxychalcone inhibits the TNF-α-induced expression of adhesion molecules such as intercellular cell adhesion molecules (ICAM)-1, vascular CAM (VCAM)-1 and E-selectin in human umbilical vein endothelial cells [16].

Previously, we reported that 2′-hydroxychalcone derivatives inhibit TPA-induced prostaglandin E_2 (PGE_2) production through the inhibition of COX-2 induction in rat peritoneal macrophages [17]. On the basis of our findings, we examined the effects of various 2′-hydroxychalcone derivatives on the LPS-induced production of NO and TNF-α, and attempted to clarify the mechanism of action in cultures of the murine macrophage cell line RAW 264.7.

2. Materials and methods

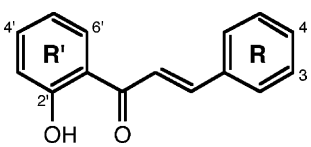
2.1. 2′-Hydroxychalcone derivatives

Four kinds of 2′-hydroxychalcone derivatives, 2′-hydroxy-4′-methoxychalcone (compound 1), 2′,4-dihydroxy-4′-methoxychalcone (compound 2), 2′,4-dihydroxy-6′-methoxychalcone (compound 3) and 2′-hydroxy-4,4′-dimethoxychalcone (compound 4) were synthesized as described previously [18]. Their chemical structures are shown in Table 1.

2.2. Cell culture

RAW 264.7 cells were obtained from RIKEN Gene Bank and cultured at 37° under 5% CO_2–95% air in Eagle's minimal essential medium (EMEM, Nissui) containing 10% fetal bovine serum (FBS, Sigma), penicillin G potassium (18 μg/mL) and streptomycin sulfate (50 μg/mL) (Meiji Seika). The cells at passage number 10 or lower were used for experiments.

Table 1
Chemical structures of 2′-hydroxychalcone derivatives



Compound	R′	R
1	4′-OCH_3	H
2	4′-OCH_3	4-OH
3	6′-OCH_3	4-OH
4	4′-OCH_3	4-OCH_3

2.3. Measurement of nitrite

RAW 264.7 cells (5×10^5 cells) were preincubated at 37° for 1 hr in 0.5 mL of medium containing each 2′-hydroxychalcone derivative, the tyrosine kinase inhibitor genistein (Wako) or the non-specific inhibitor of NOS N^G-monomethyl-L-arginine acetate (L-NMMA, Wako). After three washes with phosphate-buffered saline (PBS, pH 7.4), the cells were further incubated at 37° for 12 hr in 0.5 mL of EMEM containing 10% FBS in the presence of LPS (0.1 μg/mL) (Wako) and the corresponding concentration of each drug. After incubation for 12 hr, nitrite levels in the conditioned medium were determined using Griess reagent [19].

2.4. Measurement of TNF-α

RAW 264.7 cells (5×10^5 cells) were preincubated at 37° for 1 hr in 0.5 mL of medium containing each 2′-hydroxychalcone derivative or genistein. After three washes with PBS, the cells were further incubated at 37° for 6 hr in 0.5 mL of EMEM containing 10% FBS in the presence of LPS (0.1 μg/mL) and the corresponding concentration of each drug. After 6 hr incubation, TNF-α levels in the conditioned medium were determined using a TNF-α enzyme-linked immunosorbent assay (ELISA) kit (BioSource) according to the manufacturer's instructions.

2.5. Measurement of cell viability

RAW 264.7 cells (1×10^5 cells) were incubated at 37° for 12 hr in 0.1 mL of medium containing various concentrations of each 2′-hydroxychalcone derivative in the presence or absence of LPS (0.1 μg/mL), then 10 μL of 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) solution in PBS (5 mg/mL) was added, and the cells were further incubated at 37° for 4 hr. After the removal of the medium, 100 μL of DMSO was added, and the absorbance at 595 nm was determined [20–22].

2.6. Reverse transcription (RT)-polymerase chain reaction (PCR) for TNF-α mRNA

RAW 264.7 cells (2×10^6 cells) were preincubated at 37° for 1 hr in 2 mL of medium containing various concentrations of compound 1, 2 or 3. After three washes with PBS, the cells were further incubated at 37° for 4 hr in 2 mL of EMEM containing 10% FBS in the presence of LPS (0.1 μg/mL) and the corresponding concentrations of each compound. After incubation, the total RNA was extracted using a VIOGENE DNA/RNA Extraction kit (Viogene) according to the manufacturer's instructions. The extracted RNA (1 μg) was reverse transcribed at 37° for 1 hr by adding 5 μM of random hexamer oligonucleotides (Gibco BRL), 200 units of reverse transcriptase (Takara), 0.5 mM deoxyribonucleotide triphosphates (dNTP) (Takara) and 10 mM

dithiothreitol (Takara). The PCR primers used were 5′-TTG ACC TCA GCG CTG AGT TG-3′ (sense) and 5′-CCT GTA GCC CAC GTC GTA GC-3′ (antisense) for TNF-α, and 5′-TGA TGA CAT CAA GAA GGT GGT GGA-3′ (sense) and 5′-TCC TTG GAG GCC ATG TAG GCC AT-3′ (antisense) for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PCR for TNF-α was performed for 27 cycles of 0.5 min of denaturation at 94°, 1 min of annealing at 58° and 1.5 min of extension at 72° using a DNA thermal cycler (Takara), and for GAPDH, with 27 cycles of 1 min of denaturation at 94°, 1 min of annealing at 57° and 1 min of extension at 72°. PCR was carried out with 10 μL of template DNA and 40 μL of PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$) containing each primer (0.2 μM), dNTP (0.2 mM) and *Taq* DNA polymerase (1.25 units) (Takara). After PCR, 10 μL of the reaction mixture was subjected to electrophoresis on a 1.5% agarose gel, and the PCR products were visualized by ethidium bromide staining. The levels of mRNA for TNF-α and GAPDH were quantified by scanning densitometry.

2.7. Western blot analysis

RAW 264.7 cells (2×10^6 cells) were preincubated at 37° for 1 hr in 2 mL of medium containing various concentrations of compound 1, 2 or 3. After three washes with PBS, the cells were further incubated at 37° for 12 hr for induction of iNOS, 30 min for phosphorylation of JNK and c-jun, or 20 min for degradation of IκB-α, in 2 mL of EMEM containing 10% FBS in the presence of LPS (0.1 μg/mL) and the corresponding concentrations of each compound. After incubation, the cells were washed three times with PBS, dipped in 150 μL of ice-cold lysis buffer (20 mM HEPES, pH 7.4, 1% Triton-X 100, 10% glycerol, 1 M sodium fluoride, 2.5 mM *p*-nitrophenylene phosphate, 10 μg/mL of phenylmethylsulfonylfluoride, 1 mM Na_3VO_4, 5 μg/mL of leupeptin, and 1 mM EDTA) for 15 min, and disrupted with a Handy Sonic Disrupter (UR-20P, TOMY). The lysis buffer containing the disrupted cells was centrifuged at 13,000 *g* and 4° for 20 min. The supernatant fraction obtained was boiled for 5 min in 3× sample buffer (50 mM Tris, pH 7.4, 4% SDS, 10% glycerol, 4% 2-mercaptoethanol, and 0.05 mg/mL of bromophenol blue) at a ratio of 2:1 (v/v), loaded on an acrylamide gel (8 or 10%) and subjected to electrophoresis (150 min at 125 V). Each antibody for iNOS, IκB-α, JNK and c-jun was purchased from Santa Cruz Biotechnology, and Western blotting was carried out as described previously [23]. The levels of each protein were quantified by scanning densitometry, and the individual band density value for each point was expressed as the relative density signal.

2.8. Preparation of nuclear extract

RAW 264.7 cells (4×10^6 cells) were preincubated at 37° for 1 hr in 4 mL of medium containing various concentrations of compound 1, 2 or 3. After three washes with PBS, the cells were further incubated at 37° for 1 hr in 4 mL of EMEM containing 10% FBS in the presence of LPS (0.1 μg/mL) and the corresponding concentrations of each compound. After incubation, the cells were scrapped off the plate using a cell scraper, and centrifuged at 2500 *g* and 4° for 5 min. The cells were suspended in 400 μL of Tris-buffered KCl solution (20 mM Tris–HCl, pH 7.8, 50 mM KCl, 10 μg/mL of leupeptin, 0.1 mM dithiothreitol, and 1 mM phenyl methylsulfonyl fluoride), and lysed by the addition of the same volume of Tris-buffered KCl solution containing 1.2% Nonidet P-40 (Sigma) with vigorous mixing for 10 s. The homogenate was centrifuged at 4° and 15,000 *g* for 30 s, and the nuclear pellet was suspended in 30 μL of cold Tris-buffered KCl solution by mixing at 4° for 15 min. The suspension was then centrifuged at 4° and 15,000 *g* for 20 min, and the resultant supernatant (nuclear extract fraction) was stored at −80° prior to use.

2.9. Electrophoretic mobility shift assay (EMSA)

EMSA was carried out according to the protocol accompanying the Gel Shift Assay System (Promega). Briefly, the double-stranded oligonucleotide probes containing NF-κB- and AP-1-binding sequences were end-labeled with 1.85 MBq of [γ-^{32}P] ATP (111 TBq/nmol, Du Pont New England Nuclear) using T4 polynucleotide kinase. The nuclear extract (4 μg) was incubated at room temperature for 20 min with 4 μL of [^{32}P]-labeled probe in a binding buffer (50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 0.25 mg/mL of poly(dI-dC), and 20% glycerol). DNA/nuclear protein complexes were separated from the DNA probe by electrophoresis on a native 4% acrylamide gel, and the gel was vacuum-dried and visualized with a GS-250 Molecular Imager (Bio-Rad).

2.10. Statistical analysis

The statistical significance of the results was analyzed using Dunnett's test for multiple comparisons and Student's *t*-test for unpaired observations.

3. Results

3.1. Effects of 2′-hydroxychalcone derivatives on LPS-induced production of nitrite and TNF-α

RAW 264.7 cells were preincubated for 1 hr at 37° in medium containing 30 μM of compound 1, 2, 3 or 4. After three washes, the cells were further incubated for 12 hr in the presence of LPS (0.1 μg/mL) and 30 μM of each compound, and nitrite concentrations in the conditioned medium were determined. Production of nitrite was

Table 2
Effects of 2′-hydroxychalcone derivatives on LPS-induced production of nitrite and TNF-α

Treatment	Nitrite (μM)	TNF-α (pg/mL)
None	5.2 ± 0.2***	39.8 ± 2.1***
LPS (0.1 μg/mL)	32.6 ± 0.4	208 ± 4.2
LPS + compound 1 (30 μM)	6.9 ± 0.6***	55.0 ± 4.5***
LPS + compound 2 (30 μM)	7.8 ± 0.7***	57.8 ± 2.9***
LPS + compound 3 (30 μM)	9.2 ± 0.4***	57.4 ± 2.9***
LPS + compound 4 (30 μM)	31.5 ± 0.4	202.7 ± 5.1
LPS + genistein (30 μM)	15.9 ± 0.6***	55.7 ± 2.8***

Values are the means from four samples with the SEM. Statistical significance: $^{***}P < 0.001$ vs. LPS control.

increased by treatment with LPS (0.1 μg/mL), and among the four derivatives, compounds 1, 2 and 3 strongly inhibited the LPS-induced production of nitrite at 12 hr, but compound 4 had no significant effect (Table 2). TNF-α production was also increased by treatment with LPS (0.1 μg/mL), and compounds 1, 2 and 3 suppressed the LPS-induced production of TNF-α at 6 hr, but compound 4 showed no significant effect (Table 2). The tyrosine kinase inhibitor genistein at 30 μM inhibited the LPS (0.1 μg/mL)-induced production of both nitrite and TNF-α at 30 μM (Table 2). In the following experiments, we analyzed the effects of compounds 1, 2 and 3 which showed the potent inhibitory activity among the four derivatives.

3.2. Effects of various concentrations of 2′-hydroxychalcone derivatives on LPS-induced iNOS expression and nitrite production

Incubation with LPS (0.1 μg/mL) for 12 hr markedly induced iNOS expression and increased nitrite production (Fig. 1A and B). Under these conditions, compounds 1, 2 and 3 inhibited the LPS-induced production of nitrite in a concentration-dependent manner at 3–30 μM (Fig. 1B). The inhibitory effect of these compounds at 3 μM was slight but significant. L-NMMA, a non-specific inhibitor of NOS, also suppressed LPS-induced nitrite production at 10–100 μM (Fig. 1B). The LPS-induced iNOS expression was suppressed by these compounds in a concentration-dependent manner as well (Fig. 1A). These findings indi-

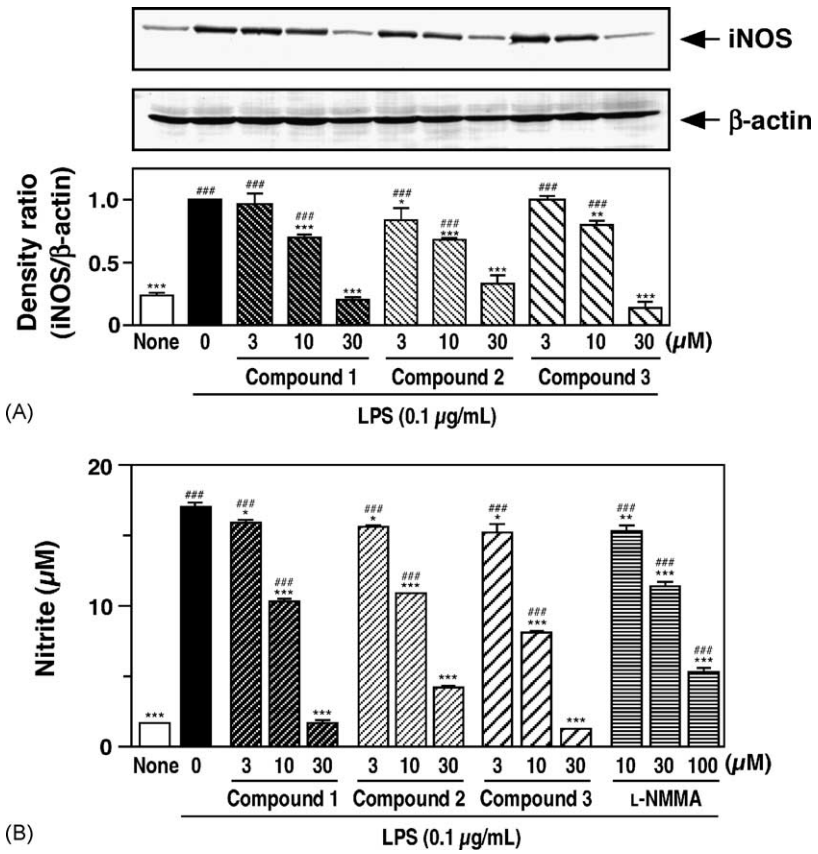


Fig. 1. Effects of 2′-hydroxychalcone derivatives on LPS-induced iNOS expression and nitrite production. RAW 264.7 cells were suspended at 1×10^6 cells/mL of medium containing the indicated concentration of each 2′-hydroxychalcone derivative or L-NMMA, and 2 mL (A) or 0.5 mL (B) of the cell suspension was preincubated at 37° for 1 hr. The cells were then washed three times with PBS, suspended in 2 mL (A) or 0.5 mL (B) of medium containing LPS (0.1 μg/mL) and the corresponding concentrations of each drug, and incubated at 37° for 12 hr. (A) The protein levels of iNOS and β-actin were determined by Western blot analysis. The density ratios of iNOS to β-actin were calculated, and the density ratio in the LPS control group is set to 1.0. (B) Nitrite concentrations in the conditioned medium were determined using Griess reagent. Values are the means from four samples with the SEM shown by vertical bars. Statistical significance: ($^{###}$) $P < 0.001$ vs. none; (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ vs. LPS control.

cate that compounds 1, 2 and 3 inhibit the LPS-induced production of nitrite through the inhibition of iNOS expression.

MTT assay at 12 hr revealed that the inhibition of the LPS-induced iNOS expression and nitrite production was not due to cytotoxicity by these compounds (data not shown).

3.3. Effects of various concentrations of 2′-hydroxychalcone derivatives on LPS-induced increases in the levels of TNF-α mRNA and TNF-α production

RAW 264.7 cells were incubated at 37° for 4 hr in medium containing LPS (0.1 μg/mL) and various concentrations of each 2′-hydroxychalcone derivative, and the levels of TNF-α mRNA were determined by RT-PCR. As shown in Fig. 2A, treatment with LPS (0.1 μg/mL) increased the level of TNF-α mRNA, and compounds 1, 2 and 3 lowered this LPS-induced increase at 10 and 30 μM.

TNF-α levels in the conditioned medium at 6 hr were also increased by LPS (0.1 μg/mL), and compounds 1, 2 and 3 suppressed the LPS-induced production of TNF-α at 3–30 μM (Fig. 2B), at which concentrations the LPS-induced increase in TNF-α mRNA levels was suppressed (Fig. 2A). These findings suggest that the inhibition of TNF-α production by compounds 1, 2 and 3 is due to the suppression of the LPS-induced expression of TNF-α mRNA.

3.4. Effects of compound 1 on LPS-induced activation of NF-κB and AP-1

To clarify the mechanism of action of the 2′-hydroxychalcone derivatives for the inhibition of the LPS-induced production of nitrite and TNF-α, effects of compound 1 on LPS-induced activation of NF-κB and AP-1 were examined. Treatment with LPS (0.1 μg/mL) for 1 hr increased the activation of both NF-κB (Fig. 3A) and AP-1 (Fig. 3B). In the presence of compound 1 at 3–30 μM, the activation of NF-κB and AP-1 was suppressed (Fig. 3A and B). Compounds 2 and 3 also suppressed the activation of NF-κB and AP-1 at 3–30 μM (data not shown). These

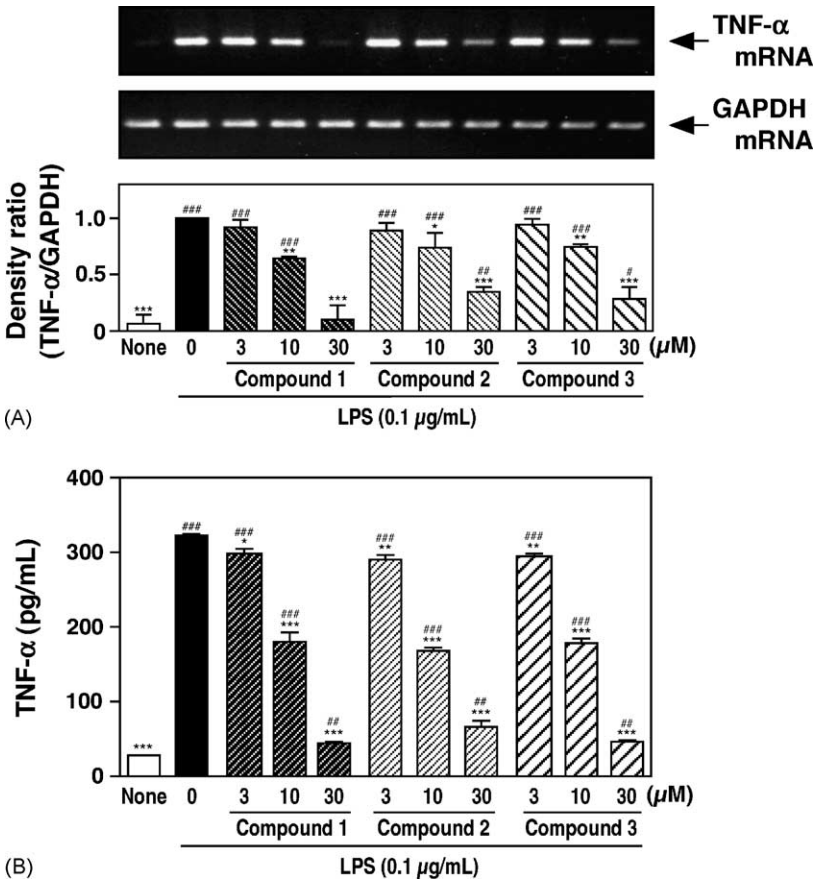


Fig. 2. Effects of 2′-hydroxychalcone derivatives on LPS-induced increases in the levels of TNF-α mRNA and TNF-α production. RAW 264.7 cells were suspended at 1×10^6 cells/mL of medium containing the indicated concentration of each 2′-hydroxychalcone derivative, and 1 mL (A) or 0.5 mL (B) of the cell suspension was preincubated at 37° for 1 hr. The cells were then washed three times with PBS, suspended in 1 mL (A) or 0.5 mL (B) of medium containing LPS (0.1 μg/mL) and the corresponding concentrations of each drug, and incubated at 37° for 4 hr (A) or 6 hr (B). (A) The levels of mRNA for TNF-α and GAPDH were detected by RT-PCR. The density ratios of TNF-α to GAPDH were calculated, and the density ratio in the LPS control group is set to 1.0. (B) TNF-α concentrations were determined by ELISA. Values are the means from three samples with the SEM shown by vertical bars. Statistical significance: ($^{\#}$) $P < 0.05$, ($^{\#\#}$) $P < 0.01$, and ($^{\#\#\#}$) $P < 0.001$ vs. none; (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ vs. LPS control.

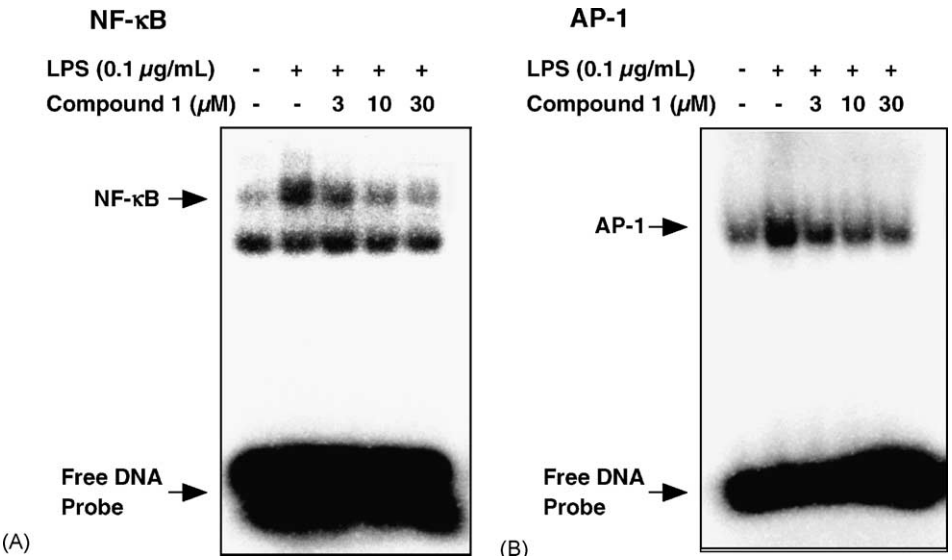


Fig. 3. Effects of 2′-hydroxy-4′-methoxychalcone (compound 1) on LPS-induced activation of NF-κB and AP-1. RAW 264.7 cells were suspended at 1×10^6 cells/mL of medium containing the indicated concentration of compound 1, and 4 mL of the cell suspension was preincubated at 37° for 1 hr. The cells were then washed three times with PBS, suspended in 4 mL of medium containing LPS (0.1 μg/mL) and the corresponding concentrations of compound 1, and incubated at 37° for 1 hr. After incubation, nuclear proteins were extracted, and the amount of NF-κB (A) or AP-1 (B) bound to the DNA probe was detected by EMSA. Similar results were obtained in three separate sets of experiments.

findings indicate that the inhibition of the LPS-induced production of nitrite and TNF-α by the 2′-hydroxychalcone derivatives is induced through the suppression of the LPS-induced activation of NF-κB and AP-1.

3.5. Effects of compound 1 on LPS-induced degradation of IκB-α

Incubation of RAW 264.7 cells at 37° for 20 min in the presence of LPS (0.1 μg/mL)-induced degradation of IκB-α (Fig. 4). Under these conditions, compound 1 significantly inhibited the LPS-induced degradation of IκB-α at 3–30 μM (Fig. 4). Compounds 2 and 3 also inhibited the LPS-induced degradation of IκB-α at 3–30 μM (data not shown). These findings suggest that the 2′-hydroxychalcone derivatives suppress the activation of NF-κB through the inhibition of IκB-α degradation.

3.6. Effects of compound 1 on LPS-induced phosphorylation of JNK and c-jun

After incubation with LPS (0.1 μg/mL) for 30 min, the phosphorylation of JNK and c-jun were significantly increased (Fig. 5A and B). Under these conditions, compound 1 inhibited the LPS-induced phosphorylation of JNK (Fig. 5A) and c-jun (Fig. 5B) in a concentration-dependent manner, at which concentrations the LPS-induced activation of AP-1 was inhibited (Fig. 3B). Compounds 2 and 3 also inhibited the LPS-induced phosphorylation of JNK and c-jun at 3–30 μM (data not shown). These findings indicate that the 2′-hydroxychalcone derivatives down-regulate the c-jun phosphorylation via the suppression of JNK phosphorylation.

4. Discussion

In cultures of the murine macrophage cell line RAW 264.7, three 2′-hydroxychalcone derivatives, compounds 1, 2 and 3, suppressed the LPS-induced production of nitrite

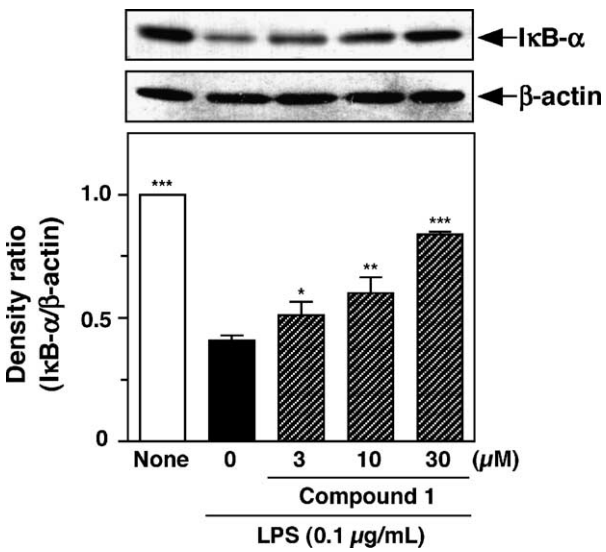


Fig. 4. Effects of 2′-hydroxy-4′-methoxychalcone (compound 1) on LPS-induced degradation of IκB-α. RAW 264.7 cells were suspended at 1×10^6 cells/mL of medium containing the indicated concentration of compound 1, and 2 mL of the cell suspension was preincubated at 37° for 1 hr. The cells were then washed three times with PBS, suspended in 2 mL of medium containing LPS (0.1 μg/mL) and the corresponding concentrations of compound 1, and incubated at 37° for 20 min. The protein levels of IκB-α and β-actin were determined by Western blot analysis. The density ratios of IκB-α protein were calculated, and the mean value of the density ratio in the unstimulated control group is set to 1.0. Values are the means from three samples with the SEM shown by vertical bars. Statistical significance: (*) $P < 0.05$, (**) $P < 0.01$ and (***) $P < 0.001$ vs. LPS control.

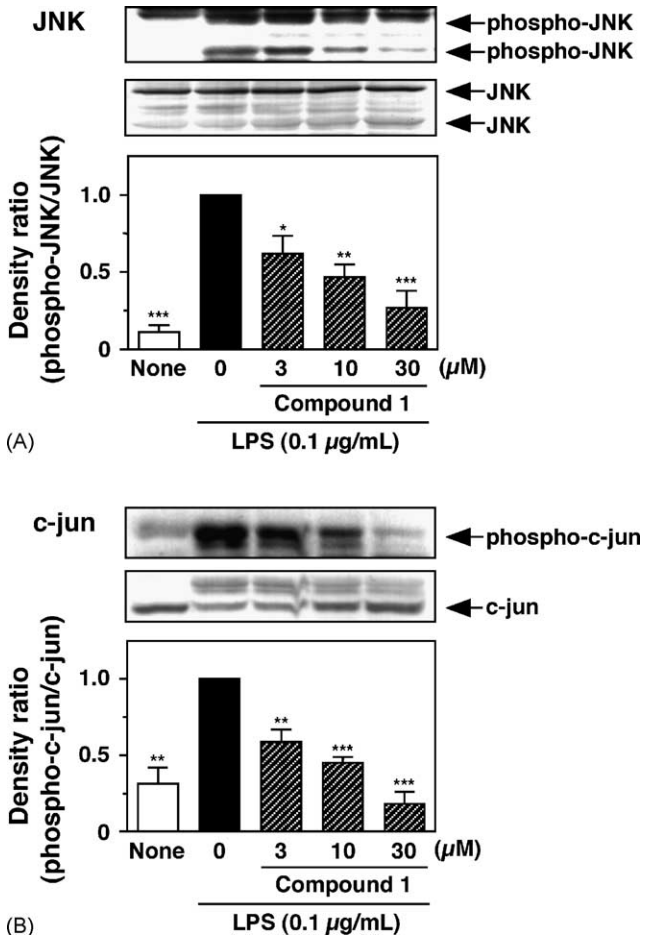


Fig. 5. Effects of 2′-hydroxy-4′-methoxychalcone (compound 1) on LPS-induced phosphorylation of JNK and c-jun. RAW 264.7 cells were suspended at 1×10^6 cells/mL of medium containing the indicated concentration of compound 1, and 2 mL of the cell suspension was preincubated at 37° for 1 hr. The cells were then washed three times with PBS, suspended in 2 mL of medium containing LPS (0.1 μg/mL) and the corresponding concentrations of compound 1, and incubated at 37° for 30 min. The protein levels of phospho-JNK, JNK, phospho-c-jun and c-jun were determined by Western blot analysis. The density ratios of phospho-JNK (A) and phospho-c-jun (B) were calculated, and the mean value of the density ratio in the LPS control group is set to 1.0. Values are the means from three samples with the SEM shown by vertical bars. Statistical significance: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ vs. LPS control.

and TNF-α, but compound 4 did not (Table 2). These findings indicated that the substitution of –OH with $–OCH_3$ at position 4 (compound 4) decreased the inhibitory activity of compound 2. The reduction of the inhibitory activity caused by the substitution of 4-OH with 4-OCH_3 was also observed in our previous study on the suppression of TPA-induced PGE_2 production in rat peritoneal macrophages [17]. These findings suggested that the inhibition of LPS-induced production of nitrite and TNF-α by 2′-hydroxychalcone derivatives is induced by a similar mechanism to the inhibition of TPA-induced PGE_2 production. To clarify the mechanism of action of 2′-hydroxychalcone derivatives for the inhibition of the LPS-induced production of nitrite and TNF-α, effects of compounds 1, 2 and 3 on the activation of NF-κB, an essential transcription factor for the expression of COX-2 [24], iNOS [25] and TNF-α [26] were examined. Our findings suggested that the three compounds blocked the LPS-induced nuclear translocation of NF-κB by preventing the degradation of IκB-α, an inhibitor of NF-κB activation [27], thus inhibiting the LPS-induced production of nitrite and TNF-α (Figs. 1 and 2).

It has been reported that 2′-hydroxychalcone down-regulates the TNF-α- and LPS-induced expression of ICAM-1 and VCAM-1 in human umbilical vein endothelial cells via inhibition of the activation of NF-κB [16], a key transcription factor for the expression of ICAM-1 and VCAM-1 in TNF-α-stimulated endothelial cells [28]. Consistent with the results shown by Madan *et al.* [16], our observations also demonstrated that compounds 1, 2 and 3 inhibited the nuclear translocation of NF-κB at a similar concentration range.

It is reported that the redox regulation is involved in the activation of NF-κB [29,30], and the antioxidant reagent pyrrolidine dithiocarbamate and *N*-acetylcysteine inhibit the activation of NF-κB [31,32]. Moreover, these inhibitors strongly suppressed the production of nitrite and TNF-α [31,33]. It is also reported that 2′-hydroxychalcone shows potent antioxidant activity [34]. Therefore, it is possible that the 2′-hydroxychalcone derivatives examined in this study inhibited the activation of NF-κB through their antioxidant property.

Next, we examined the effect of compounds 1, 2 and 3 on the activation of AP-1, because the promoter region of both the *iNOS* and *TNF*-α genes contains a binding site for the transcription factor AP-1 [25,26]. As shown in Fig. 3B, EMSA analysis revealed that the LPS-induced activation of AP-1 was inhibited by compound 1, at which concentrations the LPS-induced production of nitrite and TNF-α was inhibited (Figs. 1 and 2). Activation of JNK leads to the phosphorylation of serine 63 and 73 in c-jun, a component of AP-1, and an increase in the transcriptional activity of AP-1 [35]. Thus, the activation of AP-1 is mainly dependent on the activation of the JNK signaling pathway. Our findings that compounds 1, 2 and 3 inhibited the LPS-induced phosphorylation of JNK and its downstream substrate c-jun indicate that the inhibition of AP-1 activation by these compounds is due to inhibition upstream of JNK. To our knowledge, our report is the first to describe that 2′-hydroxychalcone derivatives have an inhibitory effect on JNK activation. Further study is necessary to elucidate the mechanism underlying the inhibition of JNK activation by 2′-hydroxychalcone derivatives.

Furthermore, to show that the inhibitory activity of 2′-hydroxychalcone derivatives is not specific to LPS stimulation, we examined the effects of 2′-hydroxychalcone derivatives on TPA (30 nM)-induced production of nitrite and TNF-α. Under the condition of TPA stimulation, the increased production of nitrite and TNF-α was also inhibited by compound 1, 2 or 3 at 30 μM through the inhibition of NF-κB and JNK activation (data not shown).

In conclusion, this study showed that 2′-hydroxychalcone derivatives suppress the LPS-induced production of nitrite and TNF-α in the macrophage cell line RAW 264.7, by inhibiting the activation of both NF-κB and AP-1. Because NF-κB and AP-1 are critical transcription factors that regulate the production of various proinflammatory proteins and cytokines in activated macrophages during the process of inflammation, the inhibition of these transcription factors might be an effective therapeutic approach for inflammatory diseases such as rheumatoid arthritis. It is possible that 2′-hydroxychalcone derivatives are lead compounds for novel anti-inflammatory drugs having inhibitory activity on the production of various inflammatory mediators such as PGE_2, NO and cytokines.

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