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Role of Prooxidants and Antioxidants in the Anti-Inflammatory and Apoptotic Effects of Curcumin (Diferuloylmethane)

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Abstract

Extensive research within last half a century has indicated that curcumin (diferuloylmethane), a yellow pigment in curry powder, exhibits antioxidant, anti-inflammatory and proapoptotic activities. Whether anti-inflammatory and proapoptotic activities assigned to curcumin, are mediated through its antioxidant mechanism was investigated. We found that TNF-mediated NF-κB activation was inhibited by curcumin; and glutathione reversed the inhibition. Similarly, suppression of TNF-induced AKT activation by curcumin, was also abrogated by glutathione. The reducing agent also counteracted the inhibitory effect of curcumin on TNF-induced NF-κB regulated antiapoptotic (Bcl-2, Bcl-xL, IAP1), proliferative (cyclin D1) and proinflammatory (COX-2, iNOS and MMP-9) gene products. The suppression of TNF-induced AP-1 activation by curcumin was also reversed by glutathione. Also, the direct proapoptotic effects of curcumin were inhibited by glutathione and potentiated by depletion of intracellular glutathione by buthionine sulfoximine. Moreover, curcumin induced the production of reactive oxygen species (ROS) and modulated the intracellular GSH levels. Quenchers of hydroxyl radicals, however, were ineffective in inhibiting curcumin mediated NF-κB suppression. Further, N-acetylcysteine partially reversed the effect of curcumin. Based on these results we conclude that curcumin mediate its apoptotic and anti-inflammatory activities through modulation of the redox status of the cell.

Keywords

Curcumin; Glutathione; Tumor necrosis factor; Nuclear factor-κB; Reactive oxygen species

INTRODUCTION

Curcumin (diferuloylmethane), a dietary pigment responsible for the yellow color of turmeric, is used as a traditional medicine, well documented in ayurveda for the treatment of numerous inflammatory conditions. Extensive research within the last half-a-decade has confirmed that curcumin mediates anti-inflammatory effects through the downregulation of transcription factor nuclear factor-κB (NF-κB) [1,2] tumor necrosis factor (TNF) [3], interleukin-6 (IL-6) [4], interleukin-8 (IL-8) [5], adhesion molecules [6], inducible nitric oxide synthase (iNOS)

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[7], matrix metalloproteinase-9 (MMP-9) [8], cyclooxygenase-2 (COX-2) [9], and 5-lipoxygenase (5-LOX) [10]. In fact, curcumin has been shown to bind to an active site in 5-LOX, and the two together have been cocrystallized [11]. This phytochemical has also been shown to suppress the proliferation of a wide variety of tumor cells by downregulating c-myc [6], cyclin D1 [12], activator protein-1 (AP-1) [13], phosphatidylinositol-3-kinase/AKT signaling [14], and epidermal growth factor receptor (EGFR) signaling [15]. Curcumin can also induce apoptosis through the modulation of antiapoptotic gene products [2,6] and BID cleavage, cytochrome c release, and caspase-9 activation, leading to caspase-3 activation [16]. More recently, curcumin was found to bind to thioredoxin reductase and alkylate a critical cysteine residue, thus converting the activity of the enzyme to NADPH oxidase [17]. Since thioredoxin reductase is overexpressed in tumor cells, authors suggested that the NADPH-oxidase mediated production of reactive oxygen species (ROS) may be responsible for the ability of curcumin to selectively kill tumor cells [18].

How curcumin mediates all these effects is not fully understood. Besides having anti-inflammatory and growth-modulatory effects, this compound is also one of the most potent antioxidants. According to some reports, curcumin is as much as 10 times more potent than even vitamin E [19]. It has been generally assumed that the antioxidant effects of curcumin are responsible for its anti-inflammatory, antiproliferative, proapoptotic, and chemopreventive effects, although there is currently no evidence to support this. However, there is no evidence so far if this is indeed the case. In the present report, we investigated whether the anti-inflammatory and proapoptotic effects of curcumin are mediated through the antioxidant mechanism. The results to be described indicate that anti-inflammatory and apoptotic effects of curcumin may be due to its ability to perturb the redox balance in the cell.

MATERIALS AND METHODS

Reagents

Curcumin (>95% pure) was purchased from LKT Laboratories (St. Paul, MN). A 25 mM solution of curcumin was prepared in dimethyl sulfoxide, stored as small aliquots at –20 °C, and diluted as needed in cell culture medium. Bacteria-derived human recombinant TNF, purified to homogeneity with a specific activity of 5×10^7 U/mg, was kindly provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, Iscove's modified Dulbecco's medium, Dulbecco's modified Eagle's medium, and fetal bovine serum were obtained from Invitrogen (Grand Island, NY). Buthionone sulfoximine, glutathione, mannitol N-acetylcysteine and antibody against β-actin were obtained from Sigma-Aldrich (St. Louis, MO). Dichlorodihydrofluorescein diacetate and [5-(and-6)-carboxy-2, 7-dichlorofluoresceindiacetate were purchased from Molecular Probes (Eugene, OR). Antibodies against cyclin D1, iNOS, MMP-9, poly(ADP-ribose) polymerase (PARP), inhibitor of apoptosis protein-1 (IAP1), IAP2, Bcl-2, and Bcl-xL, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against COX-2 was obtained from BD Biosciences (San Diego, CA). Antibodies against IKK-α, and IKK-β, were kindly provided by Imgenex (San Diego, CA).

Cell Lines

Human chronic myeloid leukemia (KBM-5) and human embryonic kidney carcinoma (A293) cells were obtained from the American Type Culture Collection (Manassas, VA). KBM-5 cells were cultured in Iscove's modified Dulbecco's medium with 15% fetal bovine serum. A293 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Culture media were also supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

Electrophoretic Mobility Shift Assay

To examine NF-κB activation, we performed electrophoretic mobility shift assay (EMSA) as described previously[20]. Briefly, cells were washed with ice-cold phosphate-buffered saline and suspended in 0.4 mol of lysis buffer (10 mM HEPES, pH 7.9,10 mM KC1, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml benzamidine). The cells were allowed to swell on ice for 15 min, after which 25 μl of 10% Nonidet P-40 was added. The tubes were then agitated on a vortex for 10 *s* and then microcentrifuged for 30 s. The nuclear pellets were resuspended in 25 μl of ice-cold nuclear extraction buffer (20mM HEPES, pH 7.9,0.4M NaCl, 1m M EDTA, 1m M EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2.0 pg/ml leupeptin, 2.0 pg/ml aprotinin, and 0.5 mg/ml benzamidine), and the tubes were incubated on ice for 15 min with intermittent agitation. This nuclear extract were then microcentrifuged for 5 min at 4°C, and the supernatant was frozen at −70°C. Electrophoretic mobility shift assays (EMSAs) were performed by incubating 15 μg of nuclear extract with 16 fmol of ^{32}P-end-labeled, 45-mer double-stranded NF-κB oligonucleotides from the human immunodeficiency virus long terminal repeat (5′-TTGTTACAA **GGGACTTTC** CGCTG **GGGACTTTC** CAGGGAGGCGTGG-3′; boldface indicates NF-κB binding sites) in the presence of 0.5 μg of poly(dI-dC) in a binding buffer (25 mM HEPES, pH 7.9,0.5mM EDTA, 0.5 mM dithiothreitol, 1% Nonidet P-40, 5% glycerol, and 50 mM NaCl) for 30 min at 37 °C. The DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels using buffer containing 50 mM Tris, 200 mM glycine, and 1 mM EDTA, pH 8.5.

The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with antibodies against either p50 or p65 of NF-κB for 15 min at 37 °C before the complex was analyzed by EMSA. The dried gels were visualized, and radioactive bands were quantified with a PhosphorImager (Amersham Biosciences, Piscataway, NJ) using ImageQuant software.

IKK Assay

To determine the effect of glutathione (GSH) on curcumin-mediated suppression of TNF-induced IKK activation, IKK assay was performed as described previously [21]. To determine the total amounts of IKK-α and IKK-β in each sample, 50 μg of the whole-cell protein was resolved on 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), electrotransferred to a nitrocellulose membrane, and blotted with antibodies against IKK-α or IKK-β.

Western Blot Analysis

To determine the levels of protein expression, we prepared whole cell extracts [22] and fractionated them by SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with the appropriate antibodies, and detected by enhanced chemiluminescence (Amersham Biosciences). The bands obtained were quantified using NIH imaging software (Bethesda, MD).

NF-κB-Dependent Reporter Gene Expression Assay

NF-κB-dependent reporter gene expression was assayed as described [23]. To examine TNF-induced reporter gene expression, we transfected the cells with 0.5 μg of the SEAP expression plasmid and 2 μg of the control plasmid pCMVFLAG1 DNA for 24 h. We then treated the cells for 2 h with GSH and added curcumin at various concentrations. TNF (1 nM) was added after 4 h, and the cell culture medium was harvested collected after 24 h of TNF treatment.

The culture medium was then analyzed for SEAP activity, essentially as described by the manufacturer's instructions (Clontech, Palo Alto, CA), using a Victor 3 microplate reader (Perkin Elmer Life & Analytical Sciences, Boston, MA) with excitation at 360 nm and emission at 460 nm.

AP-1 Activation Assay

To assay AP-1 activation by EMSA, 10 μg of nuclear extract protein was incubated with 16 fmol of ^{32}P-end-labeled AP-1 consensus oligonucleotide (5′-CGCTTGA**TGACTCA**GCCGGAA-3′; bold indicates AP-1 binding site) for 30 min at 37 °C. The resulting DNA-protein complexes were resolved from free oligonucleotide on 6% native polyacrylamide gels [20]. The specificity of binding was examined by competition with unlabeled oligonucleotide. The radioactive bands were visualized and quantified as indicated above.

Live and Dead Assay

To measure apoptosis, we also used a Live and Dead viability/cytotoxicity kit (Molecular Probes, Eugene, OR), which determines intracellular esterase activity and plasma membrane integrity. This assay was performed as indicated [22].

Annexin V Assay

An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected using the binding properties of annexin V. To identify apoptosis, cells were stained with anti-annexin V antibody conjugated with fluorescein isothiocyanate (FITC). Briefly, KBM-5 cells were preincubated with various concentrations of GSH for 2 h, and then curcumin (50 μM) was added. After treatment with curcumin for 24 h at 37 °C, [24] cells were washed in phosphate-buffered saline, resuspended in 100 μl of binding buffer containing FITC-conjugated anti-annexin V antibody, and analyzed by flow cytometry (FACSCalibur; BD Biosciences). Data were collected from at least 10,000 cells at a flow rate of 250–300 cells/s.

TUNEL Assay

We also assayed cytotoxicity by the TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling) method, which examines DNA strand breaks during apoptosis, using an *in situ* cell death detection reagent (Roche Diagnostics, Mannheim, Germany). Briefly, KBM-5 cells were preincubated with various concentrations of GSH for 2 h, and then curcumin (50 μM) was added. After treatment with curcumin for 24 h at 37 °C [21], cells were incubated with reaction mixture for 60 min at 37 °C. Stained cells were analyzed by flow cytometry. Data were collected from at least 10,000 cells at a flow rate of 250–300 cells/s.

PARP Cleavage Assay

We examined caspase-3 activation by assaying PARP cleavage. Whole-cell extracts were prepared from treated cells in lysis buffer (20 mM Tris [pH 7.4], 250 mM NaCl, 2 mM EDTA [pH 8.0], 0.1% Triton X-100, 0.01 μg/ml aprotinin, 0.005 μg/ml leupeptin, 0.4 mM phenylmethylsulfonyl fluoride, and 4 mM sodium orthovanadate) [25]. The lysates were spun at 14,000 rpm for 10 min to remove insoluble material, resolved by 10% SDS-PAGE, and probed with anti-PARP antibodies.

Intracellular GSH Assay

To measure intracellular GSH, KBM-5 cells were incubated with the indicated concentrations of buthionine sulfoximine (BSO) or treated with curcumin. Monobromobimane (final concentration, 40 μM) was loaded into cells [26]. Fluorescence emission from cellular sulfhydryl-reacted monobromobimane was recorded using a flow cytometer. Monobromobimane is also known to react with small molecular weight thiols other than GSH but GSH forms the majority of monobromobimane reactive thiols and, for clarity, we address it as GSH levels in the subsequent text. There are several reports in the literature measuring GSH levels using this dye [27–29]. Data were collected from at least 10,000 cells at a flow rate of 250–300 cells/s.

Prooxidant measurements

To detect intracellular ROS, KBM-5 cells were preincubated with 20 μM oxidation-sensitive dichlorofluorescein diacetate (DCF-DA) or oxidation insensitive [5-(and-6)-carboxy-2, 7-dichlorofluorescein diacetate] for 15 min at 37 °C before being treated with various concentrations of curcumin. The oxidized form of the dye (DCF) acts as a control for changes in uptake, ester cleavage, and efflux [30].

After 2 h of incubation, the increase in fluorescence resulting from oxidation of H_2DCF to DCF was measured by flow cytometry [31]. Measurements with the oxidation insensitive probe failed to detect any differences in the amount of fluorescence between the different treated groups. The mean fluorescence intensity at 530 nm was calculated. Data were collected from at least 10,000 cells at a flow rate of 250–300 cells/s.

RESULTS

The goal of this study was to investigate the mechanism by which curcumin exhibits anti-inflammatory and proapoptotic effects. Anti-inflammatory effects were examined by investigating the effect of curcumin on NF-κB activation pathway induced by TNF, one of the most potent proinflammatory cytokine.

Glutathione Abrogates Curcumin-Mediated Suppression of TNF-Induced NF-κB Activation Pathway

Since curcumin mediates its anti-inflammatory effects primarily through the downregulation of NF-κB, we investigated whether GSH can modulate the effect of curcumin on TNF-induced NF-κB activation. KBM-5 cells were exposed to curcumin in the presence of various concentrations of GSH, and NF-κB was activated by treating the cells with TNF. As indicated by the DNA-binding (EMSA), treatment of cells with TNF induced NF-κB activation, which was inhibited by the addition of curcumin (Fig. 1*A*). Pretreatment with GSH resulted in an almost-complete blockade of curcumin-mediated suppression of NF-κB activation in a dose-dependent manner. Glutathione alone did not have any effect on TNF-induced NF-κB activation at any of the concentrations used. Fig. 1B shows that curcumin did not modify the DNA-binding ability of NF-κB proteins prepared from TNF-treated cells directly up to 50 μM concentration.

When nuclear extracts from TNF-activated cells were incubated with antibodies to the p50 (NF-κB1) and p65 (RelA) subunit of NF-κB, the resulting bands was shifted to higher molecular masses, suggesting that the TNF-activated complex consisted of p50 and p65. The addition of excess unlabeled NF-κB (cold oligonucleotide, 100-fold) caused a complete disappearance of the band, whereas mutated oligonucleotide had no effect on DNA binding (Fig. 1C).

Activation of IKK is critical for activation of NF-κB by TNF, and curcumin has been shown to downregulate IKK [2]. We therefore examined whether GSH can modulate the ability of curcumin to inhibit TNF-induced IKK activity. We pretreated KBM-5 cells with various concentrations of GSH for 2 h and then exposed the cells to curcumin for 4 h. We then activated IKK by treating the cells with TNF for 15 min. TNF activated IKK activity, which was completely suppressed by curcumin; GSH blocked this inhibition in a dose-dependent manner (Fig. 1D). Neither GSH nor curcumin affected the expression of IKK-α or IKK-β.

Glutathione Blocks Curcumin-Mediated Suppression of TNF-Induced AKT Activation

TNF has also been shown to be a potent activator of AKT and curcumin has been shown to inhibit it [2]. Although AKT is essential for cell survival, its role in TNF-induced IKK activation is controversial [32]. We investigated whether GSH can affect the ability of curcumin to suppress TNF-induced AKT phosphorylation. As shown in Fig. 1E, TNF induced AKT phosphorylation, which was inhibited by curcumin. GSH blocked the effect of curcumin in a dose-dependent manner. GSH alone, however, did not affect TNF-induced AKT activation.

Glutathione Inhibits Curcumin's Ability to Suppress TNF-Induced NF-κB Reporter Activity

Although we showed by EMSA that GSH modifies the ability of curcumin to suppress NF-κB activation, DNA binding alone is not always associated with NF-κB-dependent gene transcription, suggesting that there are additional regulatory steps. In our reporter gene assay, TNF induced significant NF-κB-dependent reporter (SEAP) activity compared to the control. Curcumin inhibited this TNF-induced NF-κB reporter activity, and pretreatment with GSH significantly blocked the curcumin-mediated suppression of TNF-induced SEAP activity (Fig. 2*A*).

Glutathione Inhibits Curcumin-Mediated Suppression of TNF-Induced NF-κB-Regulated Gene Products

We also examined the effect of GSH on curcumin-mediated suppression of NF-κB down stream events. Because NF-κB regulates the expression of antiapoptotic proteins such as IAP1 [33], Bcl-2 [34], and Bcl-xL [35], we examined whether curcumin can modulate the expression of these antiapoptotic gene products and, if so, whether GSH can block the effect of curcumin. Western blot analysis showed that TNF induced these antiapoptotic proteins, whereas curcumin significantly suppressed them (Fig. 2*B*). Pretreatment with GSH inhibited the suppressive activity of curcumin. GSH alone had little effect on TNF-induced expression of these antiapoptotic proteins.

NF-κB is known to regulate the expression of proinflammatory and proliferative markers, including iNOS [36], COX-2 [37], MMP-9 [38], and cyclin D1 [39]. To determine whether GSH can inhibit the curcumin to suppress these gene products, cells were pretreated with various concentrations of GSH for 2 h, treated with curcumin for 4 h, and then exposed to TNF. TNF upregulated the levels of these proteins compared to the control, whereas curcumin significantly suppressed the expression of these gene products (Fig. 2*C*). In the cells pretreated with GSH, curcumin could not suppress gene expression by TNF.

Glutathione Inhibits Curcumin-Mediated Suppression of AP-1 Activity

The transcription factor AP-1 regulates the expression of multiple genes essential for cell proliferation, differentiation, and apoptosis [40]. TNF is one of the most potent activators of AP-1 [41] whereas curcumin has been reported to suppress AP-1 activation [42]. To determine whether GSH affects the ability of curcumin to inhibit TNF-induced AP-1 activation, KBM-5 cells were pretreated with GSH for 2 h and then with curcumin for 4 h before AP-1 was activated with TNF. Unstimulated cells showed some basal AP-1 activity, which was suppressed by

curcumin (Fig. 3A). TNF induced a several-fold increase in AP-1 levels, whereas curcumin suppressed it completely. Glutathione inhibited curcumin-mediated suppression of AP-1 activity in a concentration-dependent manner. GSH alone did not affect TNF-induced AP-1 activity at any of the concentrations used.

When nuclear extracts from TNF-activated cells were incubated with excess unlabeled AP-1 (cold oligonucleotide, 100-fold) caused a complete disappearance of the band, suggesting the specificity of probe (Fig. 3B).

Glutathione Inhibits Curcumin-Induced Cell Death

Curcumin is one of the most potent activators of apoptosis in tumor cells [16]. We investigated whether GSH can modulate curcumin's ability to induce apoptosis. KBM-5 cells were pretreated with various concentrations of GSH for 2 h, after which curcumin was added and cell death was assayed using various techniques. As indicated by esterase staining, curcumin induced apoptosis, and GSH inhibited it in a dose-dependent manner (Fig. 4*A*). Similar results were obtained using annexin V (Fig. 4*B*) and TUNEL (Fig. 4*C*) staining. For instance, TUNEL staining revealed that treatment of cells with curcumin for 24 h induced about 80% cell death, whereas adding GSH almost completely blocked it in a dose-dependent manner. We also monitored curcumin-induced apoptosis by assaying caspase-3 activation, a hallmark of apoptosis. The induction of caspase-3-mediated PARP cleavage by curcumin was significantly inhibited by GSH (Fig. 4*D*).

Downregulating Endogenous GSH Increases Curcumin-Mediated Cell Death

Since exogenous addition of GSH inhibited curcumin-mediated cell death, we determined whether the cytotoxicity of curcumin can be enhanced by downregulating endogenous GSH. Glutathione levels were measured by assaying monobromobimane fluorescence. Endogenous GSH levels were decreased by treating cells with a selective inhibitor of GSH synthesis (BSO) for 24 h (Fig. 5*A*). Curcumin induced apoptosis, as measured by annexin V-FITC staining and BSO treatment increased curcumin-induced apoptosis significantly (Fig. 5*B*). No significant differences on either GSH levels or apoptosis was observed between 100 and 250 μM BSO.

Curcumin Induces ROS Generation in Cells

The evidence presented above suggests that curcumin mediates its effects through the prooxidant pathway. We used a DCF-DA probe to examine whether this mechanism can generate ROS inside cells. Cells were labeled with DCF-DA, treated with various concentrations of curcumin for 2 h, and analyzed by flow cytometry. Curcumin induced a significant increase in ROS levels over the control (Fig. 5*C*). This effect was observed at concentrations of curcumin as low as 1 μM and increased steadily up to 25 μM curcumin and declined slightly thereafter. ROS levels increased significantly in GSH depleted cells (BSO pretreated cells) upon treatment with curcumin as compared to control cells treated with curcumin (Fig. 5D).

Time course of changes in GSH levels upon treatment with curcumin

KBM-5 cells were treated with 10 μM curcumin for different intervals of time. Cells were stained with monobromobimane and fluorescence was measured on flow cytometer. Curcumin decreased GSH levels after 2 and 4 h of incubation but significant increase was seen at 16 and 24 h time points (Fig. 5E).

Hydroxyl Radical Quencher Mannitol and DMSO Has No Effect on Curcumin's Ability to Suppress NF-κB Activation or Induce Apoptosis

We next sought to determine whether curcumin mediates its prooxidant effects through the production of hydroxyl radicals. We pretreated KBM-5 cells with mannitol, a well-known hydroxyl radical scavenger, for 2 h and then with curcumin for 4 h. We then treated the cells with TNF and analyzed for NF-κB activation. Glutathione was used as a positive control in this experiment. Mannitol had no effect on curcumin's ability to suppress TNF-induced NF-κB activation (Fig. 6*A*). To confirm above results we used an alternative intracellular hydroxyl radical scavenger DMSO, and we found that DMSO had no effect on curcumin's ability to suppress TNF- induced NF-κB activation (Fig. 6*B*). To determine whether mannitol can inhibit curcumin-mediated cell death, cells were pretreated with mannitol for 2 h and then incubated with curcumin. As revealed by a trypan blue exclusion assay, mannitol failed to block the reduction in cell viability caused by curcumin (Fig. 6*C*).

NAC and EDTA failed to inhibit curcumin mediated suppression of NF-κB

NAC, a precursor for GSH synthesis was incubated with KBM-5 cells for 2 and 24h before treating the cells with curcumin for 4h. In another set of experiment, cells were treated with EDTA for 2 h prior to the addition of curcumin. We then treated the cells with TNF and analyzed for NF-κB activation. Pretreatment for 24 h with NAC (that increases intracellular GSH levels) lead to about 45% inhibition of curcumin mediated NF-κB suppression (Fig. 6D). However, 2 h prior treatment with NAC failed to inhibit curcumin's NF-κB suppressive ability (Data not shown). EDTA also could not inhibit curcumin mediated suppression of NF-κB (Fig. 6E).

DISCUSSION

The goal of this study was to determine whether the anti-inflammatory and proapoptotic effects of curcumin are mediated through the antioxidant or prooxidant mechanism. Our results suggest that glutathione can block the ability of curcumin to suppress the TNF-induced activation of NF-κB, IKK, AKT, NF-κB reporter activity, and expression of antiapoptotic, proinflammatory, and proliferative gene products. We also found that curcumin-induced apoptosis can be inhibited by glutathione. Curcumin treatment also led to the production of ROS and changed the intracellular GSH levels. The proapoptotic activity of curcumin has been reported to be inhibited by superoxide dismutase and N-acetyl cysteine in leukemia cells [43], suggesting the involvement of superoxide radicals. In agreement with this report, a specific hydroxyl radical quencher mannitol had no effect on the proapoptotic activity of curcumin. All of this evidence suggests that the proapoptotic effects of curcumin are mediated through the prooxidant pathway. Similarly, the anti-inflammatory activity of curcumin (suppression of NF-κB) was unaffected by mannitol, EDTA or DMSO. Curcumin's ability to suppress NF-κB was intercepted either by pretreatment of cells with exogenous GSH or through elevating endogenous GSH levels.

The mechanism by which curcumin mediates its prooxidant effects remains unclear. Mitochondria are the major source of ROS in the cell. Evidence from our laboratory and other suggest the role of mitochondria in curcumin induced apoptosis [16,44]. Thus, it is possible that curcumin activates mitochondrial enzymes that lead to production of ROS. The induction of ROS by curcumin could occur through its interaction with thioredoxin reductase [17] and thus changing its activity to NADPH oxidase which could then lead to the production of ROS. Moreover, glutathione has been shown to suppress curcumin-induced ROS production [45]. Several reports suggest that curcumin can induce ROS [18,45,46]. There are also reports which suggest that curcumin quenches ROS production [47,48] and thus acts as an antioxidant. Other reports suggest that curcumin quenches ROS production at low concentrations and induces ROS production at high concentrations [49].

It is not clear which structural group of curcumin is responsible for inducing ROS production. The finding that tetrahydrocurcumin is unable to produce ROS [45] suggests a role for the α,β-unsaturated carbonyl moiety of curcumin in the production of ROS. Curcumin is a Michael acceptor and thus can react with sulfhydryl groups [50]. Curcumin has been shown to be a thiol-modifying agent [51], although it does not oxidize protein thiols but rather alkylates them via a Michael addition [50]. Fang et al. showed that curcumin irreversibly inactivates thioredoxin reductase by alkylating a critical cysteine residue in the catalytic site of the enzyme [17]. This enzyme catalyzes NADPH-dependent reduction of thioredoxins, which play essential roles in substrate reduction, defense against oxidative stress, and redox regulation. Another recent report showed that curcumin also inhibits interleukin-1 receptor-associated kinase (IRAK) by modifying the protein's cysteinyl sulfhydryl groups *in vivo* [52].

Whether the effects of GSH on the ability of curcumin to suppress inflammation and induce apoptosis occur through quenching of cellular ROS or through thiol modification is less clear. Curcumin has been shown to induce GSH biosynthesis [5]. It is unlikely that curcumin reacts with GSH directly under our conditions, as Oetari et al., 1996 showed that GSH prevents the instability of curcumin in phosphate buffer at pH 7.4 [53]. These authors have thoroughly studied the stability of curcumin in aqueous solvents in the presence of thiols. We used cell culture medium containing 10% FBS; and these conditions have been shown also to stabilize curcumin [54]. Curcumin has been shown to induce mitochondrial membrane-permeability transition pores through thiol oxidation [55]. Awasthi et al. demonstrated that curcumin forms conjugates with GSH by separating mono- and diglutathionyl adducts of curcumin [56]. This suggests that formation of curcumin-GSH adducts lead to inactivation of parent curcumin's activity. This is, however, unlikely because glutathionylated curcumin has been reported to be more active than curcumin [57]. We also found that NAC reverses the effect of curcumin, most likely by increasing intracellular GSH contents. That NAC prevents the instability of curcumin in phosphate buffer pH 7.4, has been shown. Consistant with these finding, we found that depletion of intracellular GSH by BSO enhanced curcumin-induced apoptosis. These results are in agreement with Syng-ai et al [18].

Our results are in agreement with previous findings suggesting that ROS are needed for the apoptotic effects of curcumin [18,58–62]. Indeed, we found that depletion of endogenous GSH augmented curcumin-induced cell death in tumor cells. We also ruled out the involvement of hydroxyl radicals in our system. The complexity, however, lies with the suppression of NF-κB activation. It has been shown that ROS are needed for TNF-induced NF-κB activation [63]. Thus, it is not clear how both suppression and activation of NF-κB are mediated through ROS production. It may be that lower levels of ROS result in NF-κB activation, whereas higher levels of ROS suppress NF-κB activation. Another possibility is that the apoptotic effects of curcumin are mediated through ROS generation, whereas the NF-κB-suppressive effects are mediated through thiol modification. NAC, a ROS scavenger, failed to reverse the effect of curcumin mediated NF-κB suppression in 2h (Data not shown). However, 45% reversal was observed at 24 h which correlates with increase in intracellular GSH levels. Indeed, IRAK, a kinase needed for NF-κB activation by interleukin-1 and lipopolysaccharide, has been shown to be modified by curcumin [52]. A third possibility is that curcumin, like vitamin C, acts both as a prooxidant and an antioxidant. While the prooxidant mechanism mediates apoptotic effects, the antioxidant mechanism mediates NF-κB-suppressive effects.

Glutathione also abrogated the ability of curcumin to suppress TNF-induced activation of AP-1, the transcription factor implicated in induction of a number of genes involved in cell proliferation, differentiation, and immune and inflammatory responses [40]. Park et al. showed that curcumin inhibits AP-1 independent of their conserved cysteine residue [64]. Thus, curcumin may exert its antiproliferative effects through the downregulation of AP-1 and cyclin D1, as shown here; these effects also require the production of ROS. Whether in vitro

concentrations of curcumin employed here are related to that in vivo, is not clear. Exposure of cells to a drug in vivo is usually much longer than that in vitro. Cheng et al., 2001 showed serum concentration of 1.75 μM [65]. There is little information on curcumin concentrations in tissues but biological responses both in rodents and humans, have been reported. Overall, our results suggest that the intracellular levels of GSH will influence curcumin's anti-inflammatory and proapoptotic activities. Curcumin has been well proven to be pharmacologically safe in humans. Its proapoptotic and antininflammatory activities described here is applicable to a wide variety of diseases.

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ABBREVIATIONS

GSH
: Glutathione

TNF
: Tumor necrosis factor

NF-κB
: Nuclear factor-κB

ROS
: Reactive oxygen species

EMSA
: Electrophorectic mobility shift assay

DCF-DA
: Dichlorodihydrofluorescein diacetate

FITC
: Fluorescein isothiocyanate

BSO
: Buthionine sulfoximine

SEAP
: Secretory alkaline phophatase

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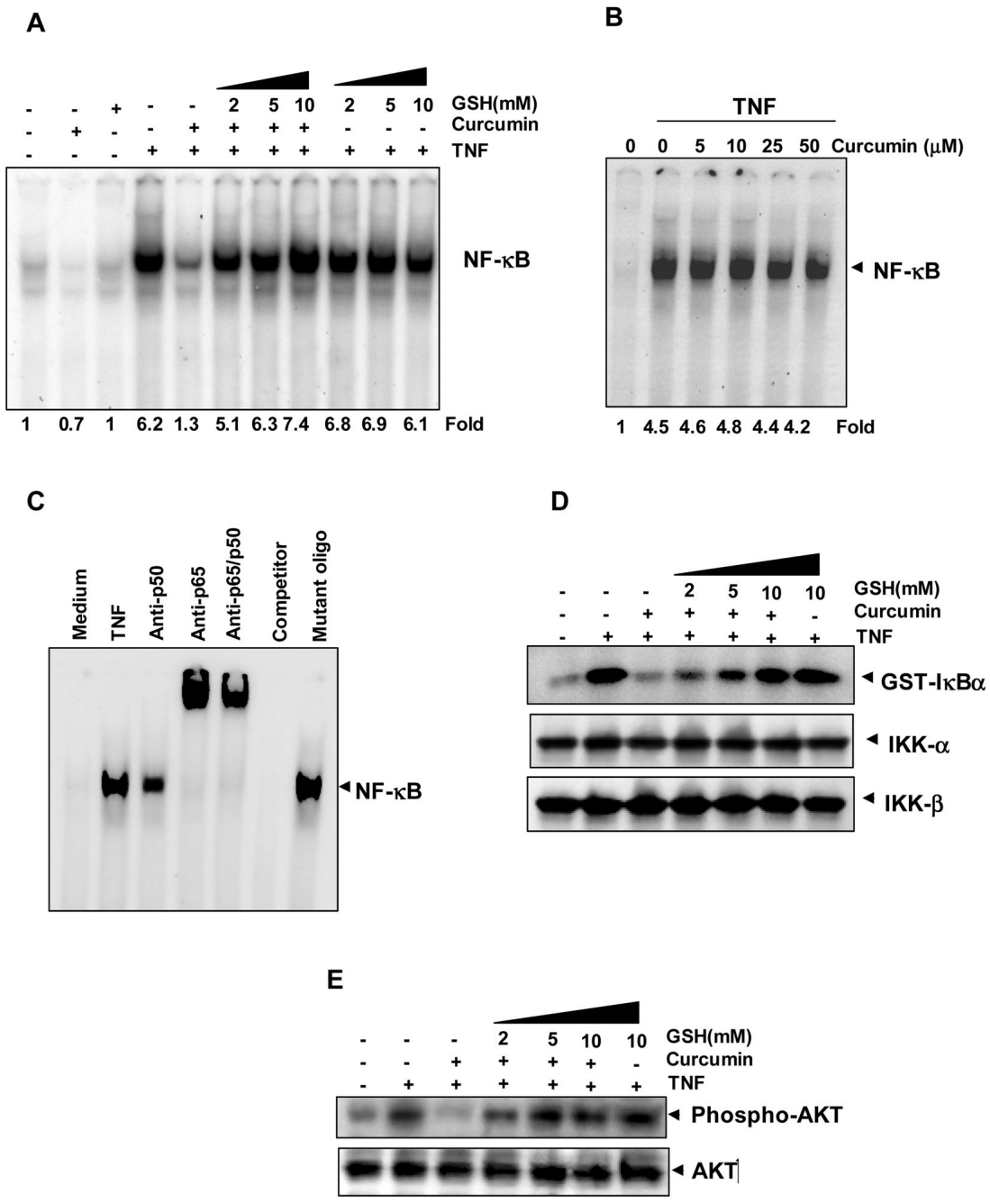

FIGURE 1. Glutathione abrogates curcumin-mediated inhibition of NF-κB

A, Curcumin inhibited TNF-induced activation of NF-κB and GSH blocked the effect of curcumin in a dose-dependent manner. KBM-5 cells were incubated with indicated concentrations of GSH for 2 h, treated with 50 μM curcumin for 4 h. Then cells were activated with 0.1 nM of TNF for 30 min, and then subjected to EMSA to assay for NF-κB activation. **B**, The direct effect of curcumin on the NF-κB complex was investigated. Nuclear extracts were prepared from untreated cells or cells treated with 0.1 nM TNF and incubated for 30 min with the indicated concentrations of curcumin. They were then assayed for NF-κB activation by EMSA. **C**, NF-κB induced by TNF is composed of p65 and p50 subunits. Nuclear extracts from untreated or TNF-treated cells were incubated with the indicated antibodies, unlabeled

NF-κB oligo-probe, or mutant oligo-probe and then assayed for NF-κB activation by EMSA. **D,** Glutathione inhibited curcumin-mediated suppression of IKK activity. KBM-5 cells were preincubated with indicated concentrations of GSH for 2 h, treated with 50 μM concentration of curcumin for 4 h. Then cells were activated with 1 nM TNF for 15 min. Whole-cell extracts were immunoprecipitated with antibody against IKK-α and analyzed by an immune complex kinase assay. To examine the effect of curcumin ad GSH on level of IKK proteins, whole-cell extracts were fractionated on SDS-PAGE and examined by Western blot analysis using anti-IKK-α and anti-IKK-β antibodies. **E**, Curcumin suppressed TNF-mediated phosphorylation of AKT, as shown by Western blotting; GSH significantly blocked the effect of curcumin. KBM-5 cells were preincubated with indicated concentrations of GSH for 2 h, treated with 50 μM concentration of curcumin for 4 h. Then cells were activated with 1 nM TNF for 10 min. Whole cell extracts were prepared, separated on 10% SDS-PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was performed using phospho AKT antibody. Anti-AKT was used as a loading control.

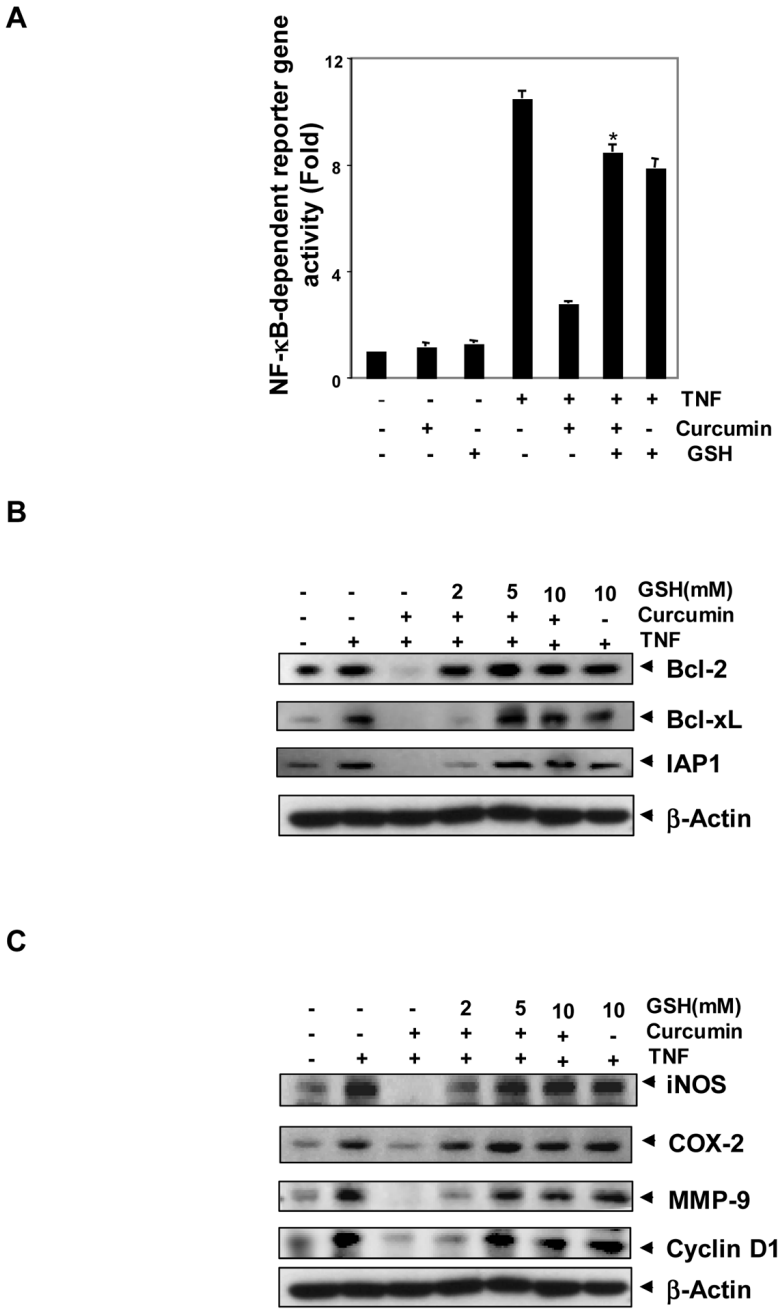

FIGURE 2. Glutathione blocks curcumin-mediated suppression of NF-κB regulated gene expression

A, Curcumin significantly suppressed TNF-induced NF-κB reporter gene (SEAP) expression in A293 cells; GSH inhibited the effect of curcumin. A293 cells were transiently transfected with a NF-κB-containing plasmid for 24 h. After transfection, the cells were incubated with 2 mM GSH for 2 h, treated with 10 μM curcumin. Then cells were treated with 1 nM TNF for an additional 24 h. The supernatants of the culture medium were assayed for SEAP activity. Each bar represents mean ± S.D. from three replicates and two independent experiments were carried out. $^{*}p < 0.01$, as compared to curcumin plus TNF treated group. **B** and **C**, Glutathione blocked curcumin-mediated repression of TNF-induced NF-κB-dependent expression of

antiapoptosis (*B*); proliferation and metastasis-related (*C*) gene products as shown by Western blotting. KBM-5 cells were incubated with indicated concentrations of GSH for 2 h, treated with 50 μM curcumin for 4 h. Then cells were activated with 1 nM TNF for 16 h. Whole-cell extracts were prepared and subjected to Western blot analysis using the relevant antibodies.

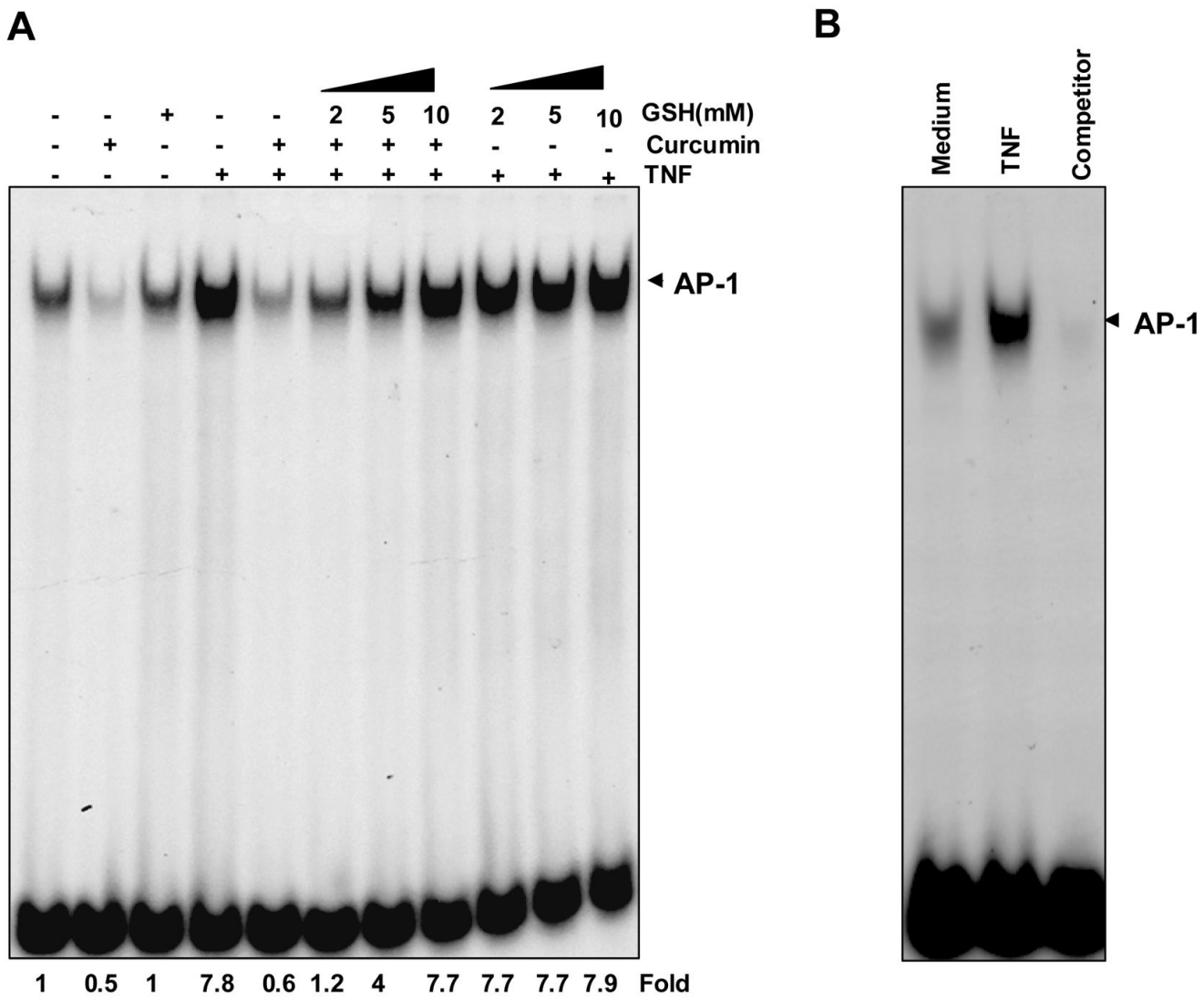

FIGURE 3. Effect of GSH on curcumin-mediated suppression of TNF-induced AP-1 activity

A, Curcumin suppressed TNF-induced AP-1 activity completely and GSH significantly inhibited the effect of curcumin. KBM-5 cells were incubated with indicated concentrations of GSH for 2 h, treated with 50 μM curcumin for 4 h. Then cells were activated with 0.1 nM of TNF for 30 min, and then subjected to EMSA to assay for AP-1 activation. **B,** The specificity of binding was examined by competition with unlabeled oligonucleotide. Nuclear extracts from untreated or TNF-treated cells were incubated with the cold unlabeled AP-1 oligo-probe then assayed for AP-1 activation by EMSA.

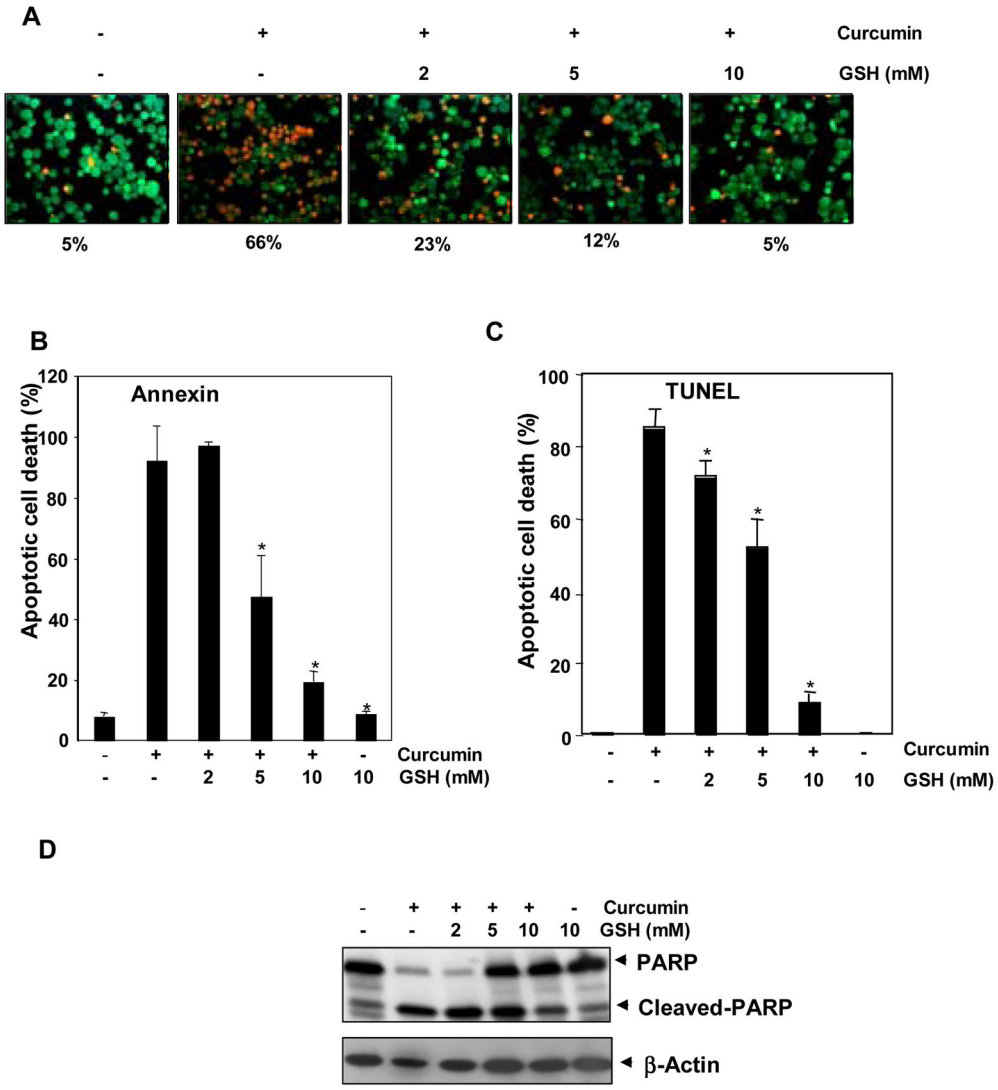

FIGURE 4. Effect of GSH on curcumin-induced cell death

Glutathione inhibited curcumin-induced cell death in a concentration-dependent manner. **A**, KBM-5 cells were pretreated with indicated concentrations of GSH for 2 h and then treated with 50 μM concentration of curcumin for 16 h. Cells were stained with Live and Dead assay reagent for 30 min and then analyzed under a fluorescence microscope. Red color highlights dead cells, and green color highlights live cells. **B**, Flow cytometric analysis of annexin V-FITC staining to detect early apoptotic effects. KBM-5 cells were pretreated with indicated concentrations of GSH for 2 h and then treated with 50 μM concentration of curcumin for 24 h. Cells were incubated with anti-annexin V antibody conjugated with FITC and then analyzed with a flow cytometer for early apoptotic effects. Each bar represents mean ± S.D. from three replicates and two independent experiments were carried out. $^{*}p < 0.01$, as compared to curcumin treated group. **C**, Flow cytometric analysis of TUNEL staining to detect apoptotic effects. KBM-5 cells were pretreated with indicated concentrations of GSH for 2 h and then treated with 50 μM concentration of curcumin for 24 h. Cells were fixed, stained with TUNEL reagent. Each bar represents mean ± S.D. from three replicates and two independent experiments were carried out. $^{*}p < 0.01$, as compared to curcumin treated group. **D**, Western blot analysis of PARP cleavage. KBM-5 cells were pretreated with indicated concentrations

of GSH for 2 h and then treated with 50 μM concentration of curcumin for 24 h. Whole-cell extracts were prepared and subjected to Western blot analysis using anti-PARP antibody.

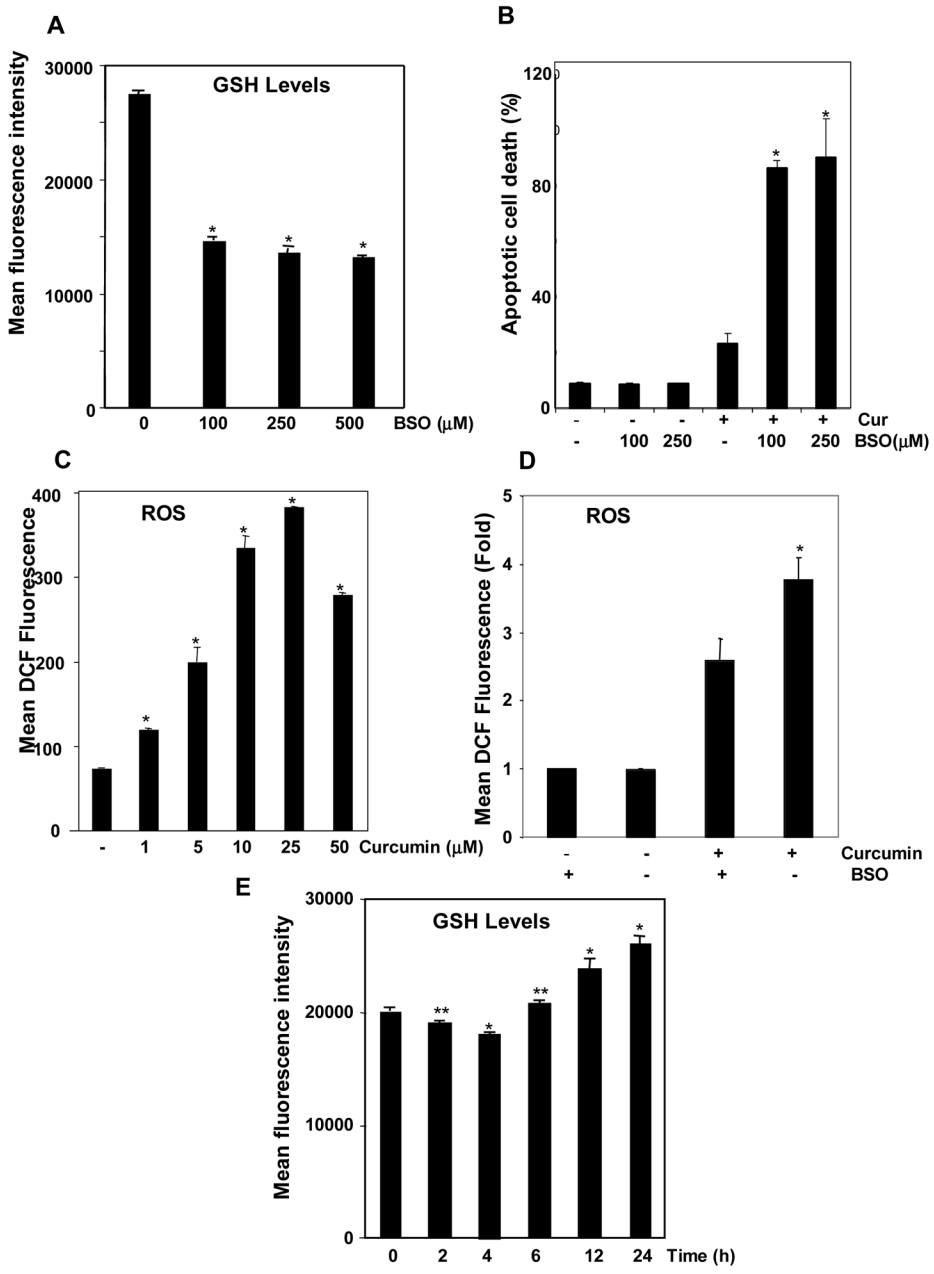

FIGURE 5. Depletion of endogenous GSH by pretreatment with BSO increases curcumin-induced cell death

A, Flow cytometric analysis of monobromobimane, which becomes fluorescent after reacting with GSH, in KBM-5 cells. Glutathione levels were significantly depleted by treatment with BSO. KBM-5 cells were incubated with indicated concentrations of BSO for 24 h. Cells were stained with monobromobimane (40 μM) and acquired on flow cytometer. Each bar represents mean ± S.D. from three replicates and two independent experiments were carried out. $^{*}p <$ 0.01, as compared to control. **B,** Decrease in endogenous GSH led to increased cytotoxicity of curcumin, as measured by annexin V staining. KBM-5 cells were incubated with indicated concentrations of BSO for 24 hr, treated with 10 μM concentration of curcumin for 24 h. Cells were incubated with anti-annexin V antibody conjugated with FITC. Each bar represents mean ± S.D. from three replicates and two independent experiments were carried out. $^{*}p < 0.01$, as

compared to curcumin treated group. **C,** Curcumin induced generation of ROS in KBM-5 cells. ROS levels are measured using a dye, DCF-DA, which becomes fluorescent after reacting with ROS. Cells were stained with 20 μM DCF-DA for 20 min and then treated with different concentrations of curcumin for 2 h. Each bar represents mean ± S.D. from three replicates and two independent experiments were carried out. $^{*}p < 0.01$, as compared to control. **D,** Depletion of intracellular BSO led to further increase in ROS levels when treated with curcumin as compared to only curcumin treated group. KBM-5 cells were treated with or without BSO (250 μM) for 24 h prior to 20 μM DCF-DA addition. Then cells were incubated in presence or absence of 10 μM curcumin additional for 2h and DCF fluorescence was measured on flow cytometer. Each bar represents mean ± S.D. from three replicates. $^{*}p < 0.01$, as compared to control. **E**, Curcumin changed the intracellular GSH levels. KBM-5 cells were treated with 10 μM curcumin for indicated time intervals before staining with 40 μM monobromobimane. Cells were acquired on flow cytometer. Each bar represents mean ± S.D. from three replicates and two independent experiments were carried out. $^{*}p < 0.01$, $^{**}p < 0.05$, as compared to control.

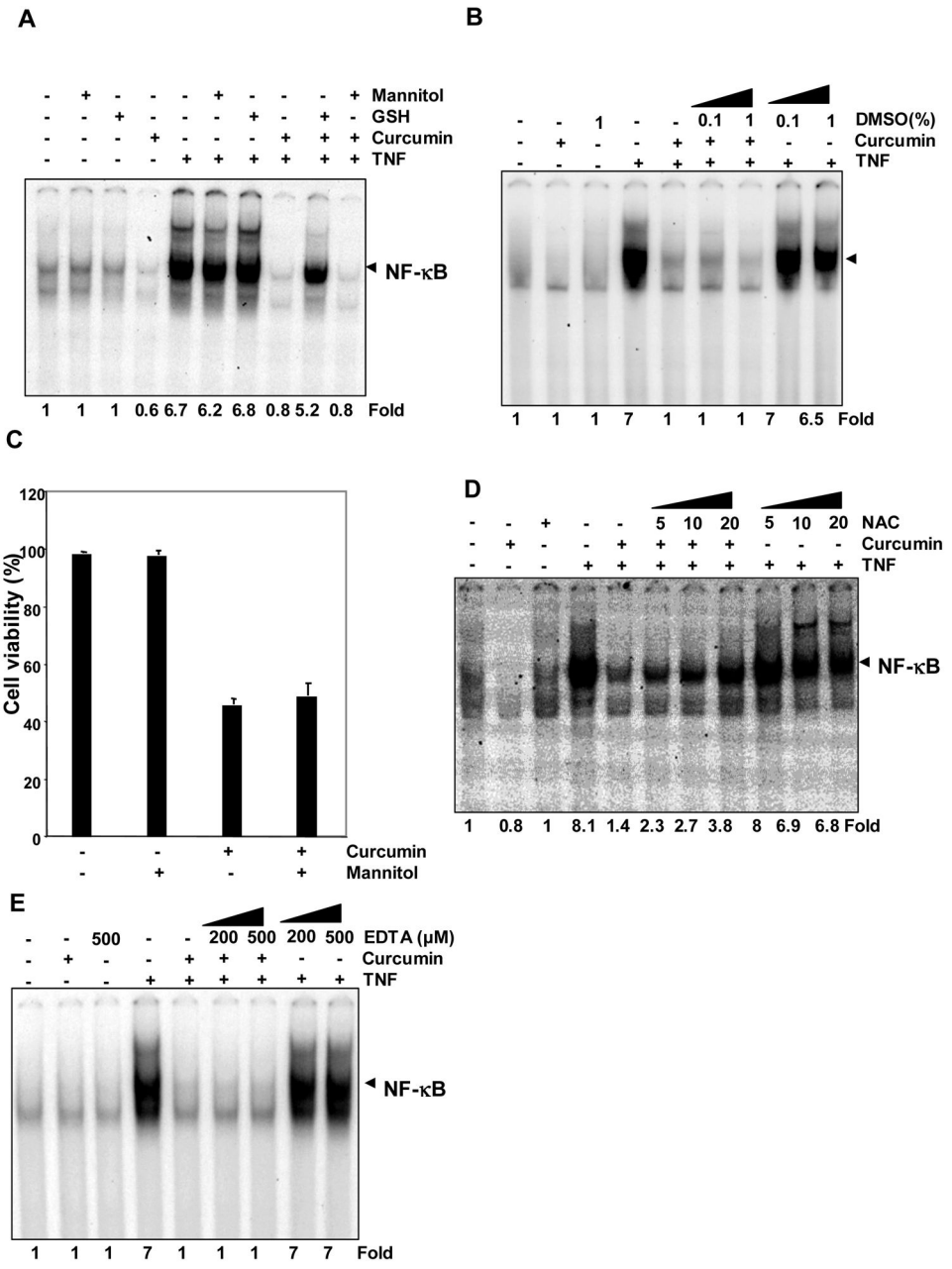

FIGURE 6. Mannitol and DMSO did not inhibit curcumin-mediated suppression of TNF-induced NF-κB activation

A, Curcumin completely suppressed TNF-induced NF-κB activation and mannitol failed to block the effect of curcumin. KBM-5 cells were incubated with 10 mM mannitol for 2 h, treated with 50 μM curcumin for 4 h. Then cells were activated with 0.1 nM of TNF for 30 min, and then subjected to EMSA to assay for NF-κB activation. **B,** Curcumin completely suppressed TNF-induced NF-κB activation and DMSO failed to block the effect of curcumin. KBM-5 cells were incubated with either 0.1or 1% DMSO, treated with 50 μM curcumin for 4 h. Then cells were activated with 0.1 nM of TNF for 30 min, and then subjected to EMSA to assay for NF-κB activation. **C**, Curcumin induced significant cell death, as shown by trypan blue exclusion, but mannitol did not rescue the cells. KBM-5 cells were pretreated with 10 mM

mannitol for 2 h and then treated with 50 μM curcumin for 24 h. Cell viability was measured by trypan blue exclusion. Each bar represents mean ± S.D. from three replicates and two independent experiments were carried out. **D and E,** Effect of NAC and EDTA on curcumin-mediated suppression of TNF-induced NF-κB activation. KBM-5 cells were incubated with either NAC for 24 h (D) or EDTA for 2 h (E). Then cells were treated with 50 μM curcumin for 4 h and were activated with 0.1 nM of TNF for 30 min. Nuclear extracts were subjected to EMSA for estimation of NF-κB.