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Curcumin Induces Glutathione Biosynthesis and Inhibits NF-κB Activation and Interleukin-8 Release in Alveolar Epithelial Cells: Mechanism of Free Radical Scavenging Activity

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ABSTRACT

Oxidants and tumor necrosis factor-α (TNF-α) activate transcription factors such as nuclear factor-κB (NF-κB), which is involved in the transcription of proinflammatory mediators, including interleukin-8 (IL-8). Curcumin (diferuloylmethane) is a naturally occurring flavonoid present in the spice turmeric, which has a long traditional use as a chemotherapeutic agent for many diseases. We hypothesize that curcumin may possess both antioxidant and antiinflammatory properties by increasing the glutathione levels and inhibiting oxidant- and cytokine-induced NF-κB activation and IL-8 release from cultured alveolar epithelial cells (A549). Treatment of A549 cells with hydrogen peroxide (H_2O_2; 100 μ*M*) and TNF-α (10 ng/ml) significantly increased NF-κB and activator protein-1 (AP-1) activation, as well as IL-8 release. Curcumin inhibited both H_2O_2- and TNF-α-mediated activation of NF-κB and AP-1, and IL-8 release. Furthermore, an increased level of GSH and glutamylcysteine ligase catalytic subunit mRNA expression was observed in curcumin-treated cells as compared with untreated cells. Curcumin interacted directly with superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($^{\bullet}OH$) as shown by electron paramagnetic resonance, quenching the interaction of the radicals with the spin trap, Tempone-H. This suggests that curcumin has multiple properties: as an oxygen radical scavenger, antioxidant through modulation of glutathione levels, and antiinflammatory agent through inhibition of IL-8 release in lung cells. *Antioxid. Redox Signal.* 7, 32–41.

INTRODUCTION

Involvement of immune and inflammatory cells has been implicated in airway inflammation in a wide variety of lung disorders, including asthma, chronic obstructive pulmonary disease, adult respiratory distress syndrome, and idiopathic pulmonary fibrosis. Activation of these cells leads to production of inflammatory mediators, including oxidants and cytokines, such as interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) (2, 20).

Oxidant challenge to the airway epithelium is normally neutralized by the antioxidants present in the alveolar fluid and within the airway lining cells. The chief intracellular antioxidant is reduced glutathione (GSH), which also plays a crucial role in the regulation of expression of several redox-sensitive antioxidant and antiinflammatory genes (19, 21). The cellular pool of GSH is maintained by the activity status of the enzyme γ-glutamylcysteine ligase catalytic subunit (GCLC; formerly known as γ-GCS), which in turn is regulated by oxidative stress (18). GSH has been reported to protect cells

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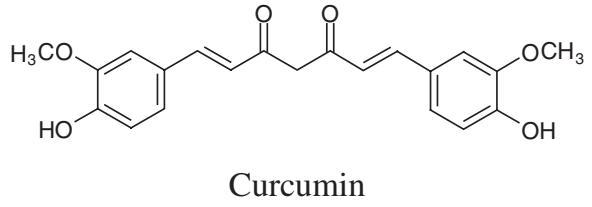
from damage due to lipid peroxidation through free radical scavenging via redox-based reactions and detoxification reactions (21). Therefore, any significant alteration in the status of GSH leads to abnormal physiological changes deleterious to the cell.

TNF-α is a pleiotropic protein that mediates a multitude of inflammatory events in the lungs (2). The transcription of inflammatory mediators is regulated by the activation of redox-sensitive transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) (19). Reactive oxygen species (ROS) and cellular redox status, particularly intracellular thiol status, are reported to be directly involved in the activation of NF-κB (18). TNF-α activates NF-κB via the classical I-κB kinase pathway and regulates IL-8 transcription (21). IL-8 is a major chemotactic and paracrine mediator for polymorphonuclear leukocyte recruitment in the lungs. Thus, modulation of its production may be a potential therapeutic target in chronic inflammatory lung diseases.

Both oxidative stress and inflammation are hallmarks of a number of lung disorders, and most current therapies are targeted to either control the oxidative stress or thwart inflammation. Hence, an effective therapeutic agent should ideally be able to control both phenomena. The use of dietary polyphenols is becoming an increasingly attractive proposition for the treatment of various inflammatory diseases in patients unresponsive to standard medications. During the last decade, a large number of dietary components, such as capsaicin, resveratrol, and gingerol, have been described as potent chemotherapeutic agents (29).

Curcumin (diferuloylmethane), a polyphenol (Fig. 1), is an active principle of the perennial herb *Curcuma longa* (commonly known as turmeric) and is largely cultivated in India, China, and other Asian and tropical countries. Turmeric has a long traditional use in the Orient for many ailments, particularly as an antiinflammatory agent (1). Recent investigations have increasingly focused on its antioxidant, hepatoprotective, anticarcinogenic and antimicrobial activity, in addition, to its use in cardiovascular disorders (3, 9, 13). More recent studies have reported that curcumin inhibits NF-κB expression/activation, cyclooxygenase, heme oxygenase-1 (HO-1), cytokines, and polymorphonuclear leukocyte recruitment in the lungs (11, 12, 16, 25, 27). Its use is documented in Ayurvedic medicine for treatment of various respiratory conditions, such as asthma, bronchial hyperactivity, and allergy. Also, there are some reports available about the beneficial effect of curcumin on free radical quenching (5, 14) and its restorative effect on GSH depletion (7, 9). However, the mechanism by which curcumin scavenges free radicals and restores oxidant/cytokine-mediated cellular GSH depletion is still poorly understood, especially in lung epithelial cells. Similarly, the molecular mechanism for the antiinflammatory action of curcumin has not been studied in lung cells. In this study, we investigated the mechanism by which curcumin modulates oxidative stress-mediated signaling of inflammatory responses and the possible mechanism of its interaction with free radicals using electron paramagnetic resonance (EPR) spectroscopy in lung alveolar epithelial cells (A549). Understanding the molecular mechanisms of action of various dietary polyphenols, including curcumin, could lead to the pharmacological development of novel therapeutic approaches for chronic lung inflammatory diseases.



Curcumin

FIG. 1. Chemical structure and features of curcumin. Curcumin belongs to the class of curcuminoids and is very similar to diarylheptanoids. The antiinflammatory action is associated with the existence of the β-dicarbonylic system, which has the conjugated double bonds (dienes) that are responsible for this activity. The presence of the diene ketone system provides a lipophilicity to the compounds, and thus probably better skin penetration. The presence of double bonds (α,β-unsaturated system) increases its potency and reactivity.

MATERIALS AND METHODS

Unless otherwise stated, all biochemical reagents used in this study were purchased from Sigma Chemical Co. (Poole, U.K.), RT-PCR reagents from Promega (Southampton, U.K.) and Invitrogen (Paisley, U.K.), and primers from MWG Biotech (Ebersberg, Germany). Curcumin was provided by Oxis Health Products (Portland, OR, U.S.A.). The human type II alveolar epithelial cell line, A549 (ECACC no. 86012804), was maintained in continuous culture at 37°C, 5% CO_2 in Dulbecco's modified minimum essential medium containing L-glutamine (2 m*M*), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum. A549 and NF-κB-transfected A549 cells grown to 80–90% confluency in six-well plates containing 10% fetal bovine serum, were washed in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS) and exposed to treatments in 2% serum-containing media. All treatments were performed in duplicate. The cells were treated with hydrogen peroxide (H_2O_2; 100 μ*M*) or TNF-α (10 ng/ml) alone or in the presence of curcumin (10 μ*M*). The cells were incubated in 2 ml of 2% serum-containing medium at 37°C, 5% CO_2 for 4 and 24 h. Following treatments, the cells were washed with cold sterile CMF-PBS. NF-κB-transfected A549 cells were lysed and used for NF-κB transactivation assay. The culture media were used for IL-8 assay. Nontransfected A549 cells were subjected to GSH assay and RT-PCR analysis for GCLC, IL-8, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. Nuclear extracts were prepared from control and treated cells and were used for NF-κB and AP-1 DNA binding assays by electrophoretic mobility gel shift assay (EMSA). Cell viability was determined by trypan blue exclusion and consistently remained >90% after all of the above treatments.

GSH assay

GSH levels in the cell extract were measured by the 5,5'-dithiobis-(2-nitrobenzoic acid) DTNB-GSSG reductase recy-

cling method described by Tietze (31) with slight modifications (23).

EPR measurement

EPR (Magnettech X-band miniscope MS-100, Berlin, Germany) was used to establish the antioxidant effect of curcumin with respect to superoxide anion ($O_2^{\bullet -}$) and hydroxyl radicals (•OH) generated from pyrogallol and menadione, respectively. Pyrogallol (100 μ*M*) or menadione (500 μ*M*) was incubated in phosphate-buffered saline (pH 7.4, 37°C) containing the spin trap, Tempone-H (1 m*M*) (8), oxidation of which generates 4-oxo-TEMPO with a characteristic three-line EPR signal centered at 3,365 G. Development of this signal was monitored for 60 min from addition of the oxidizing species and compared with parallel incubations containing curcumin (10 or 50 μ*M*, $n = 6$). The amplitude of the first line of the spectrum was measured; data are expressed in arbitrary units. To establish whether oxidation of curcumin generated a stable radical species or whether the product of this reaction was spin silent, separate experiments ($n = 6$) were also conducted with pyrogallol or menadione in the presence of curcumin without the spin trap. The EPR parameters for these experiments were as follows: microwave frequency, 9.4 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1,500 mG; center field, 3,365 G; sweep width, 50 G; sweep time, 20 s; number of passes, 1; receiver gain, 3×10^1.

Enzyme-linked immunosorbant assay (ELISA) for IL-8

An ELISA was used to measure IL-8 (10). All plates were read on a microplate reader (Dynatech MR 5000, Aldermaston, U.K.) and analyzed using a computer-assisted analysis program (Assay ZAP, Blosoft, Cambridge, U.K.). Typically, standard curves generated with this ELISA were linear in the 50–2,500 pg of IL-8/ml range. Only assays having standard curves with a calculated regression line value of >0.95 were used for further analysis.

NF-κB transactivation assay

A549 cells stably transfected with NF-κB and a previously described luciferase gene (a gift from Professor Ron Hay, University of St. Andrews) under the control of three synthetic copies of the κB consensus of the immunoglobulin κ chain promoter were grown in six-well plates, serum-starved for 24 h, and exposed to various treatments as described above. Culture medium was aspirated off, and the cells were washed twice with ice-cold phosphate-buffered saline, lysed in 400 μl of lysis buffer (25 m*M* Tris-phosphate buffer, pH 7.8 using phosphoric acid, 8 m*M* $MgCl_2$, 1 m*M*, dithiothreitol, 1% Triton X-100, and 15% glycerol), and centrifuged for 5 min at 13,000 rpm. Fifty microliters of lysate was added to a cuvette and placed in a Lumac Celcis M2500 (Celsis, Oxford, U.K.). The luciferase activity was measured by luminescence following the injection of 50 μl of luciferin buffer (1 m*M* ATP, 0.25 m*M* luciferin, and 1% bovine serum albumin, all diluted in lysis buffer). Values were expressed as relative percentage of luciferase units per milligram of protein.

Preparation of nuclear extracts and EMSA

Nuclear extracts were prepared as described previously (23). Binding reactions were established in 20 μl of binding buffer (Promega), using 4 μg of nuclear extract protein per reaction for the consensus probe. In the binding reaction, nuclear extracts were incubated for 20 min at room temperature with [γ-^{32}P]ATP end-labeled double-stranded AP-1 probe consensus from Promega according to the manufacturer's instructions. The sequences of the oligonucleotide used for the EMSA for NF-κB were radiolabeled NF-κB-specific oligonucleotide 5'-AGT TGA GGG GAC TTT CCC AGG C-3' and 3'-TCA ACT CCC CTG AAA GGG TCC G-5' (Promega), and for AP-1 they were 5'-CGC TTG ATG AGT CAG CCG GAA-3' and 3'-GCG AAC TAC TCA GTC GGC CTT-5' (Promega). Samples were then loaded and electrophoresed through a 6% polyacrylamide gel at a constant voltage of 180 V. The gels were dried and scanned using a PhosphorImager system (STORM, Molecular Dynamics). To monitor the specificity of the binding reaction, the assay was performed in the presence of 1,000-fold excess of the nonlabeled oligonucleotide (23).

RT-PCR

After treatment, RNA was isolated from control and treated cells using TRIzol reagent (Gibco BRL, Paisley, U.K.), as per the manufacturer's instructions, and dissolved in 50 μl of diethylpyrocarbonate-treated water. Moloney murine leukaemia virus RT (Promega) was used to reverse-transcribe cDNA from 2 μg of mRNA according to the manufacturer's instructions. The genes studied were the housekeeping gene GAPDH, GCLC, and IL-8. The primer pairs (obtained from MWG Biotech, Ebersberg, Germany) used in this study are described in Table 1.

TABLE 1. PRIMER SEQUENCE AND RT-PCR CONDITIONS

Gene	*Primer sequence (sense/antisense)*	*Annealing temperature/cycles*
GAPDH	5' CCACCCATGGCAAATTCCATGGCA 5' TCTAGACGGCAGGTCAGGTCAACC	60°C/20
GCLC	5' GTGGTACTGCTCACCAGAGTGATCCT 5' TGATCCAAGTAACTCTGGGCATTCACA	55°C/32
IL-8	5' ATGACTTCCAAGCTGGCCGTGCCT 5' TCTCAGCCCTCTTCAAAAACTTCTC	60°C/30

The primers were diluted to 100 pmol/µl with diethylpolycarbonate-treated water. For each PCR reaction, 5 µl of reverse-transcribed RNA (cDNA) was added directly to a PCR reaction mixture set to a final volume of 50 µl, containing 1× Taq DNA polymerase reaction buffer (Promega), 2.5 m*M* $MgCl_2$, 0.2 m*M* dNTP mixture, 1 unit of Taq DNA polymerase (Promega), and 1 m*M* of the appropriate primer pair. The conditions for GAPDH were 20 thermal cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min 30 s, followed by final extension for 10 min at 72°C, resulting in a product 600 bp in size. GCLC was subjected to 32 thermal cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min, followed by an extension at 72°C for 10 min, which resulted in a final product of 534 bp. The conditions for IL-8 were 30 thermal cycles of 92°C for 1 min, 60°C for 1 min, 72°C for 1 min, followed by final extension at 72°C for 5 min, resulting in a product of 180 bp. The resultant PCR products were separated by electrophoresis through 1.5% agarose gel, and the resolved bands were visualized and scanned by a white/UV transilluminator (Ultra Violet Products, Cambridge, U.K.) and quantified by densitometry.

Statistical analysis

All data are expressed as the means ± SEM. Means were compared by ANOVA and a two-way unpaired Student's *t* test (Skewness analysis showed the data to be normally distributed), followed by Tukey's post hoc test for multigroup comparisons. EPR data were analyzed by a two-way ANOVA, followed by Bonferroni post-hoc analyses. $p < 0.05$ was considered significant.

RESULTS

Curcumin restores TNF-α- and H_2O_2-mediated GSH levels by inducing the expression of GCLC in A549 cells

TNF-α (10 ng/ml) and H_2O_2 (100 µ*M*) significantly decreased GSH levels after 4 h in A549 cells compared with the control value (Fig. 2a). Cotreatment of A549 cells for 24 h with curcumin significantly prevented TNF-α- and H_2O_2-mediated decline in GSH levels (data not shown). We also examined the levels of GCLC mRNA expression in A549 cells in response to TNF-α (10 ng/ml) and H_2O_2 (100 µ*M*) in the absence and presence of curcumin (Fig. 2b and c). TNF-α and H_2O_2 inhibition of GCLC expression was significantly reversed by curcumin. Curcumin alone significantly induced the basal expression of GCLC in untreated A549 cells, suggesting that curcumin may be a direct inducer of the GCLC gene.

Curcumin exerts antioxidant properties by scavenging free radicals

EPR spectroscopy revealed that addition of curcumin (10 µ*M*) to the $O_2^{\bullet -}$ generator, pyrogallol (100 µ*M*; $n = 6$), or the $^{\bullet}OH$ generator, menadione (500 µ*M*; $n = 6$), in the absence of a spin trap failed to generate a spin signal over a 60-min period. This indicated that curcumin either interacted with the free radicals covalently and therefore yielded no paramagnetic signals or did not interact at all. However, three-line spin signals characteristic of formation of the stable radical 4-oxo-TEMPO developed in a time-dependent manner when the same oxidant-

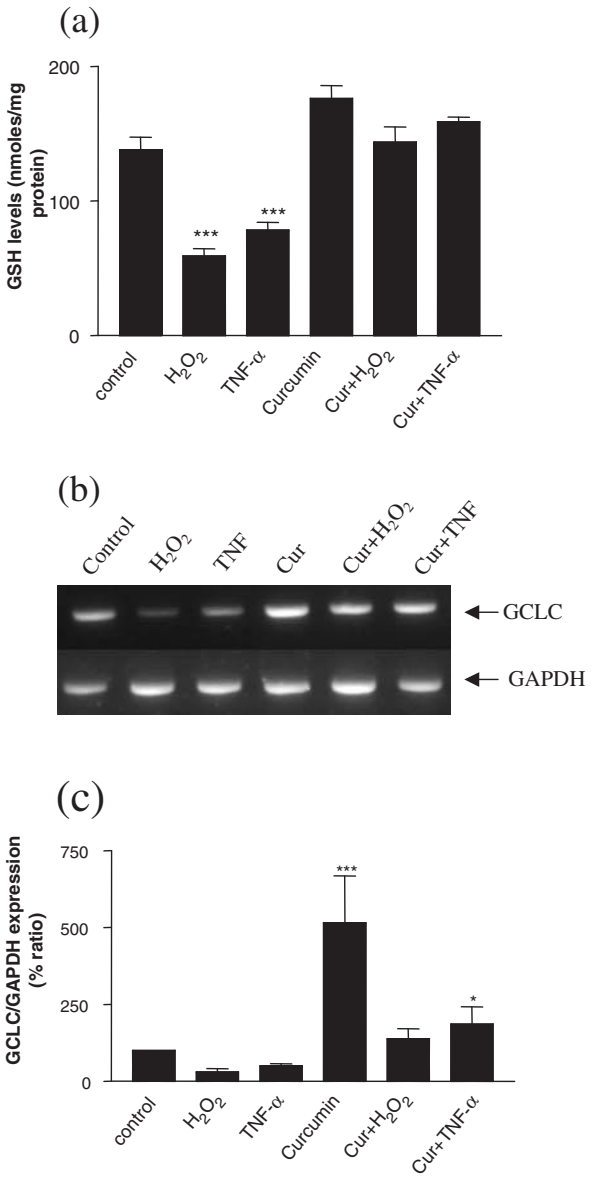


FIG. 2. Effect of H_2O_2, TNF-α, and curcumin on GSH levels and GCLC mRNA expression in A549 cells. (**a**) Treatment of cells with H_2O_2 (100 µ*M*) and TNF-α (10 ng/ml) significantly reduced the GSH levels after 4 h in A549 cells (***$p < 0.001$ versus control). Cotreatment of the cells with 10 µ*M* curcumin imparted significant protection against H_2O_2- and TNF-α-dependent decrease in GSH ($n = 12$). (**b**) A549 cells were treated with H_2O_2 (100 µ*M*) and TNF-α (10 ng/ml) for 4 h. The mRNA extracted was subjected to RT-PCR for GCLC expression. Individual band densities were calculated as described in Materials and Methods. (**c**) The extent of GCLC mRNA expression was expressed as the percentage of the ratio of GCLC/GAPDH. The histogram is representative of the densities obtained from seven individual experiments (*$p < 0.05$, ***$p < 0.001$ versus control).

generating substances were incubated with the recognized spin trap, Tempone-H (1 m*M*). Coincubation of curcumin (10 or 50 μ*M*) with pyrogallol (100 μ*M*) in the presence of Tempone-H caused a significant inhibition of spin signal development over a 60-min time period (Fig. 3A; $p < 0.001$, two-way ANOVA followed by Bonferroni post-hoc analyses, shown on the figure; $n = 6$). Curcumin at 50 μ*M* also inhibited the generation of a spin signal with menadione (50 μ*M*; Fig. 3B; $p < 0.001$, two-way ANOVA; $n = 5$), but 10 μ*M* curcumin failed to cause significant inhibition ($p > 0.05$; $n = 5$).

Expression and release of IL-8 in A549 epithelial cells is suppressed by curcumin

Increased IL-8 release was observed in A549 cells following 24 h of exposure to TNF-α and H_2O_2 compared with untreated cells ($p < 0.001$) (Fig. 4a). Approximately five- to sevenfold increase in the IL-8 release was recorded in the H_2O_2- and TNF-α-treated cells (Fig. 4a). Treatment of A549 cells for 24 h with curcumin inhibited both TNF-α and H_2O_2-induced IL-8 release in A549 cells ($p < 0.001$).

To determine the antiinflammatory properties of curcumin, A549 cells were subjected to treatment with TNF-α and H_2O_2, and the expression of the IL-8 gene was measured in the presence and absence of curcumin. IL-8 expression was significantly inhibited following cotreatment with curcumin ($p < 0.001$, Fig. 4b and c). Curcumin alone was found to inhibit the levels of IL-8 mRNA appreciably.

Curcumin inhibits TNF-α- and H_2O_2-mediated NF-κB transactivation in A549 epithelial cells

H_2O_2 and TNF-α treatments increased NF-κB transactivation (156% and 350%) compared with the control values (100%) in A549 cells (Fig. 5). Pretreatment of A549 cells with curcumin significantly inhibited H_2O_2- and TNF-α-induced NF-κB transactivation in A549 cells transfected with the NF-κB promoter-dependent luciferase gene. In addition, curcumin alone significantly altered the transactivation of the transcription factor.

Curcumin blocks TNF-α- and H_2O_2-dependent NF-κB DNA binding in A549 epithelial cells

TNF-α and H_2O_2 treatments increased NF-κB DNA binding (364% and 256%, respectively) at 4 h compared with the control values (100%) in A549 cells (Fig. 6). The NF-κB binding to DNA was significantly inhibited when the cells were treated with TNF-α and H_2O_2 in the presence of curcumin (curcumin, 41%; TNF-α + curcumin, 135%; H_2O_2 + curcumin, 41%).

TNF-α- and H_2O_2-enhanced AP-1 DNA binding is blocked by curcumin in A549 epithelial cells

Exposure of A549 cells to TNF-α and H_2O_2 increased AP-1 DNA binding (172% and 134%, respectively) at 4 h, compared with the control values (100%) (Fig. 7). However, decreased AP-1 binding was observed when the cells were treated with H_2O_2 and TNF-α in the presence of curcumin (curcumin, 82%; TNF-α + curcumin, 112%; H_2O_2 + curcumin, 85%).

DISCUSSION

The GSH status of a cell is critical for various biological events that include transcriptional activation of specific genes and modulation of redox-sensitive signal transduction and hence proinflammatory processes in the lungs (21). This tripeptide is the principal antioxidant in the lung and is present in large

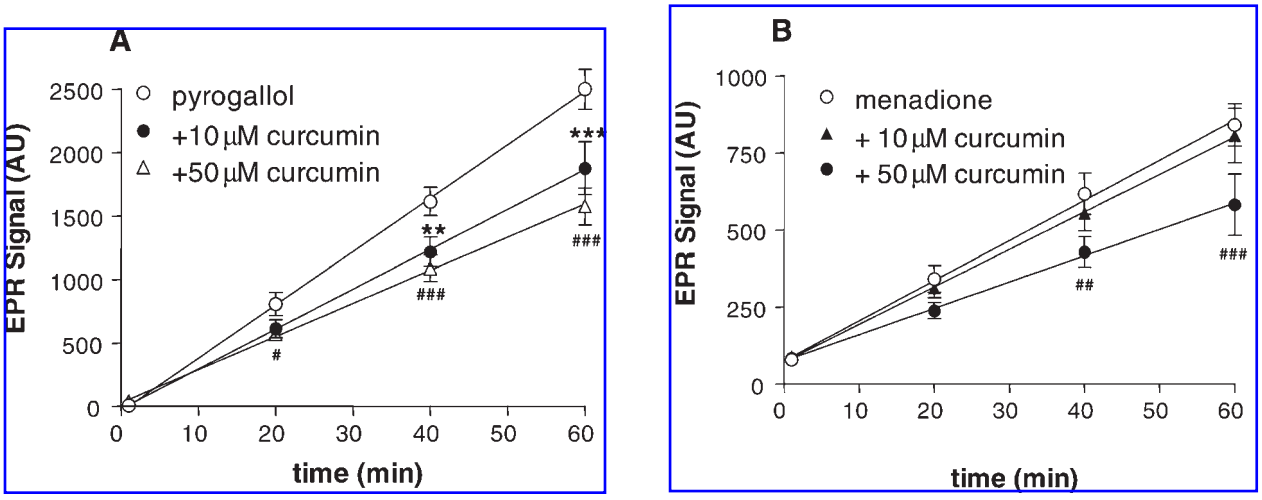


FIG. 3. EPR of curcumin interaction with certain free radicals. The effect of curcumin (10 or 50 μ*M*) on time-dependent development of EPR signals [arbitrary units (AU)] due to oxidation of Tempone-H (1 m*M*) to 4-oxo-TEMPO is shown in the presence of (**A**) pyrogallol (100 μ*M*; $O_2^{\bullet -}$ generator) and (**B**) menadione (500 μ*M*; $^{\bullet}OH$ generator) at 37°C. $^{**}p < 0.01$, $^{***}p < 0.001$ Bonferroni's post-hoc test following two-way repeated measures ANOVA for 10 μ*M* curcumin versus control; $^{\#}p < 0.05$, $^{\#\#}p < 0.001$, $^{\#\#\#}p < 0.001$ for 50 μ*M* curcumin versus control.

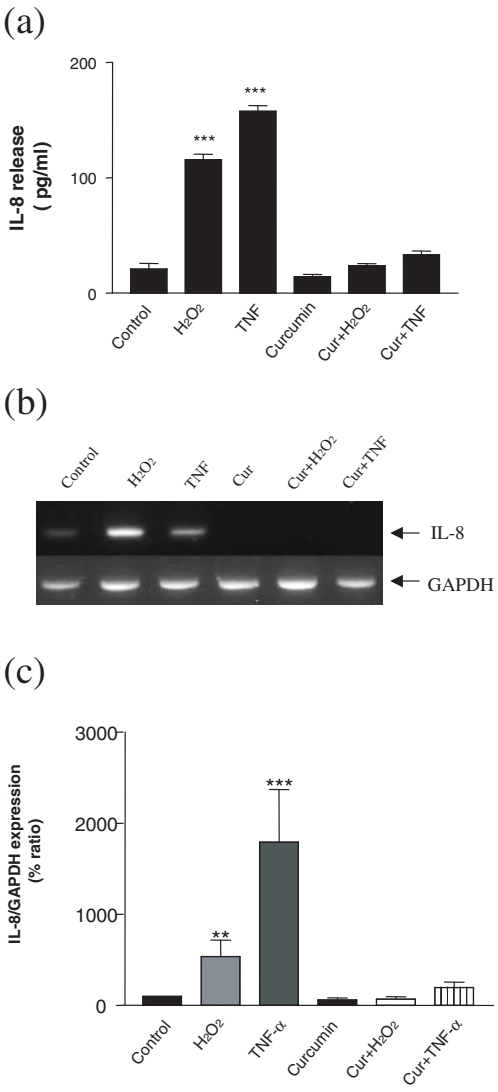


FIG. 4. Effect of H_2O_2, TNF-α, and curcumin on IL-8 release and IL-8 mRNA expression in A549 cells. (**a**) Cells were treated with H_2O_2 (100 μ*M*) and TNF-α (10 ng/ml) for 24 h with and without curcumin. IL-8 release was significantly increased in the presence of H_2O_2 and TNF-α (*** $p < 0.001$, $n = 6$). About five- to eightfold increase in % release was recorded in the presence of H_2O_2 and TNF-α as compared with control, and the trend was reversed in curcumin-treated cells. (**b**) IL-8 mRNA expression was measured in A549 cells after 24-h treatment with H_2O_2 (100 μ*M*) and TNF-α (10 ng/ml) with or without curcumin. Cotreatment of cells with H_2O_2 and TNF-α along with curcumin significantly decreased IL-8 mRNA expression. (**c**) The results are presented as an average of eight individual experiments ± SEM as ratios of IL-8/GAPDH band intensities (** $p < 0.01$, *** $p < 0.001$).

quantities in the epithelial lining fluid, presumably due to release from type II cells (20). Several disorders, such as acute respiratory distress syndrome, cystic fibrosis, and idiopathic pulmonary fibrosis, are characterized by a depletion of this essential antioxidant in the airways, suggesting a role for oxidative stress in the pathogenesis of these chronic inflammatory lung diseases (for review, see 20). The underlying causes of these diseases are unknown, and an effective antioxidant/antiinflammatory treatment strategy remains to be developed. Cytokines such as TNF-α and oxidants like H_2O_2 have been shown to activate NF-κB associated with a decrease in GSH levels in A549 cells (15, 23). Curcumin is a polyphenol of the curcuminoid family and has been reported to possess antioxidant properties (1, 16, 26). In this study, we tested the hypothesis that curcumin protects TNF-α- and H_2O_2-mediated depletion of GSH levels and inflammatory events by up-regulation of GSH biosynthesis. Cotreatment of A549 cells with curcumin and TNF-α or H_2O_2 protected the cells against GSH depletion, suggesting that curcumin either scavenges the oxidant species responsible for GSH consumption or increases the biosynthesis of GSH.

In view of its chemical structure, curcumin has the potential to accommodate extra electrons in its polyaromatic nuclei. Therefore, it seems likely that curcumin may be involved in direct interaction with the free radicals to protect the cells against the fall in GSH levels. To characterize the antioxidant/free radical scavenging activity and the nature of interaction of curcumin with free radicals, EPR spectrometry was applied. Our experiments with curcumin (used as a spin trap) and oxidant generators in the absence of a recognized spin trap showed that no spin signal is generated under these conditions. These results indicate that any oxidation of curcumin that might occur on exposure to $O_2^{\bullet -}$ or $^{\bullet}OH$ radicals does not generate a stable radical adduct of curcumin. However, interaction that may not necessarily generate paramagnetic signals was not ruled out at this juncture because a covalent interaction may yield a sta-

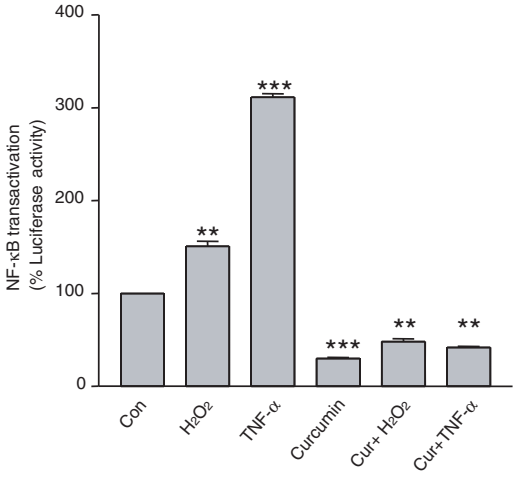


FIG. 5. Curcumin inhibits TNF-α- and H_2O_2-mediated increase in NF-κB transactivation in A549 cells. NF-κB transactivation was performed in A549 cells transfected with NF-κB-luciferase reporter system upon treatment with H_2O_2 (100 μ*M*) and TNF-α (10 ng/ml) with or without curcumin. Luciferase activity was measured in the cytosolic extracts of the cells 24 h post treatment. Curcumin significantly diminished the NF-κB transactivation by H_2O_2 and TNF-α (** $p < 0.01$, *** $p < 0.001$, versus control; $n = 3$).

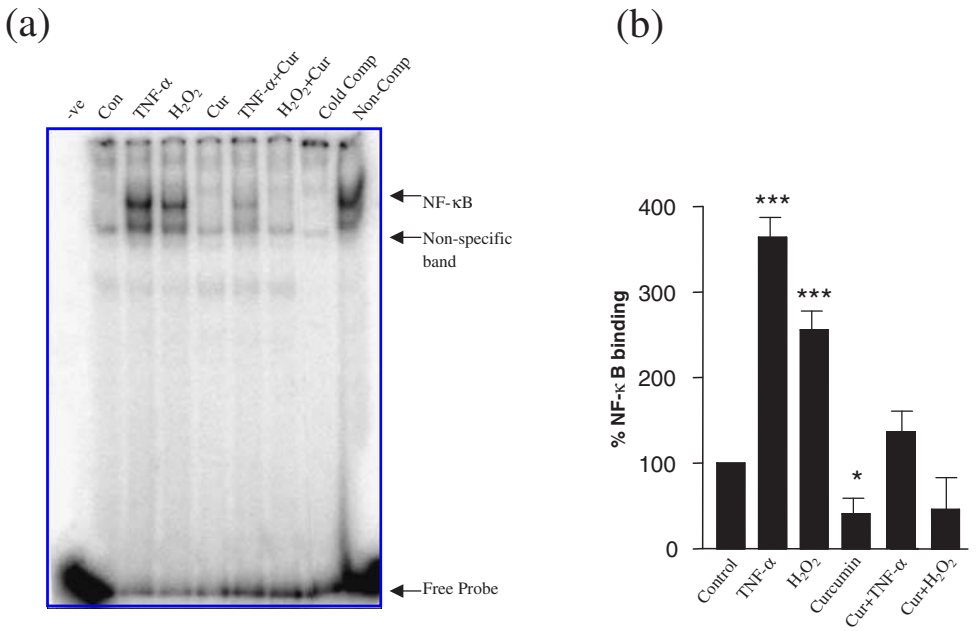


FIG. 6. Curcumin inhibits TNF-α- and H_2O_2-dependent increase in DNA binding by NF-κB in A549 cells. (**a**) EMSA of nuclear extracts isolated from A549 cells showing nuclear binding of NF-κB following 4-h treatment with TNF-α and H_2O_2, with and without curcumin. Nuclear extracts were prepared and analyzed by EMSA using (γ^{32}P)-labeled NF-κB oligonucleotide. The DNA–protein complexes formed are indicative of the NF-κB specific band. Results of negative water control, positive HeLa nuclear extract control, and cold competitor using 100-fold molar excess of unlabeled oligonucleotides are also shown. Autoradiographs shown are representative of at least three separate experiments and are also represented as histograms in terms of % densities relative to controls. (**b**) Densitometric quantitation of specific NF-κB binding was compared with the control values set at 100%. The histograms represent the mean values + SEM of the relative intensities of the bands of three experiments (*$p < 0.05$, ***$p < 0.001$, compared with control).

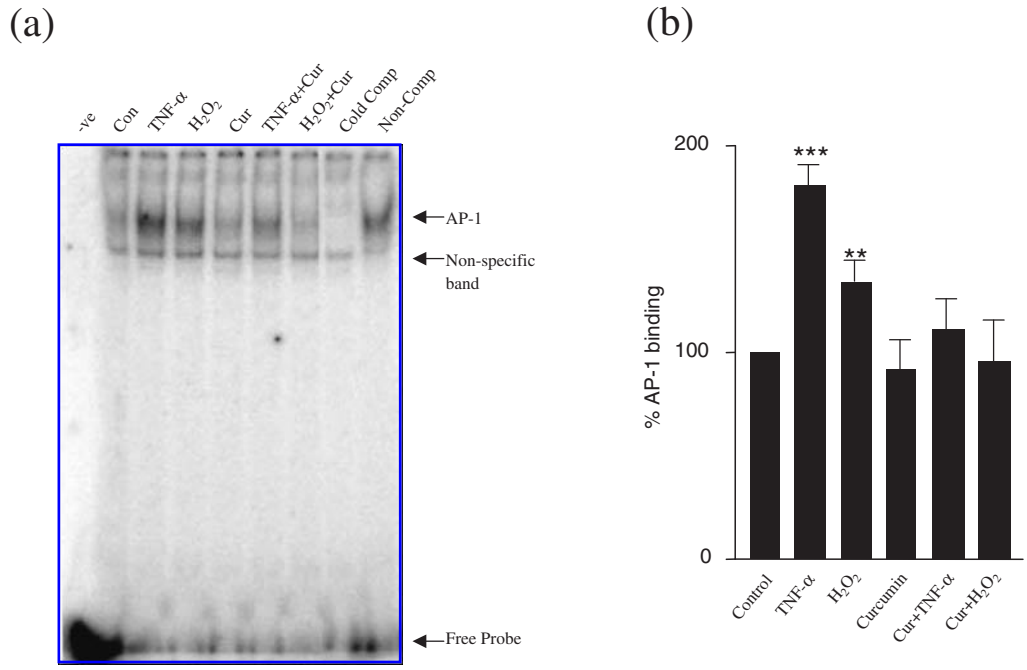


FIG. 7. Curcumin inhibits TNF-α- and H_2O_2-dependent increase in DNA binding by AP-1 in A549 cells. (**a**) Curcumin also inhibited TNF-α- and H_2O_2-dependent increase in AP-1 DNA binding following 4-h treatment as visualized by EMSA of nuclear extracts isolated from A549 cells showing nuclear binding of AP-1. Autoradiographs shown are representative of at least three separate experiments and are also represented as histogram in terms of % densities relative to controls. (**b**) Densitometric quantitation of specific AP-1 binding was compared with the control values set at 100%. The histograms represent the mean values + SEM of the relative intensities of the bands of three experiments (**$p < 0.01$, ***$p < 0.001$, compared with controls).

ble product not conducive to electron spin signal analysis. Furthermore, our results for equivalent experiments in the presence of the spin trap, Tempone-H, indicate that curcumin (50 μ*M*) inhibits the generation of the corresponding oxidation product, 4-oxo-TEMPO, by ~25%. Interestingly, the results with 10 μ*M* curcumin indicate that its antioxidant activity is more pronounced with the $O_2^{\bullet -}$ generator, pyrogallol, than with the $^{\bullet}OH$ generator, menadione. Taken together, these EPR data indicate that curcumin is an effective antioxidant, particularly with respect to $O_2^{\bullet -}$, and that the product of the interaction of curcumin with oxidants is not a stable radical species. Not only is this observation new, but it also reveals in part the mechanism by which curcumin may interact with oxidant species. Thus, it appears that one of the mechanisms by which curcumin prevents GSH depletion may be due to its direct scavenging of the oxidants. Our observation is further corroborated by earlier reports of the ability of curcumin to decrease singlet oxygen, $^{\bullet}OH$, and other anion radicals in cell-free medium (5, 14). Another mechanism by which curcumin may prevent GSH depletion could be mediated via increased biosynthesis of GSH. Up-regulation of GCLC leads to increased GSH levels in the cells (20). To test this hypothesis, expression of the GCLC gene was studied wherein we found that curcumin enhanced the expression of GCLC gene alone as well as in the presence of TNF-α and H_2O_2, implying that curcumin not only protects GSH depletion via direct free radical sequestration, but also increases its synthesis by enhancing GCLC expression at least in the early stages of the oxidative stress (4 h). Furthermore, we observed that curcumin alone could increase the expression levels of GCLC, more than recorded in the control group, and that the levels of GCLC in the TNF-α and H_2O_2 groups cotreated with curcumin were also marginally higher than in the control set. It is interesting to note that curcumin at least significantly restored the GSH levels throughout the oxidative challenge. Recent data have revealed that low concentration of curcumin, a naturally occurring antioxidant, potently induces HO-1 expression in endothelial cells, leading to increased resistance to oxidative stress-mediated damage (16). HO-1 is a stress response gene and is induced as a phase II detoxifying protein during oxidative stress. Thus, induction of HO-1 by curcumin may be an additional mechanism whereby it exerts its cytoprotective role during oxidative stress. Thus, curcumin may exhibit its antioxidant potential at both the biochemical and the molecular level. However, further studies are required to dissect the actual mechanism of action of curcumin at the molecular level.

It is known that GCLC gene expression is regulated by AP-1 and antioxidant response element (ARE) in various cells depending on the stimuli (17, 20, 23). Because curcumin is a polyphenol, we examined whether it affects AP-1-mediated GCLC expression in A549 cells. Treatment of cells with H_2O_2 and TNF-α in the presence of curcumin decreased AP-1 binding to the DNA, suggesting that curcumin may not increase GCLC expression via this route. Instead, it is possible that curcumin increases GCLC expression via an ARE-Nrf2-mediated mechanism, as has been shown previously (7). This hypothesis requires further investigations.

It is well known that ROS and cellular redox status, particularly intracellular thiol status, can be directly involved in the activation of NF-κB (18, 21). Expression of several proinflammatory cytokines and mediators is regulated by NF-κB activation in a wide variety of cells (2, 15, 19). Thus, any signal leading to NF-κB activation is likely to be proinflammatory. In this study, we investigated whether curcumin inhibits NF-κB activation and thereby IL-8 release in A549 epithelial cells. We found that curcumin inhibited H_2O_2- and TNF-α-mediated activation of NF-κB in lung epithelial cells, which is in agreement with earlier similar observations in other cell types (27). However, the mechanism by which curcumin inhibits NF-κB activation remains unclear. The activation of NF-κB involves phosphorylation of one of its subunits by I-κB kinase. The phosphorylated subunit then rapidly degrades, and the p65 unit of NF-κB translocates to the nucleus. Interestingly, Shishodia *et al.* (27) and Jobin *et al.* (12) recently reported the inhibitory effect of curcumin on I-κB kinase, an enzyme essential for the activation of NF-κB. Furthermore, our data show that the constitutive levels of NF-κB transactivation and DNA binding activity were lowered in the curcumin alone group. This may be due to direct interference of NF-κB DNA binding by curcumin as a result of covalent modification of NF-κB subunits in the nucleus (30). This direct interference of NF-κB DNA binding by curcumin has been shown in an *in vitro* study where the NF-κB DNA binding was inhibited in curcumin-treated nuclear extracts (4). The other plausible explanation for basal inhibition of NF-κB activation would be due to elevated levels of GSH because curcumin increases GSH levels in the cells.

The induction of inflammatory mediators such as IL-8 can be regulated by the activation of redox-sensitive transcription factors, including NF-κB and AP-1, in response to oxidants and TNF-α (19). IL-8 is a chemokine released during lung inflammation and is important in the recruitment and activation of immune and inflammatory cells. Moreover, oxidative stress has been shown to mediate IL-8 synthesis (6). IL-8 induction is associated with the activation of the nuclear transcription factors, such as NF-κB, AP-1, and NF-IL6, in response to diverse stimuli in various cell types (24, 28). In this study, we have shown that H_2O_2 and TNF-α induced an increase in IL-8 release with a corresponding increase in the activation of NF-κB in A549 cells. Both IL-8 release and NF-κB transactivation were inhibited by curcumin, suggesting that curcumin acts at the transcription level to inhibit IL-8 release in A549 cells. Another important transcriptional factor is the activator protein AP-1 that is required for the transcription of IL-8 gene (24). Curcumin also inhibited the oxidant- and TNF-α-mediated activation of AP-1, suggesting that curcumin acts at multiple signalling pathways to inhibit IL-8 release in A549 cells. The induction of IL-8 protein secretion and mRNA synthesis by H_2O_2 and TNF-α occurs as a result of increased gene transcription in A549 cells and other cell types (6). Therefore, we investigated the effect of curcumin on IL-8 gene expression in A549 epithelial cells. As expected, H_2O_2 and TNF-α upregulated the expression of IL-8 gene, which was inhibited by cotreatment with curcumin in A549 cells, suggesting that curcumin inhibits IL-8 release at the transcriptional level in A549 cells. This observation is in agreement with our observation of inhibition of NF-κB activation by curcumin. This has an implication in inflammatory lung disease states where IL-8 is increased (19, 20). In these cases, ROS and TNF-α would lead to an augmented inflammatory response from the tissue. This strongly suggests that the curcumin has antiinflammatory

properties and may have the potential to attenuate lung inflammation.

In conclusion, this study suggests a plausible mechanism of curcumin action. Curcumin increases GSH biosynthesis by increasing GCLC expression and therefore appears to have a good potential for therapeutic enhancement of GSH synthesis. Antiinflammatory properties of curcumin were attributable to its ability to inhibit NF-κB and AP-1 activation and IL-8 release and gene expression. In addition, curcumin directly scavenges free radicals such $O_2^{\bullet -}$, suggesting an additional mode of its antioxidant action. Thus, curcumin is a potential antioxidant/antiinflammatory therapy to inhibit the chronic inflammatory responses, leading to the development of chronic inflammatory lung diseases.

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ABBREVIATIONS

A549, alveolar epithelial cell; AP-1, activator protein-1; ARE, antioxidant response element; CMF-PBS, calcium- and magnesium-free phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; EPR, electron paramagnetic resonance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCLC, γ-glutamylcysteine ligase catalytic subunit; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; HO-1, heme oxygenase-1; IL-8, interleukin-8; NF-κB, nuclear factor-κB; $O_2^{\bullet -}$, superoxide anion; $^{\bullet}OH$, hydroxyl radical; ROS, reactive oxygen species; RT-PCR, reverse transcription–polymerase chain reaction; TNF-α, tumor necrosis factor-α.

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