

Curcumin and its major metabolites inhibit the inflammatory response induced by lipopolysaccharide: Translocation of nuclear factor- κ B as potential target

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Abstract. The aim of the present study was to investigate and compare the anti-inflammatory activities of curcumin and its three metabolites, tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. The results demonstrated that overproduction of nitric oxide (NO) was potentially inhibited following treatment with curcumin and its three metabolites. In addition, curcumin and tetrahydrocurcumin significantly inhibited the release of prominent cytokines, including tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6); however, hexahydrocurcumin and octahydrocurcumin did not significantly alter cytokine release. Furthermore, the present study investigated the effect of curcumin and its metabolites on the expression of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2) and activated-nuclear factor kappa B (NF- κ B); the results showed that curcumin and its three metabolites significantly inhibited LPS-mediated upregulation of iNOS and COX-2 as well as NF- κ B activation. However, curcumin exerted a more potent effect on LPS-stimulated RAW 264.7 cells compared to that

of its three metabolites, of which tetrahydrocurcumin was found to be the most pharmacologically active. In conclusion, the results of the present study demonstrated that curcumin and its major metabolites inhibited the LPS-induced inflammatory response via the mechanism of inhibiting NF- κ B translocation to the nucleus.

Introduction

Previous studies have reported that curcumin exhibited a wide spectrum of biological activities, including anti-inflammatory (1,2), anti-tumor (3-6), antioxidant (7,8), anti-microbial (9,10), choleric (11), neuroprotective (12,13) and anti-mutagenic (14) activities; curcumin was also found to be involved in numerous drug-drug interactions (15,16). Evidence suggested that curcumin was markedly efficacious and safe at high doses (17,18); however, due to low absorption and rapid metabolism, the bioavailability of curcumin was not ideal (19). Previous studies on the pharmacokinetics of curcumin led to the identification of key metabolites, including tetrahydrocurcumin, curcumin-O-glucuronide, curcumin-O-sulfate, hexacurcumin, monoacetylcurcumin and octahydrocurcumin (20), of which tetrahydrocurcumin was characterized as the primary metabolite and has been widely investigated for its biological activities.

Tetrahydrocurcumin was reported to induce autophagic cell death through coordinated modulation of the phosphoinositide 3-kinase/Akt-mechanistic target of rapamycin and mitogen-activated protein kinase signaling pathways in human leukemia HL-60 cells (21). Tetrahydrocurcumin was reported to reduce HT1080-cell invasion and migration via downregulation of extracellular matrix (ECM)-degrading enzymes and inhibition of cell adhesion to ECM proteins (22). The anti-cancer and anti-angiogenic effects of curcumin and tetrahydrocurcumin were also demonstrated in implanted hepatocellular carcinoma in nude mice (23). Furthermore, tetrahydrocurcumin was shown to be more effective than curcumin in preventing azoxymethane-induced colon carcinogenesis (24).

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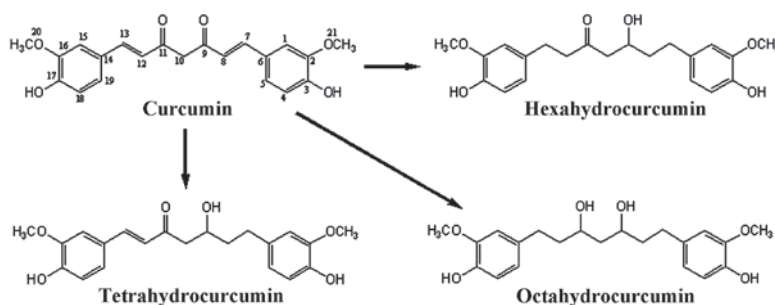


Figure 1. Chemical structures of curcumin and its metabolites, tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin.

However, previous studies on the anti-inflammatory effect of tetrahydrocurcumin and the biological activities of other curcumin metabolites are limited (25). One of these studies identified metabolites of curcuminoids in the feces and urine of rats following oral administration (26). The aim of the present study was to investigate and compare the anti-inflammatory activities of curcumin and its three metabolites, tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. Chemical structures of curcumin and its three metabolites are shown in Fig. 1.

Materials and methods

Curcumin and its metabolites. A mixture of curcuminoids (curcumin, 55.2%; demethoxycurcumin, 22.9%; and bisdemethoxycurcumin, 21.8%) was purchased from Tianjin Jian Feng Pharmaceutical Co., Ltd (Tianjin, China). Male Wistar-derived rats (200–250 g body weight, 8–10 weeks old, provided by the Laboratory Animal Center of the Shenyang Pharmaceutical University, Shenyang, China) were housed under controlled temperature ($22\pm 2^{\circ}\text{C}$), humidity ($55\pm 10\%$) and light (8:00 a.m. to 8:00 p.m.) conditions in a breeding room. Normal food and water were available *ad libitum* but were withdrawn 24 h prior to administration. Curcuminoids were administered *per os* as a 30% aqueous 1,2-propylene glycol solution. Urine and feces were collected for 48 h from the animals housed in stainless steel metabolism cages equipped with a urine and feces separator (B6-10, Suhang Technology Equipment Co., Ltd., Suzhou, China). Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Shenyang Pharmaceutical University (approval date: 08/10/2010, no. 100810; Liaoning, China). Tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin (Fig. 1) were isolated from feces and urine of rats over 48 h following oral administration of the curcuminoid mixture, as previously described (26). The purity of tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin was determined using high-performance liquid chromatography (HPLC; Waters 600; Waters Corp., Milford, MA, USA) and a ultraviolet detector (Waters 490; Waters Corp.); peak areas were normalized and the purities of the metabolites were 98.2, 98.6 and 98.5%, respectively.

Reagents. RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Mouse tumor necrosis factor- α (TNF- α) and

interleukin-6 (IL-6) ELISA kits as well as a bicinchoninic acid (BCA) protein concentration assay kit were obtained from Yantai Science and Biotechnology Co., Ltd (Shandong, China). Anti-NOS2 antibody (sc-651, rabbit polyclonal IgG, dilution 1:1,000), anti-Cox-2 antibody (sc-1746, goat polyclonal IgG, dilution 1:1,000), anti-I κ B- α antibody (sc-371, rabbit polyclonal IgG, dilution 1:1,000), anti-NF κ B p65 antibody (sc-372, rabbit polyclonal IgG, dilution 1:1,000) and anti-actin antibody (sc-1616, goat polyclonal IgG, dilution 1:1,000), as well as horse-radish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Phenylmethylsulfonyl fluoride, dithiothreitol, *E. coli*, LPS and all other chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA). A 50 mM solution of tested samples was prepared in 100% cell culture grade dimethyl sulfoxide, stored as small aliquots at -20°C , and then diluted to the required concentrations prior to use. Hydrocortisone (H4001; purity, $\geq 98\%$; Sigma-Aldrich) was used as the positive control drug for all experiments performed.

Cell culture. Mouse monocyte-macrophage RAW 264.7 cells (TIB-71; American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI 1640 medium supplemented with penicillin (100 U/ml; Yantai Science and Biotechnology Co., Ltd.), streptomycin (100 $\mu\text{g}/\text{ml}$; Yantai Science and Biotechnology Co., Ltd.) and 10% heat-inactivated FBS at 37°C in a humidified incubator with 5% CO_2 and 95% air. Medium was routinely replaced every two days and cells were passaged using 0.25% trypsin (Yantai Science and Biotechnology Co., Ltd.) until they attained confluence.

Nitric oxide (NO) analysis. The amount of nitrite in the cell culture supernatant was determined using a commercial Griess reagent kit (a mixture of Griess reagent A and Griess reagent B, ratio 1:1, A: 1% sulphanilamide in 5% H_3PO_4 and B: 0.1% naphthylethylene diamine dihydrochloride) (Yantai Science and Biotechnology, Co., Ltd.) as previously described (27). RAW 264.7 cells were treated with LPS (1 $\mu\text{g}/\text{ml}$) and curcumin or its respective metabolites (12.5–100 μM) for 24 h. Cell culture supernatant (100 μl) was added to 100 μl Griess reagent and then incubated at room temperature for 10 min. Absorbance was measured at 540 nm (Biotek Synergy HT; BioTek Instruments, Inc., Winooski, VT, USA) and inhibitory rates were calculated using a standard calibration curve of different concentrations of sodium nitrite as previously prepared (28).

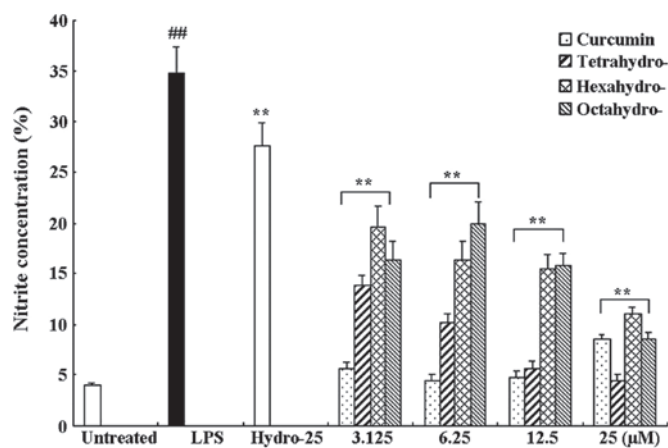


Figure 2. Inhibitory effects of curcumin and its metabolites on NO production in RAW 264.7 cells. RAW 264.7 cells were treated with 1 μ g/ml LPS only or in combination with different concentrations of curcumin and its metabolites (3.125, 6.25, 12.5 and 25 μ M) or hydro-25 for 24 h. The concentration of nitrite was then determined using Griess reagent and absorbance values to detect NO production levels. Values are presented as the mean \pm standard deviation (n=3). **P<0.01 vs. LPS only group, ###P<0.01 vs. untreated group. Curcumin and its three metabolites significantly inhibited the LPS-induced NO overproduction in a dose-dependent manner (P<0.01). NO, nitric oxide; LPS, lipopolysaccharide; hydro-25, 25 μ M hydrocortisone; tetra/hexa/octahydro-, tetra/hexa/octahydrocurcumin.

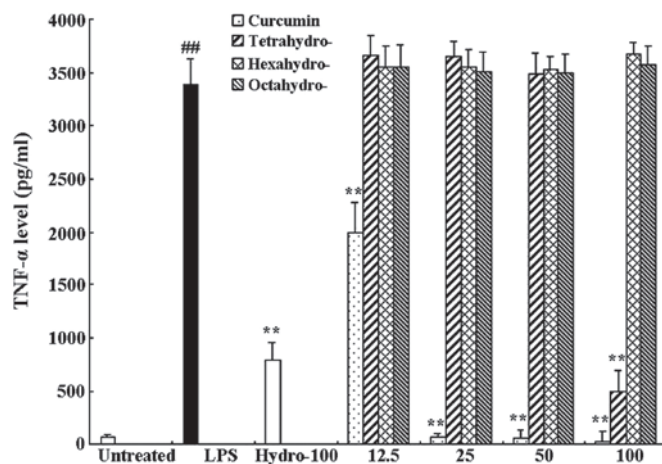


Figure 3. Inhibitory effects of curcumin and its metabolites on TNF- α release in RAW 264.7 cells. RAW 264.7 cells were treated with 1 μ g/ml LPS only or in combination with different concentrations of curcumin and its metabolites (12.5, 25, 50 and 100 μ M) or hydro-100 for 6 h. ELISA assays were used to determine the protein expression levels of TNF- α . Values are presented as the mean \pm standard deviation (n=3). **P<0.01 vs. LPS only group, ###P<0.01 vs. untreated group. TNF- α , tumor necrosis factor- α ; LPS, lipopolysaccharide; hydro-100, 100 μ M hydrocortisone; tetra/hexa/octahydro-, tetra/hexa/octahydrocurcumin.

Determination of cytokines, TNF- α and IL-6 expression. RAW 264.7 cells were treated with LPS (1 μ g/ml) and curcumin or its respective metabolites (12.5-100 μ M) for 6 h. Mouse tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) ELISA kits (Yantai Science and Biotechnology Co., Ltd.) were used according to the manufacturer's instructions in order to determine the protein expression levels of TNF- α or IL-6 using 100 μ l culture supernatant as previously described (29).

Total protein, cytoplasmic and nuclear protein extraction. Total protein was extracted by using western cell lysis buffer (Yantai Science and Biotechnology Co., Ltd.) in order to determine the protein expression levels of iNOS and COX-2. Cytoplasmic and nuclear proteins were separated using a nuclear and cytoplasmic protein extraction kit according to the manufacturer's instructions (Yantai Science and Biotechnology Co., Ltd.) for further use for the western blot analysis of I κ B- α and nuclear factor kappa B (NF- κ B) p65 subunit (30).

Western blot analysis. Protein concentrations were determined using a BCA protein concentration assay kit (Yantai Science and Biotechnology Co., Ltd.). Each respective protein (30 μ g) was boiled in SDS-PAGE loading buffer (Yantai Science and Biotechnology Co., Ltd.), subjected to gel electrophoresis and then electrophoretically transferred onto nitrocellulose membranes (Pall Corporation, Port Washington, NY, USA). The membranes were blocked using 5% nonfat dried milk in Tris-buffered saline with Tween 20 (TBST; 20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20) at room temperature for 1 h. Samples were then washed and incubated in their respective primary antibody solution (anti-iNOS, anti-COX-2, anti-I κ B- α , anti-p65 or anti- β -actin, dilution 1:1,000) overnight at 4°C. Membranes were then washed with TBST and incubated with HRP-conjugated secondary antibody solution for 1 h at room temperature. Blots were then washed three times in TBST, detected using enhanced chemiluminescence (Beyotime Institute of Biotechnology, Haimen, China) and

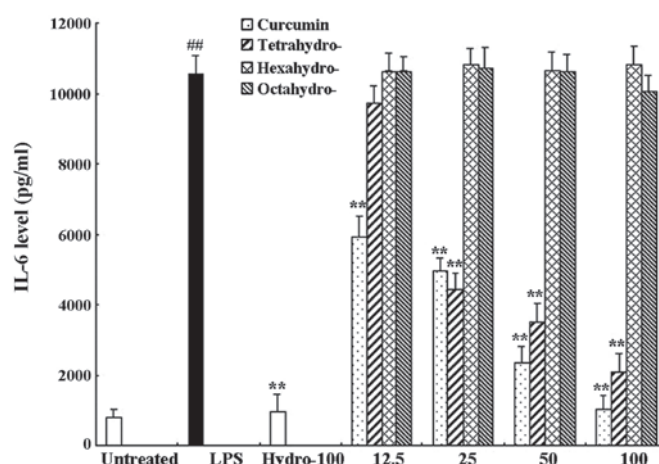


Figure 4. Inhibitory effects of curcumin and its metabolites on IL-6 release in RAW 264.7 cells. RAW 264.7 cells were treated with 1 μ g/ml LPS only or in combination with different concentrations of curcumin and its metabolites (12.5, 25, 50 and 100 μ M) or hydro-100 for 6 h. ELISA assays were used to determine the protein expression levels of IL-6. Values are presented as the mean \pm standard deviation (n=3). **P<0.01 vs. LPS only group, ##P<0.01 vs. untreated group. IL-6, interleukin-6; LPS, lipopolysaccharide; hydro-100, 100 μ M hydrocortisone; tetra/hexa/octahydro-, tetra/hexa/octahydrocurcumin.

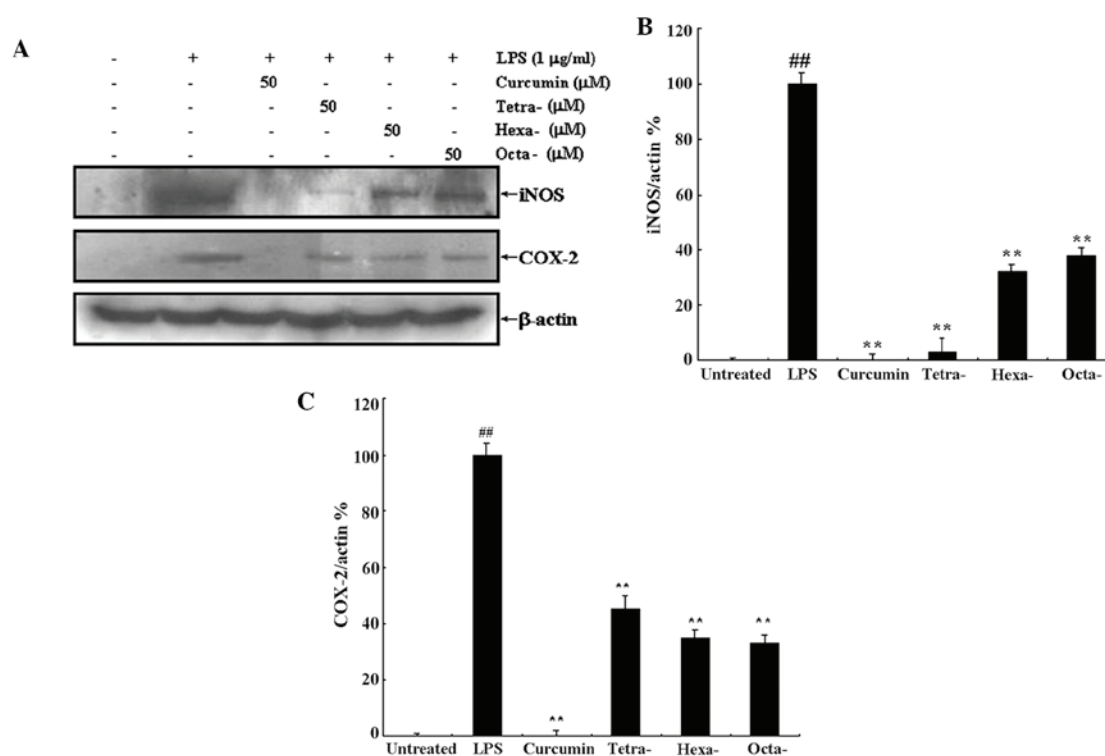


Figure 5. Curcumin and its metabolites inhibit LPS-induced overexpression of iNOS and COX-2 in RAW 264.7 cells. RAW 264.7 cells were treated with 1 μ g/ml LPS only or in combination with 50 μ M curcumin or its metabolites for 24 h. (A) Western blot analysis was then used to determine the protein expression levels of iNOS and COX-2. Densitometric analysis of (B) iNOS and (C) COX-2 protein expression. β -actin was used as an internal control. Values are presented as the mean \pm standard deviation (n=3). **P<0.01 vs. LPS only group, ##P<0.01 vs. untreated group. iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; LPS, lipopolysaccharide; tetra-/hexa-/octa-, tetra/hexa/octahydrocurcumin.

exposed to photographic films (Kodak, Tokyo, Japan). Images were collected and the corresponding protein bands for iNOS, COX-2, I κ B- α , p65 and β -actin were quantified by densitometric analysis using DigDoc100 software (Alpha Innotech Corporation, San Leandro, CA, USA). β -actin was used as the internal control.

Statistical analysis. Data were analyzed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Values are expressed as the

mean \pm standard deviation. Statistical comparisons were conducted using a one-way or two-way analysis of variance followed by a two-way post hoc test. P<0.05 was considered to indicate a statistically significant difference between values.

Results

Curcumin and its metabolites do not affect cell viability. A preliminary MTT assay showed that curcumin and its

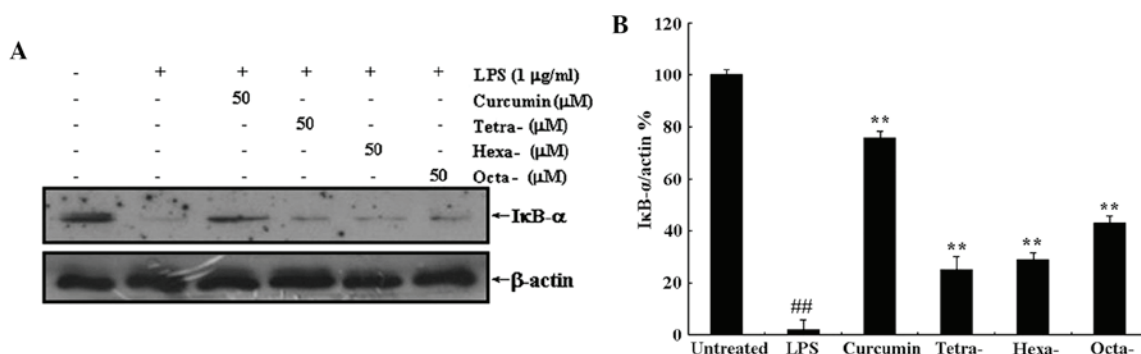


Figure 6. Curcumin and its metabolites inhibit the LPS-induced degradation of IκB-α in RAW 264.7 cells. RAW 264.7 cells were treated with 1 μg/ml LPS only or in combination with 50 μM curcumin or its metabolites for 10 min. (A) Western blot analysis was used to determine the cytoplasmic protein expression of IκB-α. (B) Densitometric analysis of cytoplasmic IκB-α protein expression. β-actin was used as an internal control. Values are presented as the mean ± standard deviation (n=3). **P<0.01 vs. LPS only group, ##P<0.01 vs. untreated group. IκB-α, inhibitor of κB-α; LPS, lipopolysaccharide; tetra-/hexa-/octa-, tetra/hexa/octahydrocurcumin.

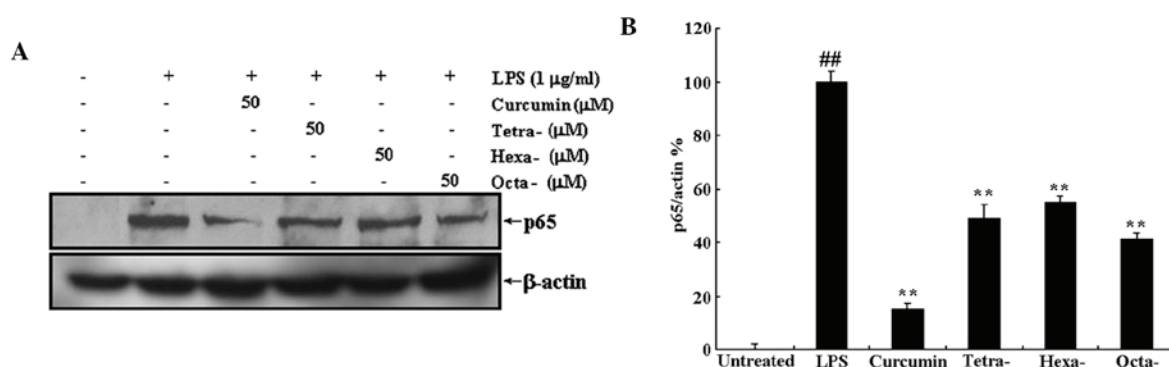


Figure 7. Curcumin and its metabolites inhibit the LPS-induced degradation of p65 in RAW 264.7 cells. RAW 264.7 cells were treated with 1 μg/ml LPS only or in combination with 50 μM curcumin or its metabolites for 45 min. (A) Western blot analysis was used to determine the nuclear protein expression of the nuclear factor kappa B p65 subunit. (B) Densitometric analysis of nuclear p65 protein expression. β-actin was used as an internal control. Values are presented as the mean ± standard deviation (n=3). **P<0.01 vs. LPS only group, ##P<0.01 vs. untreated group. LPS, lipopolysaccharide; tetra-/hexa-/octa-, tetra/hexa/octahydrocurcumin.

metabolites (3.125-100 μM) did not affect the viability of RAW 264.7 cells within 24 h.

Curcumin and its metabolites inhibit LPS-induced NO overproduction in RAW 264.7 cells. RAW 264.7 cells were treated with 1 μg/ml of LPS only or in combination with different concentrations of curcumin and its metabolites or hydrocortisone as the positive control. The concentration of nitrite was determined by detecting NO production levels 24 h following treatment (Fig. 2). LPS induced a significant increase in nitrite concentration compared with that of the untreated group (P<0.01; 34.78±2.54 and 3.96±0.29 μM, respectively). In addition, curcumin and its three metabolites significantly inhibited the LPS-induced NO overproduction in a dose-dependent manner (P<0.01); however, curcumin exhibited a more potent inhibition of nitrate concentrations compared to that of its metabolites.

Curcumin and tetrahydrocurcumin significantly inhibit the release of TNF-α and IL-6 in RAW 264.7 cells. RAW 264.7 cells were treated with 1 μg/ml LPS only or in combination with different concentrations of curcumin and its metabolites or hydrocortisone as the positive control. Following

6 h of incubation, an ELISA assay was used to determine the levels of TNF-α and IL-6 in the supernatants. As shown in Figs. 3 and 4, compared to the untreated control group, LPS significantly induced the release of proinflammatory cytokines TNF-α (63.57±6.41 and 3389.97±39.11 pg/ml, respectively) and IL-6 (811.95±32.84 and 10536.81±833.99 pg/ml, respectively). Curcumin exerted a significant inhibitory effect on TNF-α as well as IL-6 release compared to that of the LPS-only group; LPS-induced TNF-α and IL-6 release were almost completely inhibited following 100 μM curcumin treatment. In addition, the curcumin metabolite tetrahydrocurcumin significantly inhibited the release of TNF-α and IL-6, whereas hexahydrocurcumin and octahydrocurcumin did not exhibit any marked inhibitory effects on the release of TNF-α and IL-6 at concentrations of 12.5-100 μM.

Curcumin and its metabolites inhibit LPS-induced iNOS and COX-2 overexpression. As demonstrated above, curcumin and its metabolites were able to inhibit LPS-induced NO overproduction (Fig. 2). NO overproduction is associated with the upregulation of iNOS expression; therefore, in the present study, western blot analysis was used to determine the expression levels of iNOS and COX-2 following treatment

with LPS in the presence or absence of 50 μ M curcumin or its metabolites (Fig. 5A). Densitometric analysis revealed that 50 μ M curcumin completely inhibited LPS-induced expression of iNOS and COX-2 (Fig. 5B and C, respectively); in addition, curcumin metabolites significantly inhibited iNOS and COX-2 expression compared to that of the LPS only treatment. Furthermore, the metabolite tetrahydrocurcumin appeared to exhibit a more potent effect on iNOS expression compared to that of the other metabolites; however, statistical analyses were not performed to confirm this observation.

Curcumin and its metabolites prevent the translocation of NF- κ B to the nucleus via inhibition of LPS-induced I κ B- α protein degradation. Translocation of NF- κ B to the nucleus is the result of I κ B- α protein degradation. Western blot analysis was used to determine the effect of curcumin and its metabolites on LPS-induced I κ B- α degradation and translocation of NF- κ B to the nucleus. As shown in Figs. 6 and 7, treatment with 50 μ M curcumin or its metabolites significantly inhibited the LPS-induced decrease in cytoplasmic I κ B- α expression as well as the LPS-induced increase in nuclear protein expression of the NF- κ B subunit p65. This therefore indicated that curcumin and its metabolites inhibited I κ B- α degradation and, as a result, prevented the translocation of NF- κ B to the nucleus. Furthermore, the three metabolites of curcumin produced significant results; however, they appeared to have a less potent effect on LPS-induced degradation of I κ B- α and p65 expression than that of curcumin.

Discussion

Elucidation of metabolite structures is among the most challenging tasks of drug metabolism studies. At present, comparisons of electrospray ionization multi-stage tandem mass spectrometry data and HPLC retention times with synthetic standards are generally used to identify the structures of metabolites (31). However, when metabolites are difficult to obtain or synthesize, biological studies into the activities of these drug metabolites may be challenging or even impossible.

In the present study, the anti-inflammatory activities of three major metabolites of curcumin were investigated and compared to those of curcumin. Among these metabolites, tetrahydrocurcumin was previously reported to be the primary metabolite of curcumin and has been investigated for its biological activities (21-23); however, these studies primarily focused on the anti-tumor activities of tetrahydrocurcumin, whereas studies into the other metabolites of curcumin and their numerous biological activities are rare and insufficient. The results of the present study indicated that three major metabolites of curcumin, including tetrahydrocurcumin, hexahydrocurcumin and octahydrocurcumin (25), inhibited various LPS-induced responses of RAW 264.7 macrophage cells, including excess NO production, increased iNOS and COX-2 protein expression as well as LPS-induced degradation of I κ B- α and overexpression of nuclear p65; in addition, tetrahydrocurcumin significantly inhibited the LPS-induced release of pro-inflammatory cytokines TNF- α and IL-6. However, the results also determined that curcumin exerted more potent inhibitory effects on the LPS-induced responses of RAW 264.7 cells.

In conclusion, the results of the present study confirmed the anti-inflammatory effects of curcumin and its metabolites in RAW 264.7 macrophage cells and, to the best of our knowledge, the present study was the first direct study to compare the anti-inflammatory activities of curcumin and its main metabolites. In addition, the results indicated that the anti-inflammatory mechanism of curcumin and its metabolites may proceed via the inhibition of I κ B- α degradation, which in turn prevented the translocation of NF- κ B to the nucleus. These results may therefore provide the theoretical basis for further studies into the pharmacokinetics of curcuminoids.

Acknowledgements

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