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Comparative Studies on the Suppression of Nitric Oxide Synthase by Curcumin and Its Hydrogenated Metabolites through Down-regulation of IκB Kinase and NFκB Activation in Macrophages

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ABSTRACT. Nitric oxide (NO) plays an important role in inflammation and in the multiple stages of carcinogenesis. In this study, we investigated the inhibitory effects of curcumin and its metabolites, tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin, on the induction of NO synthase (NOS) in RAW 264.7 cells activated with lipopolysaccharide (LPS). Western blotting and northern blotting analyses demonstrated that curcumin strongly reduced 130-kDa protein and 4.5-kb mRNA levels of iNOS in LPS-activated macrophages compared with its metabolites, tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin. Moreover, electrophoretic mobility shift assay (EMSA) experiments indicated that curcumin blocked the LPS-induced binding of nuclear factor-κB (NFκB), a transcription factor necessary for iNOS induction to its ^{32}P-labeled double-stranded oligonucleotide probe. The inhibition of NFκB activation occurred through the prevention of inhibitor κB (IκB) degradation. Transient transfection experiments also showed that curcumin inhibited NFκB-dependent transcriptional activity. Curcumin blocked the disappearance of inhibitory κBα (IκBα) and p65 from the cytosolic fraction, and inhibited the phosphorylation of IκBα. Furthermore, we showed that curcumin could inhibit the IκB kinase 1 (IKK1) and IκB kinase 2 (IKK2) activities induced by LPS, but tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin were less active. These results suggest that curcumin may exert its anti-inflammatory and anti-carcinogenic properties by suppressing the activation of NFκB through inhibition of IKK activity. BIOCHEM PHARMACOL **60**;11:1665–1676, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. curcumin; inducible NO synthase (iNOS); NFκB; IKK; RAW 264.7 monocytes; macrophages

Curcumin (diferuloylmethane), a dietary pigment from *Curcuma longa L.*, gives the golden-yellow color and unique flavor to curry. The anti-carcinogenic effects of this compound are demonstrated by its ability to inhibit tumor initiation by azoxymethane, benzpyrene, and 7,12-dimethylbenz[*a*]anthracene, and to suppress tumor promotion by phorbol esters [1–3]. In addition, curcumin inhibited the induction of iNOS§ in activated macrophages and the production of NO by mouse peritoneal macrophages [4–6]. It has been shown that curcumin strongly inhibits both c-Jun and NFκB activation by phorbol 12-myristate 13-acetate or tumor necrosis factor-α treatment [7]. This inhibitory effect may account for the anti-inflammatory and antioxidant properties of curcumin [8, 9].

NO is produced endogenously by a family of NOSs, with a wide range of physiological and pathophysiological actions [10, 11]. At higher concentrations, NO is implicated in the pathogenesis of stroke and other degenerative diseases, such as demyelinating conditions and ischemic and traumatic injury [12]. NOS enzymes are classified into two groups, one type (cNOS) is constitutively present in several cell types (e.g. neurons and endothelial cells) and is regulated predominantly at the post-transcriptional level by calmodulin in a Ca^{2+}-dependent manner [13]. In contrast, the inducible form (iNOS), expressed in various cell types, including vascular smooth muscle cells, macrophages, hepatocytes, and astrocytes, is induced in response to proinflammatory cytokines and bacterial LPS [14–18].

Increased NOS expression and/or activity was reported in human gynecological [19], breast [20], and central nervous system [21] tumors. Also, nitrotyrosine accumulation in inflamed mucosa of patients with ulcerative colitis and gastritis indicates the production of NO and its involvement in the pathogenesis of these diseases [22]. The

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§ *Abbreviations*: DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; IκB, inhibitor κB; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; and PMSF, phenylmethylsulfonyl fluoride.

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mechanism of the signal transduction cascade involved in the induction of iNOS in response to LPS and cytokines has become an active area of investigation [23]. Previous reports have shown a potential role for tyrosine kinase in LPS promoter containing 24 transcriptional factor binding sites, including those for the NFκB family, which appear to be essential for the enhanced iNOS gene expression seen in macrophages exposed to LPS [24]; the p65 NFκB also seems to be responsible for iNOS induction in astrocytes [25]. Activation of NFκB by LPS is induced by a cascade of events leading to the activation of IKK, which phosphorylates IκB, leading to its degradation and translocation of NFκB to the nucleus [26]. Importantly, iNOS has been shown to be involved in the regulation of cyclooxygenase-2 (COX-2), which plays a pivotal role in colon tumorigenesis [27]. These observations clearly suggest that iNOS may enhance turmorigenesis. The anti-inflammatory properties of curcumin have been attributed to its direct inhibition of COX-2 [28].

Although the anti-inflammatory and anti-carcinogenic characteristics of curcumin have been studied, the action mechanism of curcumin is complicated. We previously reported that curcumin is unstable at neutral and basic pH values and is degraded to ferulic acid and feruloyl methane [29]. In addition, we have found that most of curcumin administered in mice is reduced to dihydrocurcumin, tetrahydrocurcumin, and trace hexahydrocurcumin by an endogenous reductase system in a stepwise manner and subsequently glucuronidated by UDP-glucuronosyltransferase [30]. In recent studies, tetrahydrocurcumin exhibited stronger antioxidative activities than curcumin in several *in vitro* systems [31, 32]. Thus, tetrahydrocurcumin was thought to be one of the metabolites with higher physiological and pharmacological activities than curcumin in the intestine. Tetrahydrocurcumin has been reported recently to be a less effective chemopreventive agent in mouse skin than curcumin [33]. In contrast to the case of skin carcinogenesis, mice fed 0.5% tetrahydrocurcumin in the diet showed a stronger inhibition of 1,2-dimethylhydrazine-induced mouse colon carcinogenesis than mice fed curcumin [34]. These conflicting findings prompted us to determine the efficacies of curcumin, tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin to inhibit iNOS activity in activated macrophages.

In this study, we examined the effects of curcumin, tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin on NO generation, cytosolic iNOS protein, steady-state mRNA levels, gene promoter activity, and IKK activity to assess the mechanisms of action of these compounds.

MATERIALS AND METHODS

Reagents

LPS (*Escherichia coli* 0127: E8), sufanilamide, naphthylethylenediamine dihydrochloride, and DTT were purchased from the Sigma Chemical Co. Acrylamide was purchased from the E. Merck Co. Synthetic curcumin was provided by the Yung Shin Pharmaceutical Ind. Co. Tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin (Fig. 1) were prepared by hydrogenation of curcumin with PtO_2 as described in our previous report [30].

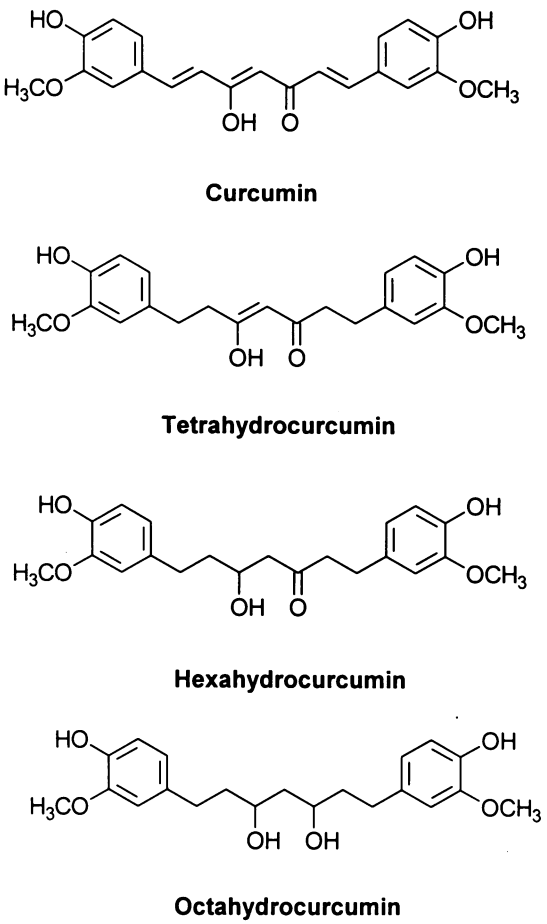

FIG. 1. Structures of curcuminoids.

Preparation of Curcumin Derivatives

Curcumin was converted to tetrahydrocurcumin (4H), hexahydrocurcumin (6H), and octahydrocurcumin (8H) by hydrogenation, with PtO_2 as the catalyst, according to the method of Uehara *et al.* [35]. TLC analysis on silica gel plates (Silica Gel 60 $F_{254,}$ 20 × 20 cm; thickness, 2 mm; Catalog No. 5717; E. Merck) was performed in a rectangular or cylindrical glass chamber covered with a glass plate. The inner side of the chamber contained Whatman No.1 filter paper to prompt and ensure the saturation of solvent vapor in the chamber. After hydrogenation, tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin were purified by preparative TLC (4% ethanol in $CHCl_{3;}$ the R_f values of tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin were 0.83, 0.63, and 0.13, respectively), and the appropriate fractions were collected and combined. HPLC analysis of hexahydrocurcumin and octahydrocurcumin revealed a single peak; tetrahydrocurcumin

revealed two peaks. The identity and purity of tetrahydrocurcumin were confirmed by examining the mass spectrometry (MS), UV, and NMR spectra. Tetrahydrocurcumin appeared as off-white crystals with a melting point of 98–99°. NMR analysis in $CDCl_3$ (^1H NMR) revealed: δ 2.57 (4H, *t*, $J = 2$ Hz), 2.81 (4H, *t*, $J = 2$ Hz), 3.78 (6H, *s*, 2 × OMe), 5.63 (1H, *s*), 6.64 (2H, *s*), 6.71 (2H, *s*), and 6.81 (2H, *q*, $J = 7.2$ Hz). Anal. Calc. for $C_{21}H_{24}O_6$: C, 67.74; H, 6.45; Found: C, 67.52; H, 6.68.

Cell Culture

RAW 264.7 cells, derived from murine macrophages and obtained from the American Type Culture Collection, were cultured in RPMI-1640 (without phenol red) supplemented with 10% endotoxin-free, heat-inactivated fetal bovine serum (Gibco), 100 U/mL of penicillin, and 100 μg/mL of streptomycin. When the cells reached a density of 2–3 × 10^6 cells/mL, they were activated by incubation in medium containing *E. coli* LPS (100 ng/mL). Various concentrations of test compounds dissolved in dimethyl sulfoxide were added together with LPS.

Cytotoxicity Assay

The RAW 264.7 cells were cultivated at a density of 2 × 10^5 cells in a 6-well plate. The polyphenols studied were added to the medium 18 hr after the inoculation. The cells were harvested after 18 hr. Viability was determined by trypan blue exclusion and microscopy examination.

Nitrite Assay

The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction [36]. One hundred microliters 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 enzyme-linked immunosorbent assay plate reader (Dynatech MR-7000; Dynatech Laboratories).

Western Blotting

Total cellular extracts were prepared in Gold lysis buffer. Aliquots containing 30–50 μg of total protein (for IKK1, IKK2, iNOS, and α-tubulin) or the cytosolic fraction (for IκBα and p65) were separated on SDS-polyacrylamide minigels (8% for IKK1, IKK2, and iNOS, and 10% for IκBα and p65) and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). The membrane was blocked overnight at room temperature with blocking solution [20 mM Tris–HCl (pH 7.4), 125 mM NaCl, 0.2% Tween 20, 1% BSA, and 0.1% sodium azide] and then incubated with anti-IκBα, anti-p65, IKK1, or IKK2 polyclonal antibodies (Santa Cruz Biotechnology), anti-macNOS monoclonal antibody (Transduction Laboratories), anti-phospho (Ser 32)-specific IκBα (New England Biolabs), or anti-α-tubulin monoclonal antibodies (Oncogene Science Inc.) at room temperature for 1 hr. iNOS, IKK1, IKK2, IκBα, p65, and α-tubulin proteins were detected by chemiluminescence (ECL, Amersham) or by incubation with nitroblue tetazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as suggested by the manufacturer (Sigma Chemical Co.).

Preparation of Probe

Polymerase chain reaction primer for mouse iNOS cDNA probe was synthesized according to the following oligonucleotide sequence: forward primer 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ (2944–2968); reverse primer 5′-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3′ (3416–3440). The probe was prepared as detailed in our previous report [37].

Northern Blotting

Total RNA (20 μg/mL) was denatured with formaldehyde/formamide and incubated at 65° for 15 min, size-fractionated on 1.2% formaldehyde-containing agarose, and transferred onto Hybond-N nylon membrane (Amersham Corp.) in 20 × standard saline citrate (3 M sodium chloride and 0.3 M sodium citrate, pH 7.0). The blotted membrane was hybridized with iNOS fragment, which was labeled with ^{32}P by using a Random Primer Labeling kit (Amersham). After hybridization, the membrane was washed, dried, and autoradiographed with Kodak x-ray film using intensifying screens at −80°.

EMSAs for NFκB

Nuclear and cytoplasmic extracts were prepared according to a modification of the method described by Lin and Lin [37]. Briefly, at the end of the culture, the cells were suspended in hypotonic buffer A [10 mM HEPES (pH 7.6), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF] for 10 min on ice, and vortexed for 10 sec. Nuclei were pelleted by centrifugation at 12,000 *g* for 20 sec, and the supernatants, containing cytosolic proteins, were collected. Then the pellets were suspended in buffer C [20 mM HEPES (pH 7.6), 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF] for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 12,000 *g* for 20 min and stored at −70°.

For electrophoretic mobility shift assays, 6 μg of each nuclear extract was mixed with the ^{32}P-labeled double-stranded NFκB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) probe, and incubated at room temperature for 20 min. The incubation mixture included 1 μg of poly(dI-dC) in a binding buffer [25 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, and 50 mM NaCl]. The DNA/protein complex was electrophoresed on 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. Radioactive bands were detected by autoradiography.

Transient Transfection and Luciferase Assay

The luciferase assay was performed as described by George *et al.* [38] with some modifications. RAW 264.7 cells were seeded in a 60-mm dish. When the cells reached confluence, the medium was replaced with serum-free Opti-MEM (Gibco). The cells then were transfected with the pNFκB-Luc plasmid reporter gene (Stratagene) using LipofectAMINE™ reagent (Gibco, NRL, Life Technologies, Inc.). After another 24 hr of incubation, the medium was replaced with complete medium. After 24 hr, the cells were trypsinized, and equal numbers of cells were plated in 12-well tissue culture plates for 18 hr. Then the cells were incubated with 100 ng/mL of LPS and TF-3 for 3 hr. Next each well was washed twice with cold PBS and harvested in 150 μL of lysis buffer [0.5 M HEPES (pH 7.8), 1% Triton N-101, 1 mM $CaCl_2$, and 1 mM $MgCl_2$]. Luciferase activity was assayed by means of the LucLite™ luciferase reporter gene kit (Packard BioScience Co.) with 100 μL of cell lysate used in each assay. Luminescence was measured on a Top Counter Microplate Scintillation and Luminescence Counter (Packard 9912 V) in the single photon counting mode for 0.1 min/well, following a 5-min adaptation in the dark. Luciferase activities were determined and normalized to protein concentrations.

IκB Kinase

IκB kinase was assayed as performed by Yamaoka *et al.* [39] with some modifications. Whole cell extracts were lysed with Gold lysis buffer [10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris–HCl (pH 7.9), 100 μM β-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 μg/mL of aprotinin, and 10 μg/mL of leupeptin] for 30 min at 4°. The cell lysates were clarified by centrifugation at 12,000 *g* for 10 min at 4°. The cell extracts then were subjected to immunoprecipitation with anti-IKK1 and anti-IKK2 specific antibodies (Santa Cruz Biotechnology) in TNT buffer [200 mM NaCl, 20 mM Tris–HCl (pH 7.5), and 1% Triton X-100, supplemented with 300 μM sodium orthovanadate, 2 μM PMSF, 10 μg/mL of aprotinin, and 1 μg/mL of leupeptin]. Then the IKK–antibody complex was precipitated with protein-A Sepharose beads for 18 hr at 4°, washed three times with TNT buffer, and then washed three times with kinase buffer [20 mM HEPES, 10 mM $MgCl_2$, 300 μM sodium orthovanadate, 20 mM β-glycerophosphate, 1 mM NaF, 2 mM DTT, and 50 mM NaCl (pH 7.5) supplemented with 2 μM PMSF, 10 μg/mL of aprotinin, 1 μg/mL of leupeptin]. The purified enzyme was in kinase buffer incubated with a GST-IκBα (1–317) fusion protein (Santa Cruz Biotechnology) as the substrate. Kinase reactions were run for 30 min at 30° using 10 μCi of [γ-^{32}P]ATP, and terminated by the addition of 5x SDS–PAGE sample-buffer and boiling for 10 min. For *in vitro* kinase assay, curcumin was added into the washed immunoprecipitates for 30 min at 4° before the kinase reaction. The reaction products were resolved by SDS–PAGE in 10% gels visualized by autoradiography with Kodak X-Omat film for 3 hr at room temperature, and quantified by densitometry (IS-1000 Digital Imaging System).

RESULTS

Effects of Various Curcuminoids on iNOS Protein

Curcumin, tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin were examined to determine whether they affect iNOS protein and NO production in macrophages activated with LPS for 6–18 hr. Inhibition of iNOS protein by these compounds was detected at 10 μM. The inhibitory activity of tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin was less than that of curcumin (Fig. 2A). The inhibition of NO generation in the LPS-activated macrophages with curcumin, tetrahydrocurcumin (4H), hexahydrocurcumin (6H), and octahydrocurcumin (8H) was also investigated. Curcumin was found to inhibit NO generation markedly, whereas tetrahydrocurcumin (4H), hexahydrocurcumin (6H), and octahydrocurcumin (8H) were shown to be less active (Fig. 2B). The cytotoxicities of the curcuminoids at different concentrations (0.5, 1.0, 2.5, 5.0, and 10 μM) were evaluated by the trypan blue exclusion assay. It was apparent that no cytotoxic effect of the curcuminoids was detected even at 10 μM (data not shown).

Effects of Curcuminoids on iNOS Gene Expression in LPS-Activated Macrophages

To investigate whether the suppression of iNOS activity by curcuminoids was due to reduced iNOS mRNA, macrophages were activated with LPS in the presence or absence of curcuminoids (10 μM) for 6 hr, harvested, and assayed for iNOS mRNA expression by northern blotting analysis. Coincubation of macrophages with LPS plus curcumin caused a complete suppression of iNOS induction after 6 hr of incubation; weaker inhibition was found in the presence of LPS plus tetrahydrocurcumin (4H), hexahydrocurcumin (6H), or octahydrocurcumin (8H), respectively (Fig. 3).

Effect of Curcuminoids on LPS-Induced Nuclear Proteins with NFκB Binding Activity

Deletion and mutational analyses have demonstrated that the transcription factor NFκB is involved in the activation of iNOS by LPS. To investigate if the curcuminoids selectively inhibited activation of NFκB, analysis of NFκB binding activity of extracted nuclear proteins by gel mobil-

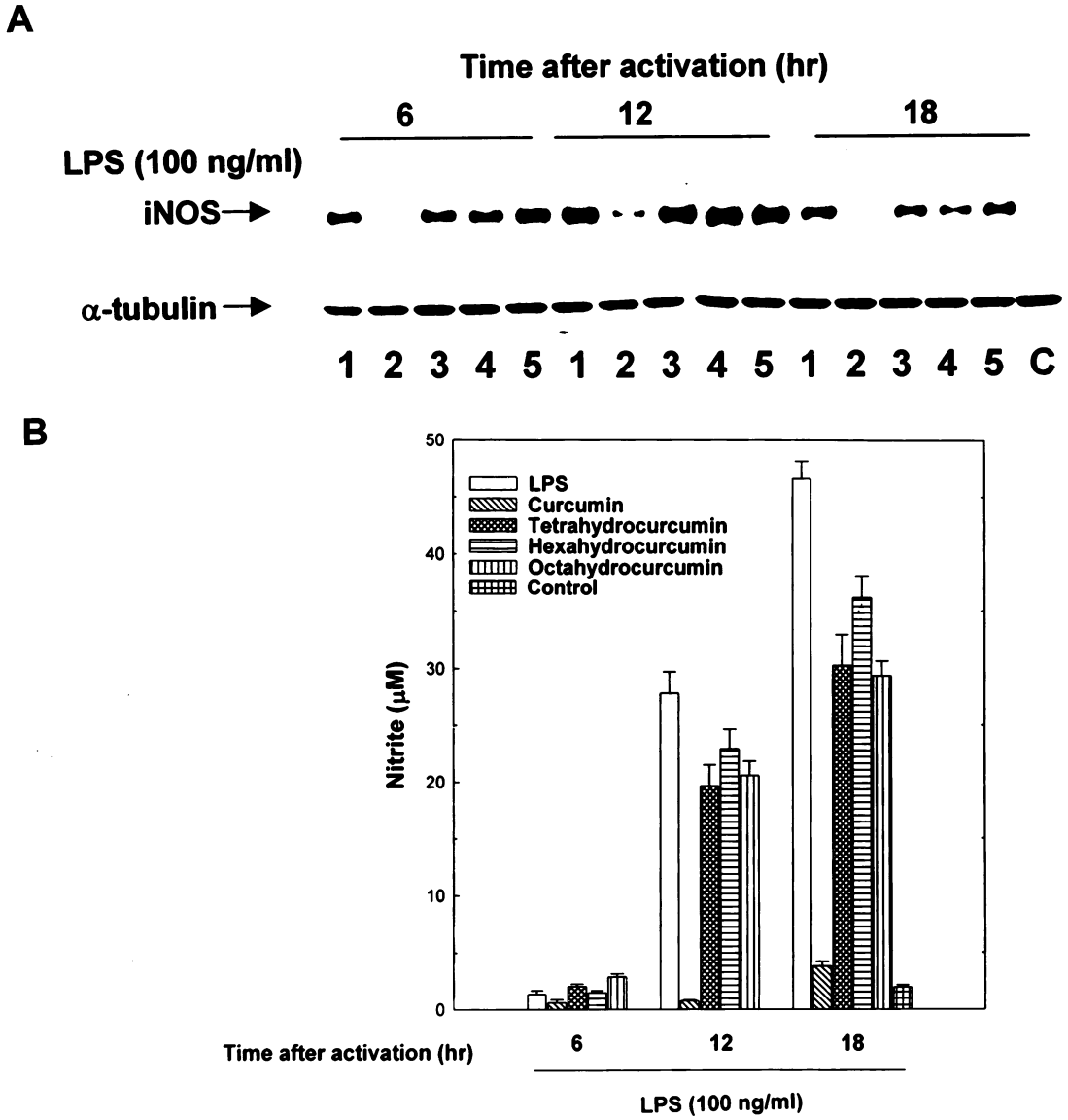

FIG. 2. Effect of curcuminoids on LPS-induced iNOS activation at 6, 12, and 18 hr. RAW 264.7 cells were cultured in the presence of LPS (100 ng/mL) with or without curcuminoids (10 μM) for 6, 12, and 18 hr. At the end of the incubation time, the culture medium was collected for (A) extraction of total protein for iNOS protein and α-tubulin analysis, and (B) nitrite assay. (A) Lane 1: LPS; lane 2: curcumin; lane 3: tetrahydrocurcumin; lane 4: hexahydrocurcumin; and lane 5: octahydrocurcumin. C = control. These experiments were repeated three times with similar results. In panel B, values are expressed as means ±.

ity shift assay was performed. As shown in Fig. 4A, the induction of specific NFκB binding activity by LPS was inhibited markedly by coincubation with curcumin (10 μM). On the other hand, inhibition by tetrahydrocurcumin (4H), hexahydrocurcumin (6H), or octahydrocurcumin (8H) was almost undetectable. The addition of excess unlabeled consensus oligonucleotide completely prevented the band shifts, demonstrating the specificity of the protein/DNA interaction. In an additional study, transient transfection with NFκB-dependent luciferase reporter plasmid was done to confirm whether curcumin inhibited NFκB binding activity in LPS-induced macrophages. As shown in Fig. 4B, curcumin inhibited LPS-induced NFκB transcriptional activity in a concentration-dependent manner.

Effect of Curcumin on the Phosphorylation and Degradation of IκBα

The translocation of NFκB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα. To determine whether the inhibitory action of curcumin was due to its effect on the phosphorylation and degradation of IκBα, the phosphorylated and cytoplasmic levels of IκBα protein were examined by immunoblot analysis. After a 15-min activation of macrophages by LPS, the serine phosphorylated IκBα protein was increased as detected by Ser32-phospho-specific IκBα antibody. The results are illustrated in Fig. 5. Curcumin had a strong ability to inhibit LPS-induced IκBα phosphorylation in a concentra-

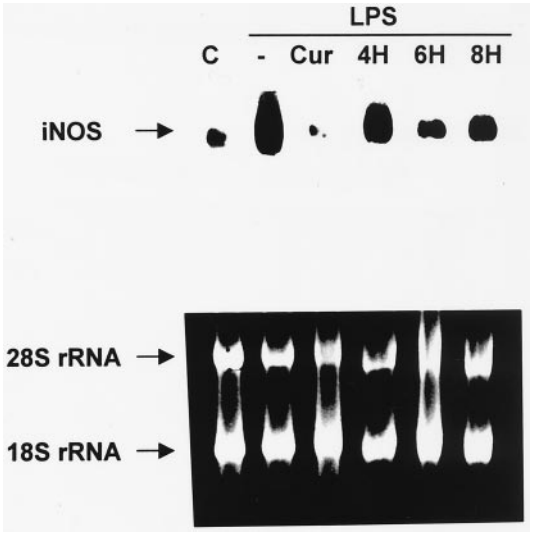

FIG. 3. Northern blot analysis of mRNA expression of iNOS. RAW 264.7 cells were incubated in the presence of LPS (100 ng/mL) with or without curcuminoids (10 μM) for 6 hr (top panel). Total RNA (20 μg) was successively hybridized to ^{32}P-labeled iNOS probe as described in Materials and Methods. To assess the variations in RNA loading, total RNA was loaded on each lane of a formaldehyde agarose gel and stained (bottom panel). C: control; Cur: curcumin; 4H: tetrahydrocurcumin; 6H: hexahydrocurcumin; and 8H: octahydrocurcumin.

tion-dependent manner. To identify the step at which NFκB signaling is affected, we examined the levels of IκBα protein in cells stimulated with LPS. As shown in Fig. 6, LPS stimulation led to a complete loss of IκBα in RAW 264.7 cells followed by the reappearance of IκBα 60 min after stimulation. However, curcumin abolished the LPS-induced IκBα degradation. We also measured the level of p65 in the cytoplasm. As expected, upon LPS treatment the level of p65 declined in the cytoplasm, and this decline was abolished by curcumin. The results suggest that curcumin inhibits either the LPS-induced translocation of p65 to the nucleus or the degradation of p65, and this is consistent with the inhibition of LPS-dependent degradation of IκBα by curcumin.

Inhibition of Kinase Activity of IKK1 and IKK2 by Curcumin in Macrophages

It has been reported that IKK1 and IKK2 need to form a heterodimer for maximal enzyme activity [40]. In an attempt to explore the effects of the curcuminoids on the inhibition of IKK activity in RAW 264.7 cells, the cells were stimulated with LPS. This stimulation caused a marked increase in IKK1 and IKK2 activity as measured after 10 min (Fig. 7). Curcumin (10 μM) markedly inhibited IKK1 and IKK2 activity induced by LPS, whereas tetrahydrocurcumin (4H), hexahydrocurcumin (6H), and octahydrocurcumin (8H) showed a less inhibitory effect at the same concentration. The curcuminoids had no effect on the level of IKK protein (data not shown). Taken together, these results suggest that inhibition of iNOS expression by curcumin may block LPS-induced NFκB activation by inhibiting IKK activity, which perturbs the degradation of IκBα.

Inhibition by Curcumin of IKK1 and IKK2 Activity in vitro

We immunoprecipitated endogenous IKK from LPS-stimulated RAW 264.7 cells and determined its kinase activity in the presence of different concentrations of curcumin (0–10 μM). IKK activity was inhibited in a concentration-dependent fashion by curcumin (Fig. 8). The IC_{50} values were 4.58 and 3.81 μM for IKK1 and IKK2, respectively. Despite extensive washing, the sample that was preincubated with curcumin remained inhibited (data not shown). These results suggest that curcumin may inhibit IKKs through direct interaction and that cellular IKKs are direct targets of curcumin.

DISCUSSION

Curcumin, a naturally occurring anti-inflammatory agent and antioxidant, has been shown to inhibit tumors in several organs, including 7,12-dimethylbenz[*a*]anthracene-induced and 12-*O*-tetradecanoylphorbol-13-acetate-promoted skin tumors [2], benzo[*a*]pyrene-induced forestomach tumors [41], and azoxymethane-induced intestinal tumors in mice [1]. Chan *et al.* [6] have shown that, *in vivo*, oral treatments of 0.5 mL of a 10 μM solution of curcumin (92 ng/g of body weight) reduced iNOS mRNA expression in the livers of LPS-injected mice by 50–70%. It has been demonstrated that curcumin inhibits the induction of iNOS in macrophages activated with LPS and interferon-γ [4]. Early studies on the tissue distribution of curcumin as well as *in vitro* studies of its absorption by everted intestinal sacs indicated that curcumin is transformed during absorption [42]. We previously reported that most of the curcumin administered is reduced by an endogenous reductase system in a stepwise manner and subsequently glucuronidated by UDP-glucuronosyltransferase [30]. Tetrahydrocurcumin is one of the major metabolites of curcumin. Only traces of hexahydrocurcumin were detected in plasma. Sugiyama *et al.* [32] reported that tetrahydrocurcumin is a stronger antioxidant than curcumin *in vitro*. However, *in vivo*, the exact mechanism for the inhibition of iNOS induction by curcumin is not clear.

In this study, we found that curcumin strongly inhibited the induction of iNOS in RAW 264.7 cells activated with LPS, whereas tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin had less effect. Nitrate can be microbiologically reduced to nitrite [43], which then can interact with dietary substrates such as amine or amides to produce *N*-nitroso compounds. The formation of carcinogenic *N*-nitrosoamines resulting from elevated NO formation has been demonstrated in cell cultures and *in vivo* [44]. Our results demonstrated that curcumin was a potent inhibitor of inducible NO synthase protein; therefore, it

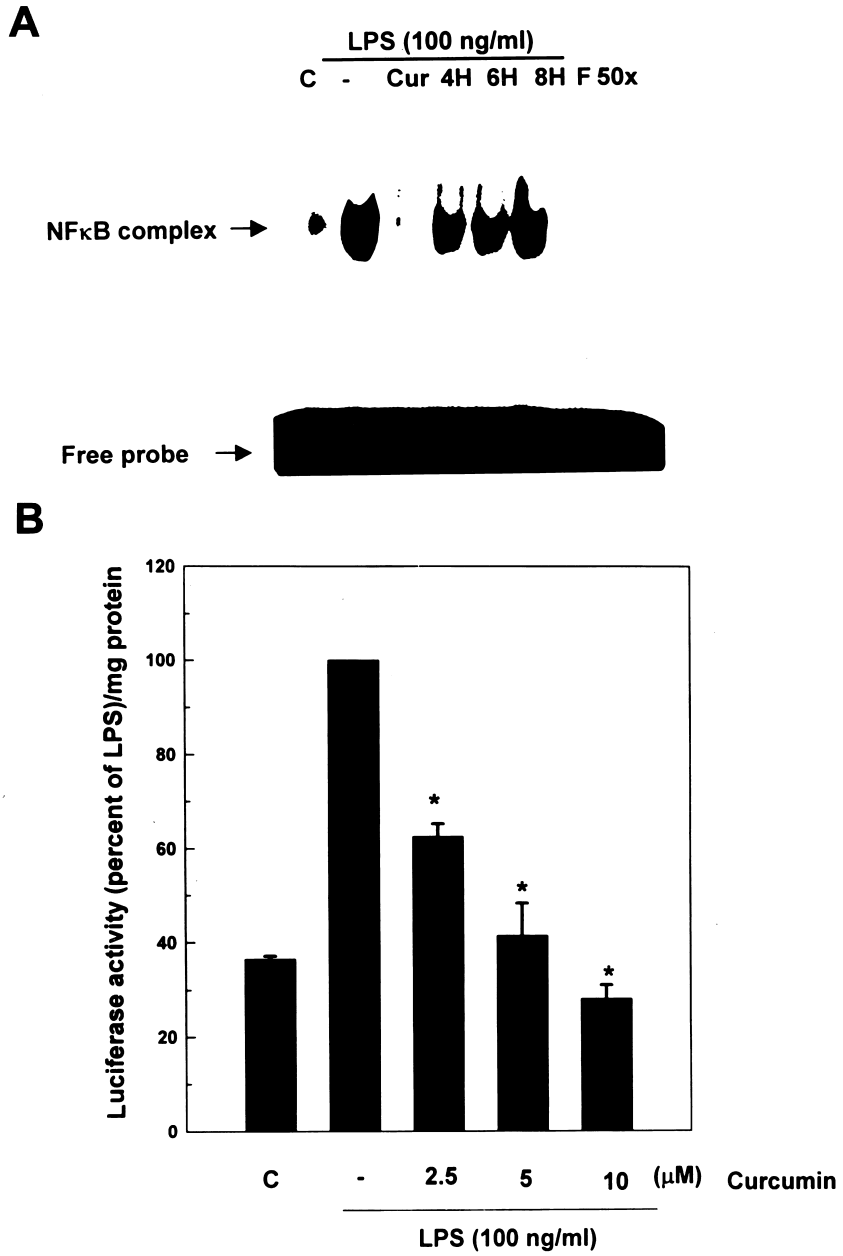

FIG. 4. Inhibition of LPS-induced NFκB activity by curcumin. Nuclear extracts were prepared from murine macrophages (RAW 264.7) and treated with 100 ng/mL of LPS and the indicated concentration of curcumin, tetrahydrocurcumin (4H), hexahydrocurcumin (6H), or octahydrocurcumin (8H) for 1 hr. EMSAs were carried out as described in Materials and Methods, and the binding of NFκB from nuclear extracts to ^{32}P-NFκB oligonucleotide is shown. The position of the NFκB–DNA complex is indicated with an arrow. The specificity of NFκB binding activities was tested by competition with a 50-fold excess of unlabeled consensus oligonucleotide. F: free probe. (B) The cells were transfected with 2.5 μg of pNFκB-Luc reporter plasmid. After transfection, cells were subcultured in 12-well plates, and then cotreated with 100 ng/mL of LPS and different concentrations of curcumin for 3 hr. Data are expressed as the means ± SEM of the percentage of maximal luciferase activity observed with LPS only, as determined in three independent experiments. Key: (*) $P < 0.001$ vs LPS treatment (Student's *t*-test).

may block the formation of *N*-nitroso compounds and peroxynitrite or hydroxyl radicals, and thus could inhibit carcinogenesis.

Activation of NFκB is necessary for LPS induction of the iNOS promoter [24]. NFκB is composed mainly of two proteins: p50 and p65. In resting cells, the NFκB heterodimer is held in the cytosol through interaction with IκB inhibitory proteins [45]. With exposure to proinflammatory stimuli, IκB becomes phosphorylated, ubiquitinated, and then degraded. Thus, the liberated NFκB dimers are translocated to the nucleus, where the transcription of the target gene is induced. Our results showed that curcumin reduces iNOS expression by blocking transcription of its gene, a conclusion supported by the observation that it reduced the steady-state of iNOS mRNA levels, and promoter activity (as assessed by gel mobility and a lucif-

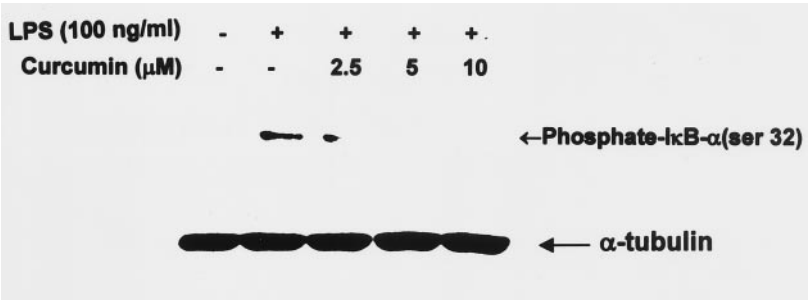

FIG. 5. Inhibition of LPS-induced IκB phosphorylation by curcumin. Murine macrophages (RAW 264.7) were incubated with LPS (100 ng/mL) without or with different concentrations (2.5 to 10 μM) of curcumin for 15 min. Control, DMSO (0.03%). Cytosolic fractions were prepared and analyzed for IκB-P protein content by western blot. This experiment was repeated three times with similar results.

erase activity assay). Surprisingly, tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin only showed weakly inhibitory activity in LPS-induced macrophages. These findings are in agreement with studies by Huang *et al.* [41] and Anto *et al.* [46], who showed that curcumin, demethoxycurcumin, and bisdemethoxycurcumin were all very potent inhibitors of TPA (phorbol ester)-induced mouse ear edema and skin carcinogenesis, respectively, while tetrahydrocurcumin was less effective. However, when tested as a direct antioxidant *in vitro*, tetrahydrocurcumin was very potent, and a mechanism was proposed that involves its β-diketone moiety [32]; we also found that hexahydrocurcumin and octahydrocurcumin have direct antioxidant activity *in vitro* (data not shown). The results of the present study indicate that the conjugated double bonds of the central seven-carbon chain of curcumin may play an important role in its biological activity.

The protein kinase complex that phosphorylates IκBs in response to a proinflammatory signal contains two catalytic subunits, IKKα and IKKβ (or IKK1 and IKK2) [40, 47, 48], and a regulatory subunit, IKKγ [or NEMO (NFκB essential modulator)] [39]. IKK activity is stimulated rapidly upon exposure of cells to proinflammatory stimuli. Two candidate IKK kinases are NFκB-inducing kinase (NIK) [49] and MAP kinase kinase kinase-1 (MEKK-1) [50, 51], but their physiological roles are not clear at the present time. NIK preferentially phosphorylates IKKα [49], whereas MEKK1 preferentially phosphorylates IKKβ [52]. We reported pre-

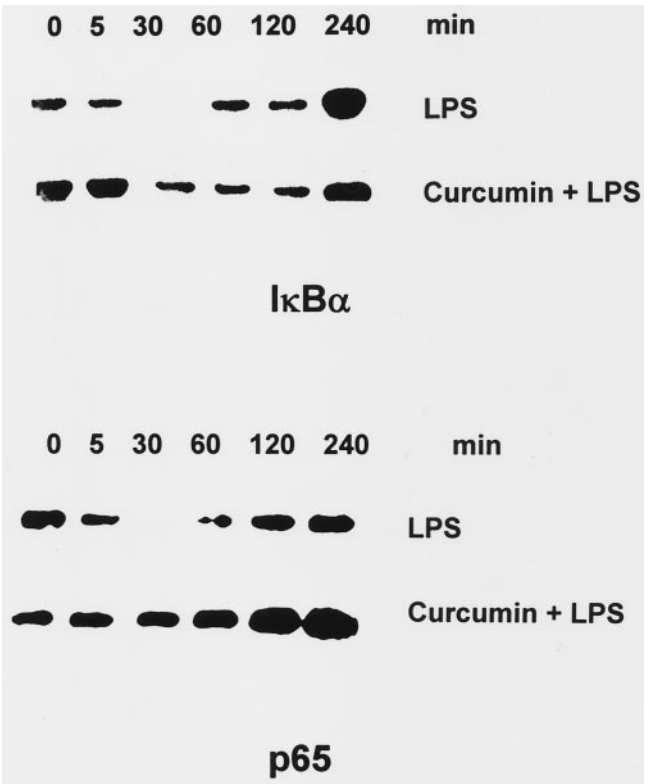

FIG. 6. Effect of curcumin on LPS-induced degradation of IκBα and on the level of p65 in cytoplasm. Cells treated with LPS (100 ng/mL) without or with curcumin (10 μM) were incubated for different times, and then assayed for IκBα and p65 in cytosolic fractions by western blotting analysis as described under Materials and Methods. This experiment was repeated three times with similar results.

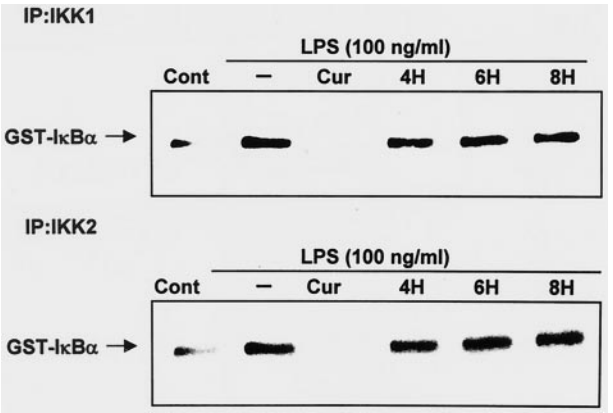

FIG. 7. Effect of curcuminoids on LPS-induced IKK activity in murine macrophages. Whole cell extracts were prepared from RAW 264.7 cells treated with LPS (100 ng/mL) without or with various curcuminoids (10 μM) or solvent only for 10 min. IKK1 and IKK2 activity assays were carried out as described in Materials and Methods. A part of each extract was used to measure IKK1 activity (top panel), and another part was used to measure IKK2 activity by immune complex kinase assay (bottom panel). Cont: control; Cur: curcumin; 4H: tetrahydrocurcumin; 6H: hexahydrocurcumin; and 8H: octahydrocurcumin. These experiments were repeated three times with similar results.

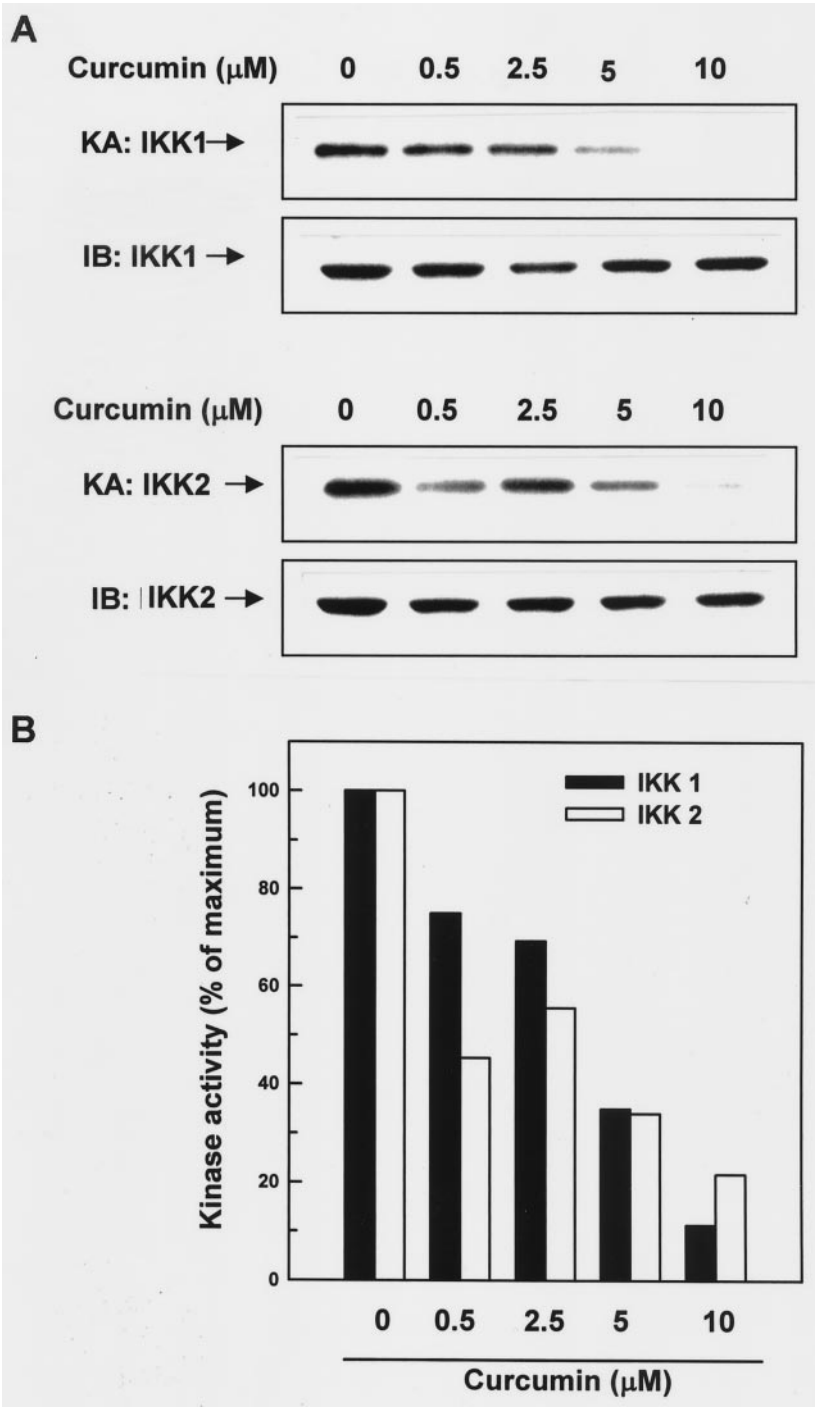

FIG. 8. Inhibition by curcumin of IKK1 and IKK2 activity *in vitro*. IKK was immunoprecipitated from LPS-stimulated RAW 264.7 cells and incubated with curcumin (0–10 μM) and then assayed for kinase activity. IKK1 and IKK2 activity assays were carried out as described in Materials and Methods. (A) IKK1 and IKK2 activities were measured by a kinase assay (KA, upper panel) or by immunoblotting (IB, bottom panel). (B) Kinase activity was quantified by densitometric analysis (IS-1000 Digital system) and expressed as a percentage of the maximal activity. This experiment was repeated three times with similar results, and the control kinase activity = 3800 cpm.

viously that some polyphenols have the potential to inhibit the activity of IKK in LPS-induced macrophages [53]. In the present study, we further demonstrated that the anti-inflammatory properties of curcumin inhibit IKK activity *in vitro*. These results suggest that inactivation of IKK is important for the prevention of inflammation.

On the basis of the current results and those of other reports [54, 55], we hypothesize that the possible site of action of curcumin on LPS-induced iNOS activation is as shown in Fig. 9. We propose that curcumin directly inhibits LPS-induced IKK activity, which prevents the phosphorylation and degradation of IκBα, thereby blocking NFκB

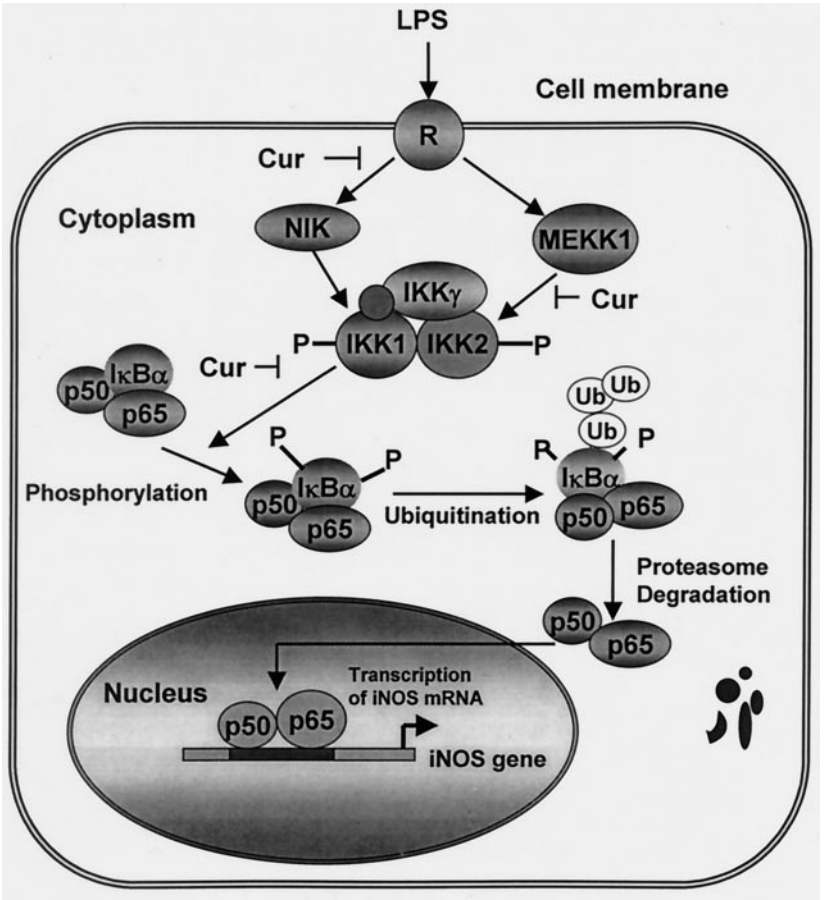

FIG. 9. Possible mechanism of action of curcumin on LPS-induced iNOS activation. Abbreviations: Cur, curcumin; LPS, lipopolysaccharide; R, LPS receptor; NIK, NFκB inducing kinase; MEKK1, MAP kinase kinase kinase-1; P, phosphate group; IKK1, IκB kinase-1; IKK2, IκB kinase-2; IKKγ, IκB kinase γ; Ub, ubiquitin; iNOS, inducible nitric oxide synthase; and ⊥, site of inhibition.

activation, which in turn leads to decreased transcription of the iNOS gene. It is possible that curcumin may inhibit IKK through down-regulating the activity of MEKK-1 or NIK (Fig. 9). More experimental data are required to confirm this concept. However, we cannot rule out the possibility that various cytokines act through different signal transduction pathways in RAW 264.7 cells. Taken together, these results suggest that inactivation of IKK is important for manifesting the antioxidation, anti-inflammation, and anti-carcinogenesis of curcumin. The reduction of IKK activity by curcumin could be mediated by a direct effect on the IKK complex or by interfering with a signal upstream from NIK in the signal transduction pathway.

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