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Research Article

Dimethoxycurcumin, a synthetic curcumin analogue with higher metabolic stability, inhibits NO production, inducible NO synthase expression and NF-κB activation in RAW264.7 macrophages activated with LPS

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Excess production of nitric oxide (NO) by inducible NO synthase (iNOS) in activated macrophages is linked to acute and chronic inflammation. Thus, it would be valuable to develop inhibitors of NO and/or iNOS for potential therapeutic use. We investigated whether dimethoxycurcumin (DiMC), a synthetic curcumin analogue with higher metabolic stability over curcumin, could inhibit NO production and iNOS expression in activated macrophages. RAW264.7 macrophages were activated with lipopolysaccharide (LPS) in the absence or presence of DiMC, which contains four methoxy groups at two aromatic rings, curcumin containing two, bis-demethoxycurcumin (BDMC) containing none, or tetrahydrocurcumin (THC) containing two but lacking conjugated double bonds in the central seven-carbon chain. NO production, iNOS expression and NF-κB activity were examined. DiMC, curcumin and BDMC inhibited NO production, iNOS expression and NF-κB activation, with DiMC being the most effective, followed by curcumin and BDMC. THC failed to inhibit NO production, iNOS expression and NF-κB activation. Our results suggest that DiMC inhibits NO production, iNOS expression and NF-κB activation in LPS-activated macrophages, which may be due not only to the conjugated double bonds but also the increased number of methoxy groups.

Keywords: Bis-demethoxycurcumin / Curcumin / Dimethoxycurcumin / Inducible nitric oxide synthase / Nitric oxide

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1 Introduction

The free radical nitric oxide (NO) is endogenously synthesized from L-arginine in a reaction catalyzed by one of three NO synthase (NOS) isoenzymes and is utilized for normal cellular functions [1]. Among three isoforms, inducible NOS (iNOS) is capable of producing high amounts of NO during inflammation [2]. In macrophages, iNOS is induced by pro-inflammatory cytokines and/or the endotoxin lipopolysaccharide (LPS), and plays an important role in non-specific host defense [3]. However, excess production of NO by iNOS in activated macrophages appears to be linked to acute and chronic inflammation [4]. Thus, it would be valuable to develop potent inhibitors of NO and/or iNOS for potential therapeutic use.

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Abbreviations: BDMC, bis-demethoxycurcumin; **COX-2**, cyclooxygenase-2; **DiMC**, dimethoxycurcumin; **iNOS**, inducible NO synthase; **MTT**, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; **THC**, tetrahydrocurcumin

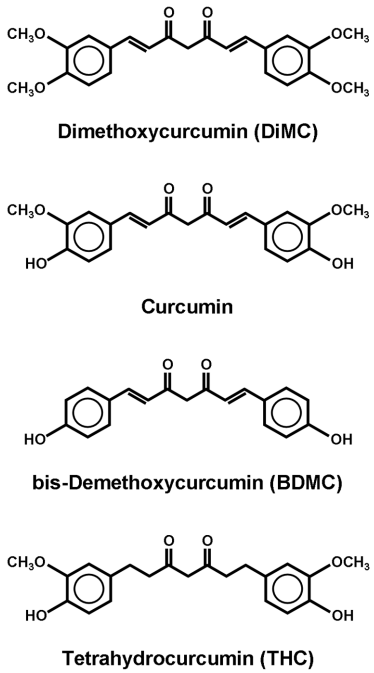

Figure 1. Chemical structures of curcumin, DiMC, BDMC and THC.

Curcumin (chemical structure shown in Fig. 1), a member of the curcuminoid family, is a yellow-colored phenolic pigment in turmeric, the other two curcuminoids being demethoxycurcumin and bis-demethoxycurcumin (BDMC; chemical structure shown in Fig. 1). Extensive investigations on pharmacological activity of curcumin have demonstrated that curcumin can inhibit NO production and iNOS expression *via* NFκB inactivation in activated macrophages [5, 6]. However, curcumin is rapidly metabolized *in vivo* into tetrahydrocurcumin (THC; chemical structure shown in Fig. 1) and other reduced forms in rats and mice and *in vitro* in human hepatic cells [7]. Moreover, it has been demonstrated that the anti-inflammatory property is lost when curcumin is reduced to THC or other metabolites [5, 7, 8]. Thus, there is a need to develop curcumin analogues with a higher metabolic stability than the original curcumin. Dimethoxycurcumin (DiMC; chemical structure shown in Fig. 1), one of several synthetic curcumin analogues, has been reported to exert an anticancer activity comparable to curcumin and to have increased metabolic stability in comparison with curcumin [9]. However, whether DiMC exerts other biological effects similar to those of curcumin remains to be investigated. Moreover, whether DiMC can inhibit NO production and iNOS expression in activated macrophages is currently unknown; and was thus investigated in this study. We also compared DiMC with curcumin, BDMC and THC for ability to inhibit NO production and iNOS expression. The chemical structures of four curcumin analogues examined in this study are shown in Fig. 1. While the original form of curcumin contains two methoxy groups at two aromatic rings, DiMC contains four and BDMC contains none. In comparison, THC contains two methoxy groups but lacks conjugated double bonds in the central seven-carbon chain.

2 Materials and methods

2.1 Chemicals and antibodies

Curcumin and BDMC were isolated from the rhizomes of turmeric, as described earlier [10]. THC was prepared from curcumin by hydrogenating the two double bonds conjugated to the β-diketone, as described previously [11, 12]. DiMC was synthetically prepared as described [13] at the College of Pharmacology, Wonkwang University (Iksan, Republic of Korea). The purity of each compound, detected by HPLC, was >90%. All solvents used in this study were LC-MS grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM, LPS (*Escherichia coli* O111:B4), Tris-reagent, Griess reagent, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Poly(dI-dC) and nuclear factor (NF)-κB specific oligonucleotide were obtained from Promega (Madison, WI, USA). Polyclonal antibodies specific for iNOS, cyclooxygenase-2 (COX-2), I-κBα kinase (IKK-α), I-κBa, phosphorylated (P)-I-κBα and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-P-p65 (Ser536) antibody from Cell Signaling Technology (Beverly, MA, USA). [γ-^{32}P]ATP was from ICN Pharmaceuticals (Costa Mesa, CA, USA). Prostaglandin E_2 (PGE_2) ELISA kit was purchased from R&D Systems (Minneapolis, MN, USA).

2.2 Cell culture and cell treatment

The mouse monocytic-macrophage cell line RAW264.7 (American Type Culture Collection, VA, USA) was cultured in DMEM containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and antibiotics (100 U/mL penicillin-G and 100 μg/mL streptomycin). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. RAW264.7 macrophages were cultured in either 6-well or 12-well flat-bottom plates at the concentration of 5×10^5 cells/mL and after 12 h of preconditioning, the cells were pre-incubated with curcumin analogues (1–10 μM) for 2 h, and stimulated with LPS (1 μg/mL) for 2 h (for NFκB assay), 6 h (for Western blot analysis) or 18 h (for nitrite and PGE_2 assays).

2.3 Cell viability assay

Cell viability was determined by a modified MTT reduction assay. MTT is a pale yellow substance that is reduced by living cells to yield a dark blue formazan product. This process

requires active mitochondria, and even fresh dead cells do not reduce significant amounts of MTT. The cells were cultured in a 96-well flat-bottom plate at concentration of 5×10^5 cells/mL and after 12 h of preconditioning, cells were treated with various concentrations of DiMC or other agents for 18 h. Thereafter, culture medium was aspirated and 100 μL MTT dye (1 mg/mL in PBS) was added; the cultures were incubated for 4 h at 37°C. The formazan crystals produced through dye reduction by viable cells were dissolved using acidified isopropanol (0.1 N HCl). Index of cell viability was calculated by measuring the OD of color produced by MTT dye reduction at 570 nm.

2.4 Western blot analysis

RAW264.7 macrophages were incubated with or without reagents. They were harvested, washed ice-cold PBS and kept on ice for 1 min. The suspension was mixed with buffer A (10 mM HEPES, pH 7.5, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 5 μg/mL aprotinin, 5 μg/mL pepstatin, and 10 μg/mL leupeptin) and lysed by three freeze-thaw cycles. Cytosolic fraction was obtained by centrifugation at 12 000 × *g* for 20 min at 4°C. The pellets were re-suspended in buffer C (20 mM HEPES, pH 7.5, 0.4 M NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 5 μg/mL aprotinin, 5 μg/mL pepstatin, and 10 μg/mL leupeptin) on ice for 40 min and centrifuged at 14 000 × *g* for 20 min at 4°C. The resulting supernatant was used as soluble nuclear fraction. Protein content was determined with BCA protein assay reagent (Pierce, Rockford, IL, USA). Total cellular or cytoplasmic fractions were separated on SDS-polyacrylamide gels (8% for iNOS and 10% for phospho-p65), and transferred to the nitrocellulose membranes (Amersham Biosciences, Inc., Piscataway, NJ, USA). The membrane was then blocked in blocking buffer containing 20 mM sodium phosphate buffer, pH 7.6, 150 mM NaCl, 0.1% Tween 20, and 5% nonfat dry milk for 1 h at room temperature. Thereafter, the membrane was incubated with antibodies against iNOS (1:1000 dilution), COX-2 (1:1000 dilution), p-p65 (1:500 dilution), IKK-α (1:500 dilution), or β-actin (1:1000 dilution) at 4°C overnight. The membrane was then washed four times with PBS-Tween 20 buffer and further incubated with secondary antibody for 1 h at room temperature. Specific bands were detected using enhanced chemiluminescence's detection system (Amersham Biosciences), and the membrane was exposed to X-ray film. For the densitometry analysis, OD (the gray-scale value of pixels; 0–255) was measured on the inverted digital images using Scion Image (Scion Corp., Frederick, MD, USA).

2.5 Electrophoretic mobility shift assay

For Electrophoretic mobility shift assay (EMSA), 6 μg of each nuclear fraction was mixed with the ^{32}P-labeled double-stranded NF-κB binding consensus oligonucleotides (top strand: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; bottom strand 3′-TCA ACT CCC CTG AAA GGG TCC G-5′) and incubated at room temperature for 20 min. The incubation mixture contains 1 μg poly(dI–dC) in a binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 1% NP-40, 5% glycerol, 50 mM NaCl). The DNA/protein complex was electrophoresed on 4.5% nondenaturing polyacrylamide gels in 0.5 × Tris/borate/EDTA buffer (0.0445 M Tris; 0.0445 M borate; 0.001 M EDTA). The specificity of binding was also examined by competition with the unlabeled oligonucleotide.

2.6 Analysis of NF-κB p65 DNA-binding activity

Cells were stimulated with LPS for 2 h in the presence or absence of DiMC. Analysis of p65 binding to its consensus oligonucleotide was performed in cell lysates using the ELISA-based Trans-AMTM NF-κB p65 kit (Active Motif, Carlsbad, CA, USA). Briefly, nuclear extract proteins (10 μg) from control or agent-treated cells were incubated with an oligonucleotide containing the NF-κB consensus binding site (5′-GGG ACT TTC C-3′) bound to a 96-well microtiter plate. After extensive washes, the NF-κB complexes bound to the oligonucleotides were incubated with rabbit anti-NF-κB p65 antibody (1:1000 dilution), followed by further extensive washings. The plates were then incubated with a secondary antibody (goat anti-rabbit horseradish peroxidase-IgG, 1:1000 dilution) and tetramethyl benzidine (substrate) was added for color development, which was read at 450 nm with a reference wavelength of 655 nm.

2.7 Analysis of IKK-α activity

The IKK complex from whole-cell extracts was precipitated with antibody against IKK-α followed by treatment with protein A/G-Sepharose beads (Pierce Chemical). After 2 h of incubation, the beads were washed with lysis buffer and assayed in a kinase assay mixture containing 50 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 2 mM dithiothreitol, 1 μCi [γ-^{32}P]ATP, 5 μM unlabeled ATP, and 2 μg GST-I-κBα (amino acids 1–54) substrate in a volume of 30 μL. After incubation at 30°C for 30 min, the reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized with an autoradiography

2.8 PGE_2 assay

RAW 264.7 cells were cultured in six-well plates and were incubated with LPS in the absence or presence of agents for 18 h. A 100-μL aliquot of culture supernatant was collected for the determination of PGE_2 concentration by ELISA.

2.9 Nitrite assay

The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction. A 100-μL aliquot of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water); absorbance of the mixture at 550 nm was determined with an ELISA plate reader.

2.10 Statistical analysis

Data were analyzed using Student's *t*-test, one-way analysis of variance or Newman-Keuls multiple comparison test. Differences were considered significant when $p < 0.01$.

3 Results

3.1 Effects of DiMC on NO production and iNOS expression

The anti-inflammatory curcumin has been reported to inhibit NO production and iNOS expression in activated macrophages [5]. Whether the curcumin analogue DiMC can also inhibit NO production and iNOS expression has not yet been investigated. In LPS-activated RAW264.7 macrophages, DiMC inhibited NO production and iNOS expression in a concentration-dependent manner (Fig. 2). At 10 μM, DiMC almost completely inhibited NO production and iNOS expression. However, DiMC exhibited cytotoxicity at concentrations higher than 20 μM in RAW264.7 macrophages (data not shown).

3.2 Comparative effects of curcumin analogues on NO production and iNOS expression

We next compared the effects of DiMC with those of curcumin analogues, and found that treatment of RAW264.7 macrophages with curcumin, DiMC or BDMC inhibited LPS-induced NO production and iNOS expression, but the potency varied. At the same concentration (10 μM), DiMC was the most potent, followed by curcumin and BDMC (Fig. 3). Interestingly, THC, which lacks conjugated double bonds in the central seven-carbon chain, failed to inhibit LPS-induced NO production and iNOS expression (Fig. 3).

3.3 Effects of curcumin analogues on NF-κB activation

Transcription factors belonging to the NF-κB family regulate a range of genes that mediate inflammation [14]. NF-κB exists in most cells as homodimeric or heterodimeric complexes of p50 and p65 subunits and remains inactive in its cytosolic form associated with its inhibitor protein, I-κBa. Upon its activation, NF-κB increases the expression

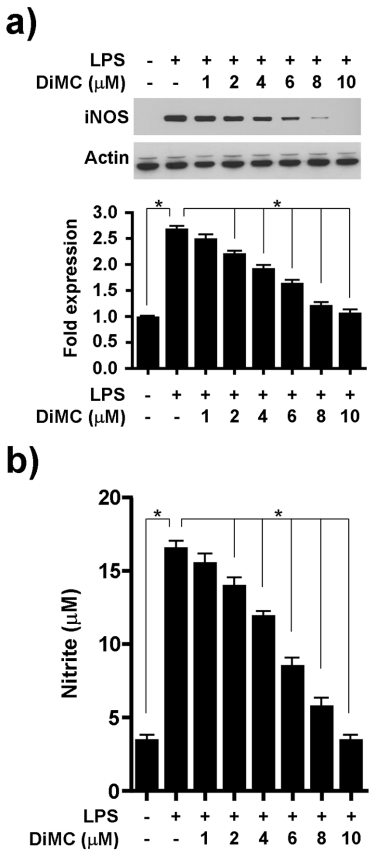

Figure 2. Effects of DiMC on NO production and iNOS expression in RAW264.7 macrophages. (a) Effects of DiMC on LPS-induced iNOS expression. Macrophages were pre-incubated for 2 h with indicated concentrations of DiMC, and then activated for 6 h with 1 μg/mL LPS. Western blot analysis for iNOS expression (upper panel) and densitometry analysis of all bands (lower panel) were carried out as described in Section 2. One of three blots is shown and each bar represents the mean ± SD from three separate experiments. *$p < 0.01$. (b) Effects of DiMC on LPS-induced NO production. Macrophages were pre-incubated for 2 h with indicated concentrations of DiMC, and then activated for 18 h with 1 μg/mL LPS. Nitrite assay were carried out as described in Section 2. Each bar represents the mean ± SD from three separate experiments. *$p < 0.01$.

of pro-inflammatory genes, including iNOS [15]. Because curcumin analogues mediate their anti-inflammatory effects primarily through the inactivation of NF-κB [5, 8, 16], we investigated the effects of DiMC on LPS-induced NF-κB activation. As indicated by the DNA-binding assay (EMSA), treatment of RAW264.7 macrophages with LPS induced NF-κB activation, which was inhibited by DiMC, curcumin and BDMC, but not by THC (Fig. 4a). Since the translocation of activated NF-κB is preceded by phosphorylation of the p65 subunit of NF-κB [17], protein extracts of macrophages were probed for the phosphorylated p65 NF-κB-subunit after stimulation with LPS in the absence or

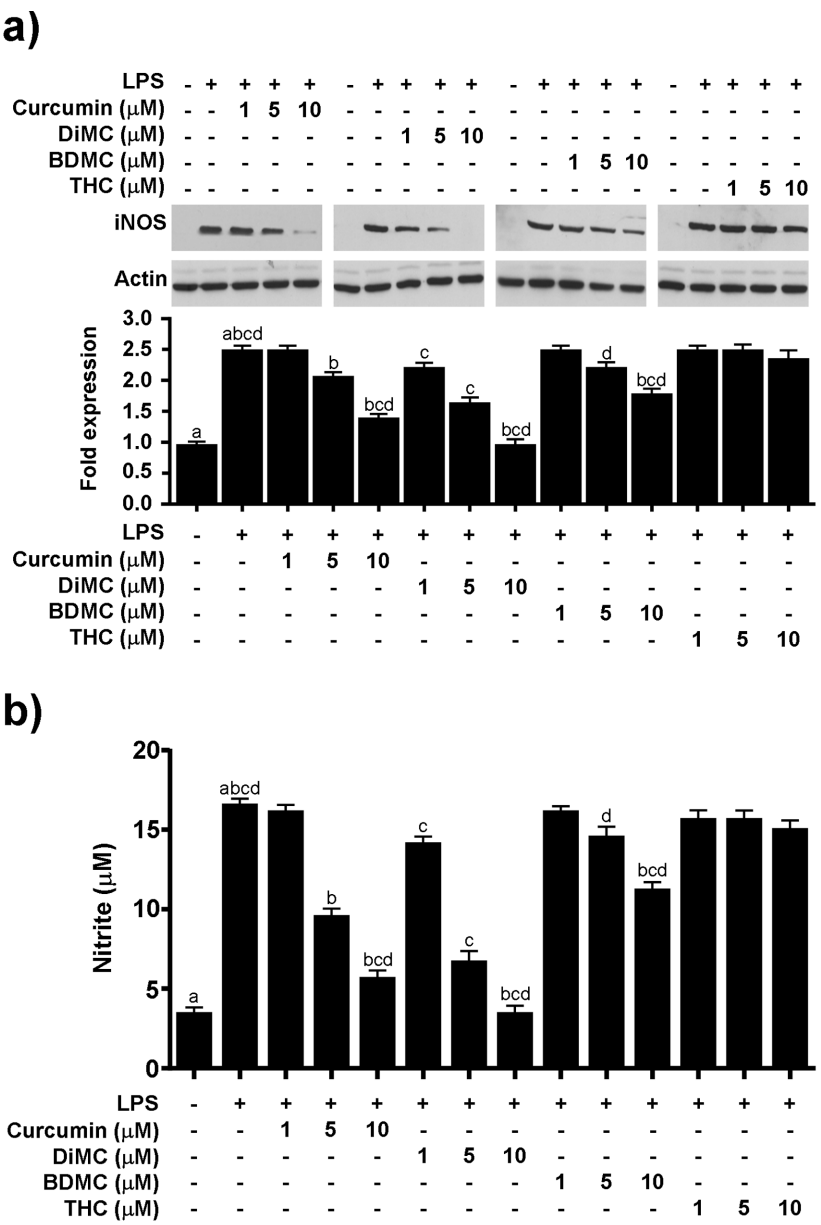

Figure 3. Comparative effects of curcumin, DiMC, BDMC and THC on NO production and iNOS expression in RAW264.7 macrophages. (a) Effects of curcumin analogues on LPS-induced iNOS expression. Macrophages were pre-incubated for 2 h with indicated concentrations of curcumin, DiMC, BDMC or THC, and then activated for 6 h with 1 μg/mL LPS. Upper panel: Western blot analysis for iNOS expression; lower panel: densitometry analysis of all bands. One of three blots is shown and each bar represents the mean ± SD from three separate experiments. Different letters indicate statistically significant differences ($p < 0.01$) among different groups. (b) Effects of curcumin analogues on LPS-induced NO production. Macrophages were pre-incubated for 2 h with indicated concentrations of curcumin, DiMC, BDMC or THC, and then activated for 18 h with 1 μg/mL of LPS. Nitrite assay were carried out as described in Section 2. Each bar represents the mean ± SD from three separate experiments. Different letters indicate statistically significant differences ($p < 0.01$) among different groups.

presence of curcumin analogues. The level of phosphorylated p65 protein was markedly up-regulated in the LPS-stimulated macrophages, but remained almost at control level when the macrophages were stimulated with LPS in the presence of 10 μM DiMC (Fig. 4b). Our ELISA data further showed that LPS treatment resulted in enhanced p65 DNA-binding activity of nuclear extract, compared to untreated control, and this enhancement in p65 DNA-binding activity was attenuated by DiMC (Fig. 4c). The highest NF-κB inhibitory activity was associated with DiMC, with curcumin showing less activity and BDMC the least. THC was almost completely ineffective in NF-κB inactivation (Fig. 4c). An essential prerequisite for the activation of NF-κB is the phosphorylation of I-κBα [17]. To test whether inhibition of LPS-induced NF-κB activation occurred through inhibition of I-κBα phosphorylation, we treated RAW264.7 macrophages with LPS in the absence or presence of curcumin analogues. Phosphorylation of I-κBα in the cytoplasm of the cells was determined by Western blot analysis using an antibody capable of recognizinge the phosphorylated form of I-κBα. We found that suppression of I-κBα phosphorylation correlated with the inhibition of DNA-binding activity of NF-κB. Again, DiMC was the most potent compound, followed by curcumin and BDMC (Fig. 4d).

IKK is required not only for I-κBα phosphorylation but also for NF-κB activation [18]. Since curcumin analogues inhibit NF-κB activation by inhibiting I-κBα phosphoryla-

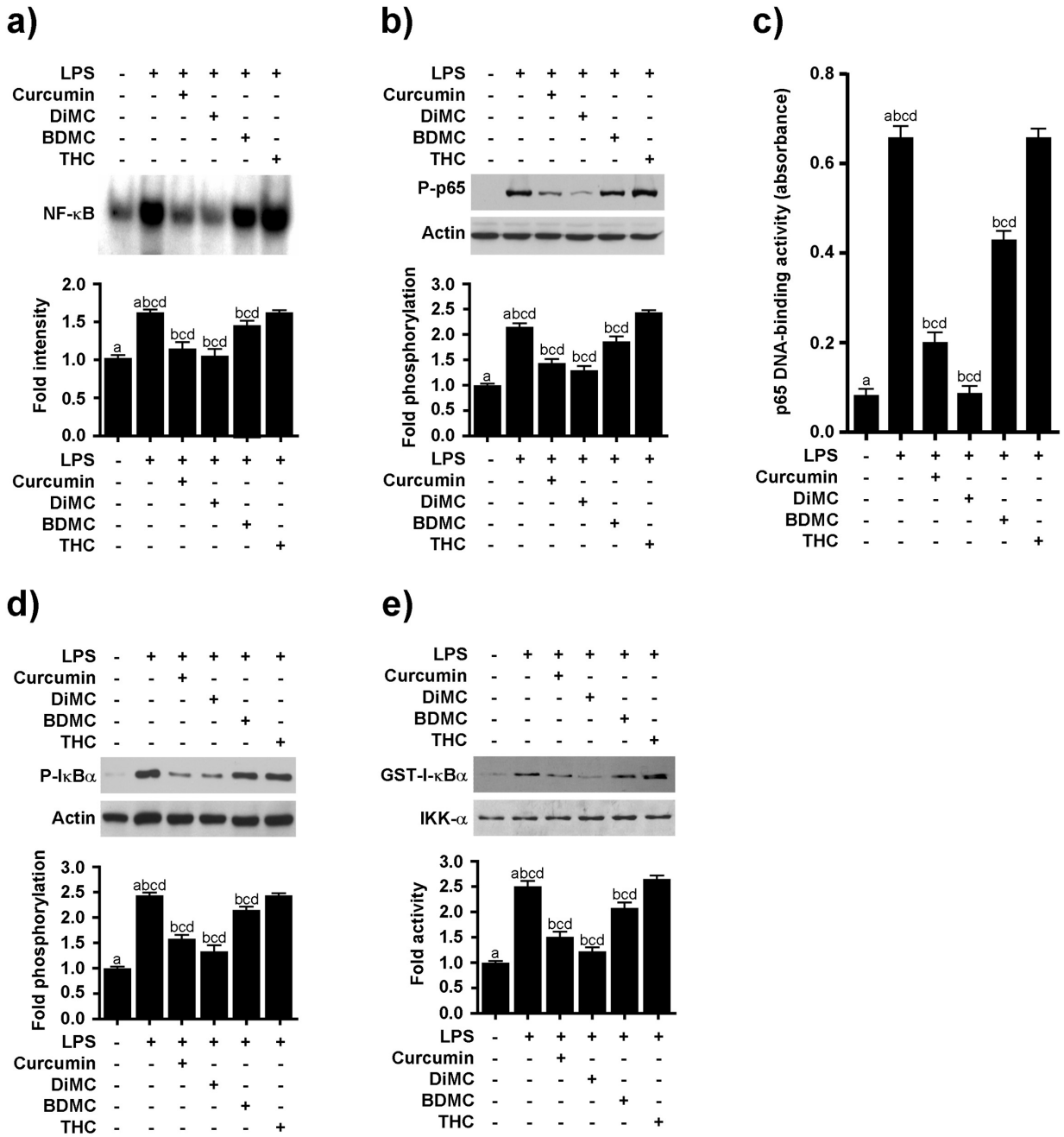

Figure 4. Comparative effects of curcumin, DiMC, BDMC and THC on NF-κB activation in RAW264.7 macrophages. (a) Effects of curcumin analogues on NF-κB activation. Macrophages were pre-incubated for 2 h with 10 μM of each compound, treated with 1 μg/mL LPS for 2 h, and subjected to EMSA to assay for NF-κB activation. (b) Effects of curcumin analogues on p65 phosphorylation. Macrophages were pre-incubated for 2 h with 10 μM of each compound, treated with 1 μg/mL LPS for 2 h, and subjected to Western blot analysis to assay for phosphorylated p65. (c) Effects of curcumin analogues on p65 DNA-binding activity. Macrophages were pre-incubated for 2 h with 10 μM of each compound, then treated with 1 μg/mL LPS for 2 h, and subjected to ELISA to assay for binding of p65 protein to DNA. (d) Effects of curcumin analogues on I-κBα phosphorylation. Macrophages were pre-incubated for 2 h with 10 μM of each compound, then treated with 1 μg/mL LPS for 1 h and subjected to Western blot analysis to assay for phosphorylated I-κBα. (e) Effects of curcumin analogues on LPS-induced IKK activity. Macrophages were pretreated with 10 μM curcumin analogues for 2 h and then activated with 1 μg/mL LPS for 1 h. Whole-cell extracts were prepared, and 200 μg protein was immunoprecipitated with antibody against IKK-α. An immune complex kinase assay was performed as described in Section 2. To examine the effect of curcumin on the level of expression of IKK-α proteins, 30 μg whole-cell protein was subjected to Western blot analysis. One of three blots is shown and each bar represents the mean ± SD from three separate experiments. Different letters indicate statistically significant differences ($p < 0.01$) among different groups.

tion, we thus examined their effects on LPS-induced activation of IKK. The IKK activity was low in unstimulated macrophages (Fig. 4e, lane 1). Stimulation of macrophages with LPS increased the kinase activity of IKK (Fig. 4e, lane 2). In contrast, pretreatment with curcumin, DiMC or BDMC (10 μg/mL) before LPS stimulation inhibited the kinase activity of IKK (Fig. 4e). THC had no effect on kinase activity of IKK (Fig. 4e, lane 6).

3.4 Effects of curcumin analogues on PGE_2 production and COX-2 expression

NF-κB activation has been also linked with the regulation of COX-2 expression [18]. We evaluated whether the suppression of LPS-induced NF-κB activation correlated with the suppression of COX-2 expression. Upon stimulation with LPS, RAW264.7 macrophages expressed a high level of COX-2 protein as compared with the untreated macrophages (Fig. 5). DiMC (10 μM) suppressed COX-2 expression and inhibited PGE_2 production in LPS-stimulated macrophages (Fig. 5). At the same concentration, DiMC was the most potent, followed by curcumin and BDMC (Fig. 5). THC failed to inhibit LPS-induced PGE_2 production and COX-2 expression (Fig. 5). These results correlated well with the data on suppression of NF-κB activation.

3.5 Effects of the mixture of curcumin and DiMC on NO production and iNOS expression

It has been reported that anti-inflammatory effects of curcumin are synergistically enhanced by curcumin analogues [8, 19]. We thus examined whether the inhibitory effect of curcumin on iNOS expression and NO production could be enhanced by DiMC. As shown in Fig. 6, treatment of RAW264.7 macrophages with 1 μM curcumin and 1 μM DiMC demonstrated a synergistic effect on suppression of LPS-induced iNOS expression, leading to a significant inhibition of LPS-induced NO production.

4 Discussion

In the present study, we investigated whether curcumin analogues differed in their ability to inhibit NO production and iNOS expression in activated macrophages. Our results demonstrate that among the curcumin analogues tested, DiMC is the most effective in inhibiting NO production and iNOS expression. In LPS-stimulated RAW264.7 macrophages, DiMC, curcumin and BDMC, but not THC, inhibited NO production by suppressing LPS-induced iNOS expression. However, despite displaying a similar basic chemical structure, in which two aromatic rings are bridged by two α,β-unsaturated carbonyl groups, the compounds differed in their ability to inhibit LPS-induced NO production and iNOS expression.

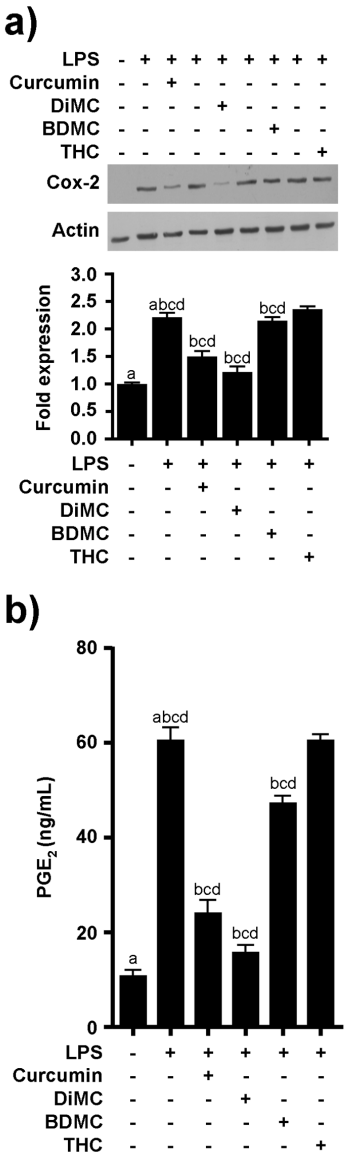

Figure 5. Comparative effects of curcumin, DiMC, BDMC and THC on PGE_2 production and COX-2 expression in RAW264.7 macrophages. (a) Effects of curcumin analogues on LPS-induced COX-2 expression. Macrophages were pre-incubated for 2 h with indicated concentrations of curcumin, DiMC, BDMC or THC, and then activated for 6 h with 1 μg/mL LPS. Western blot analysis for COX-2 expression (upper panel) and densitometry analysis for all bands (lower panel) were carried out as described in Section 2. One of three blots is shown and each bar represents the mean ± SD from three separate experiments. Different letters indicate statistically significant differences ($p < 0.01$) among different groups. (b) Effects of curcumin analogues on LPS-induced PGE_2 production. Macrophages were pre-incubated for 2 h with indicated concentrations of curcumin, DiMC, BDMC or THC, and then activated for 18 h with 1 μg/mL LPS. PGE_2 assay were carried out as described in Section 2. Each bar represents the mean ± SD from three separate experiments. Different letters indicate statistically significant differences ($p < 0.01$) among different groups.

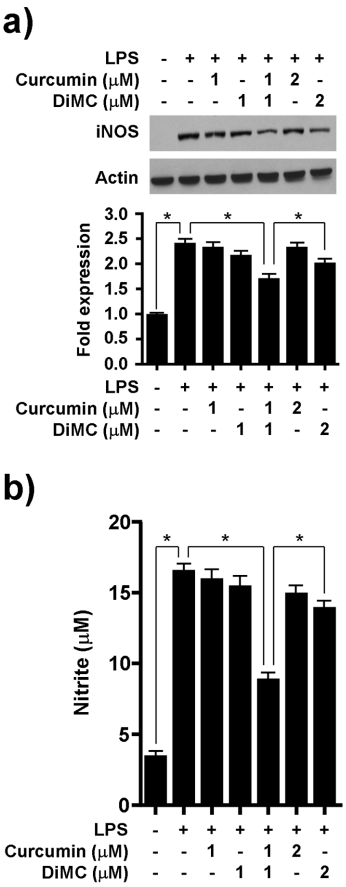

Figure 6. Effects of a combination of curcumin and DiMC on NO production and iNOS expression in RAW264.7 macrophages. (a) Effects of a combination of curcumin and DiMC on LPS-induced iNOS expression. Macrophages were pre-incubated for 2 h with indicated concentrations of curcumin, DiMC, or curcumin plus DiMC, and then activated for 6 h with 1 μg/mL of LPS. Western blot analysis for iNOS expression (upper panel) and densitometry analysis for all bands (lower panel) were carried out. One of three blots is shown and each bar represents the mean ± SD from three separate experiments. $^*p < 0.01$. (b) Effects of a combination of curcumin and DiMC on LPS-induced NO production. Macrophages were pre-incubated for 2 h with indicated concentrations of curcumin, DiMC, or curcumin plus DiMC, and then activated for 18 h with 1 μg/mL of LPS. Nitrite assay were carried out as described in Section 2. Each bar represents the mean ± SD from three separate experiments. $^*p < 0.01$.

The α,β-unsaturated carbonyl group is an important structure of curcumin analogues, because THC lacking this functional group was found to be virtually inactive in inhibiting NO production and iNOS expression. Compounds carrying this reactive group have been reported to inhibit NO production and iNOS expression through inactivation of NF-κB [20, 21]. The anti-inflammatory cyclopentenone, possessing an α,β-unsaturated carbonyl group in its chemical structure, has been shown to directly inhibit IKK by modifying the cysteine residues, thereby attenuating I-κBα phosphorylation to abolish NF-κB activity [22]. NF-κB is a key transcriptional factor for expression of pro-inflammatory mediators such as iNOS [14]. By structural analogy, curcumin analogues, except THC, have a chemical property resembling that of cyclopentenone, suggesting that active curcumin analogues may be capable of diminishing NF-κB activation. Indeed, active analogues of curcumin inhibited LPS-induced NF-κB activation by blocking I-κBα phosphorylation and IKK-α activity. Thus, one of the action mechanisms by which active curcumin analogues inhibit NO production and iNOS expression may be due to a possible abrogation of NF-κB activity. We speculate that curcumin analogues are effective, at least in part, because they bear the α,β-unsaturated carbonyl group. However, we found that, although curcumin analogues have the same functional group, they varied in their ability to inhibit iNOS-dependent NO production and NF-κB activation, with DiMC being the most active, followed by curcumin and BDMC being the least active. As DiMC contains four methoxy groups on two aromatic rings, curcumin contains two and BDMC contains none (see chemical structures shown in Fig. 1), their differential efficiency in iNOS suppression and NF-κB inactivation could be due to the increased number of methoxy groups. How the methoxy groups mediate iNOS suppression and NF-κB inactivation, however, is currently unknown. Recently, Tamvakopoulos *et al.* [9] demonstrated that in, comparison with curcumin, metabolism of DiMC is less extensive in cellular system. Thus, it is possible that the differential efficiency of curcumin analogues may be associated with their different metabolic stabilities. The differential efficiency of curcumin analogues may be caused by a different permeability of the compounds across cell membrane, probably due to the number of hydrophobic methoxy groups.

NF-κB activation has been linked with the regulation of COX-2 expression [18]. Among the analogs tested, DiMC was the most effective in suppressing COX-2 expression, followed by curcumin and BDMC. These data are in agreement with the ability of these compounds to inhibit NF-κB activation, further supporting the hypothesis that one of their action mechanisms is due to abrogation of NF-κB activity. THC was almost completely ineffective in suppressing COX-2 expression, suggesting that the conjugated bonds in the central seven-carbon chain are needed for anti-inflammatory activity.

Whether *in vitro* concentrations of curcumin analogues employed here are related to those seen *in vivo* is not clear. In a Phase I clinical trial, volunteers were shown to tolerate a curcumin dose as high as 8 g/day with no side effects [23]; however, curcumin has a very low systemic bioavailability in the plasma [7]. The dose of 8 g/day resulted in a peak serum concentration of about 2 μM [23]. Despite such a low bioavailability, therapeutic efficacy of curcumin against various human diseases, including inflammatory diseases, has been documented [24, 25]. In mice, following

administration, both curcumin and DiMC were rapidly cleared from the plasma and the resulting plasma concentrations for both compounds were low (about 1 μM) with a dose of 5 mg/kg [9]. However, a comparative study on the stability of curcumin and DiMC *in vivo* revealed that plasma levels for DiMC were higher (about threefold) in comparison with curcumin [9]. This leads us to the assumption that higher doses and/or different routes of administration would have to be considered to achieve clinical efficacy with either compound. In this regard, our finding that the mixture of DiMC and curcumin at a physiological concentration (2 μM) was more active in suppressing iNOS expression than either curcumin or DiMC alone is of interest. These results are similar to those of Tonnesan *et al.* [19] who showed that the anti-inflammatory activities of curcumin are synergistically enhanced by its analogues. Sandur *et al.* [8] have also demonstrated that the commercially available curcumin, a mixture of curcumin, demethoxycurcumin and BDMC, is more active in suppressing NF-κB activation than the individual components. The mechanism of synergism between curcumin and its analogues, however, is unknown. We speculate that a combination of curcumin and DiMC may improve the bioavailability of curcumin *in vivo*.

In summary, we have shown, for the first time, that the curcumin analogue DiMC inhibits NO production and iNOS expression *via* NF-κB inactivation in LPS-stimulated macrophages. We also demonstrated that DiMC is more potent than curcumin in the ability to inhibit iNOS-dependent NO production, COX-2-dependent PGE_2 production and NF-κB inactivation. Finally, we suggest that the inhibitory effects of DiMC on NO production, iNOS expression and NF-κB activation may be due not only to the conjugated double bonds in the central seven-carbon chain but also to the increased number of methoxy groups at two aromatic rings.

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