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Effect of Curcumin on the Aryl Hydrocarbon Receptor and Cytochrome P450 1A1 in MCF-7 Human Breast Carcinoma Cells

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ABSTRACT. We examined the interaction of curcumin, a dietary constituent and chemopreventive compound, with the carcinogen activation pathway mediated by the aryl hydrocarbon receptor (AhR) in MCF-7 mammary epithelial carcinoma cells. Curcumin caused a rapid accumulation of cytochrome P450 1A1 (CYP1A1) mRNA in a time- and concentration-dependent manner, and CYP1A1 monooxygenase activity increased as measured by ethoxyresorufin-*O*-deethylation. Curcumin activated the DNA-binding capacity of the AhR for the xenobiotic responsive element of *CYP1A1* as measured by the electrophoretic-mobility shift assay (EMSA). Curcumin was able to compete with the prototypical AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin for binding to the AhR in isolated MCF-7 cytosol, indicating that it interacts directly with the receptor. Although curcumin could activate the AhR on its own, it partially inhibited the activation of AhR, as measured by EMSA, and partially decreased the accumulation of CYP1A1 mRNA caused by the mammary carcinogen dimethylbenzanthracene (DMBA). Curcumin competitively inhibited CYP1A1 activity in DMBA-treated cells and in microsomes isolated from DMBA-treated cells. Curcumin also inhibited the metabolic activation of DMBA, as measured by the formation of DMBA–DNA adducts, and decreased DMBA-induced cytotoxicity. These results suggest that the chemopreventive effect of curcumin may be due, in part, to its ability to compete with aryl hydrocarbons for both the AhR and CYP1A1. Curcumin may thus be a natural ligand and substrate of the AhR pathway. BIOCHEM PHARMACOL **56**;2:197–206, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. curcumin; aryl hydrocarbon receptor; cytochrome P450 1A1; dimethylbenzanthracene; DNA adducts; MCF-7 cells

The AhR§ is a ubiquitous cytosolic protein that binds environmental contaminants such as the PAH, i.e. DMBA and benzopyrene, and halogenated derivatives such as TCDD. The AhR is thought to mediate the broad spectrum of biological responses that these compounds can elicit, including tetratogenesis, tumor promotion, and thymic atrophy [1]. Upon binding a ligand, the AhR translocates to the nucleus where it binds another protein, the aryl hydrocarbon receptor nuclear translocase (ARNT). This heterodimer acts as a transcription factor of the basic helix-loop-helix family of DNA binding proteins. It binds to enhancer sequences, termed the XRE (also known as the dioxin-responsive element) flanking the 5′-promoter region of several genes [2]. The most extensively studied cellular response to PAH is the transcriptional induction of the gene *CYP1A*. This gene encodes the enzyme cytochrome P450 1A, which catalyzes the oxidative catabolism of PAH. This reaction generates genotoxic metabolites that can enter the nucleus and bind to specific residues of DNA, leading to mutagenesis [3]. Ligand-bound AhR also activates the transcription of a battery of detoxification (or Phase II) enzymes such as UDP-glucuronosyl transferase. The known ligands of the AhR, with the exception of indolo[3,2-*b*]carbazole [4], a compound found in some vegetables, are man-made. Natural endogenous or exogenous ligands have been postulated but remain to be identified. Thus, the physiologic function of the AhR and the pathway it regulates have yet to be discovered.

Curcumin, a polyphenolic compound derived from the plant *Curcurma longa*-Linn., is present in the spice turmeric, which is used extensively in Indian cooking. Dietary intake of curcumin among Indians has been estimated at up to 100 mg/day [5]. Additionally, curcumin has been used in Indian and Chinese traditional medicines for centuries. Recently,

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§ *Abbreviations:* AhR, aryl hydrocarbon receptor; CYP1A1, cytochrome P450 1A1; DMBA, dimethylbenzanthracene; EMSA, electrophoretic-mobility shift assay; EROD, ethyoxyresorufin-*O*-deethylase; PAH, polycyclic aromatic hydrocarbons; RT–PCR, reverse transcription–polymerase chain reaction; TBE, Tris, borate, ethylenediaminetetraacetic acid; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and XRE, xenobiotic (or dioxin) responsive element.

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the chemopreventive potential of curcumin in animal models of cancer has been examined. Curcumin has been shown to be effective in inhibiting azoxymethane-induced colon tumorigenesis [6, 7] and in inhibiting 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion in rat skin following initiation by DMBA [8, 9]. Reports on the effects of curcumin on DMBA-induced mammary cancer, however, are conflicting. Pereira *et al.* [7] found no effect of curcumin on DMBA-induced mammary cancer in rats. Singletary *et al.* [10], on the other hand, found that curcumin inhibits DMBA–DNA adduct formation and mammary tumorigenesis in rats. The mechanism of curcumin's action, however, was not identified. Curcumin has been shown to inhibit cytochrome P450 1A1/1A2 activity in rodent hepatic microsomes [11], and to decrease the formation of benzo[*a*]pyrene or aflatoxin-generated DNA adducts in rodent liver [12, 13]. These results indicate that curcumin may interact with the carcinogen activation pathway mediated by the AhR.

In the present study, we have used the human mammary epithelial carcinoma MCF-7 cell line to study the interaction of curcumin with the AhR pathway. The MCF-7 cell line was chosen since it represents the target tissue of DMBA and because it has a well-characterized AhR pathway [14–16]. We report, for the first time, that curcumin is able to induce CYP1A1 mRNA accumulation and induce CYP1A1 enzymatic activity by activation of the AhR. Curcumin is able to compete with TCDD for binding to isolated AhR, and partially inhibits the response of the AhR to DMBA. Furthermore, curcumin decreases DMBA activation by inhibiting CYP1A1 activity in a competitive manner. These results indicate that curcumin is a ligand of the AhR and possibly a substrate of CYP1A1, and is only the second natural dietary factor to be so identified.

MATERIALS AND METHODS

Materials

RPMI 1640, glutamine, fetal bovine serum, trypsin/EDTA, PBS, and TBE buffer were obtained from BioFluids. Curcumin, DMBA, HEPES, EDTA, dithiothreitol (DTT), glycerol, poly(dI-dC), sodium molybdate, ethyoxyresorufin, resorufin, Tris–HCl, sulforhodamine, salmon sperm, and protease inhibitors were from the Sigma Chemical Co. α-Naphthoflavone was from Indofine. [^{32}P]CTP and [^{32}P]dATP were from DuPont/NEN. The RT–PCR kit was from Stratagene. TBE gels, TBE running buffer, and high-density sample buffer were from Novex. PCR primers for glyceraldehyde 3-phosphate dehydrogenase were from Clontech. Bio-Gel HT hydroxyapatite and protein assay kit were from Bio-Rad. [^{3}H]TCDD was from ChemSyn. Aquasol was from Beckman. Polyclonal antibody to AhR was a gift from Dr. Alan Poland of the University of Wisconsin. Curcumin used in all experiments was dissolved in DMSO at a concentration of 1 mM and stored at −20°.

Cell Culture

MCF-7 cells were grown in RPMI 1640 supplemented with 2 mM of glutamine and 10% fetal bovine serum. Cells were subcultured weekly using 0.25% trypsin/0.05% EDTA. All experiments were carried out on confluent cells in growth medium, except where indicated.

RT–PCR for CYP1A1

Confluent MCF-7 cells were treated with curcumin with or without 1 μM of DMBA in growth medium for the times and concentrations indicated in the figures. The cells were washed twice with PBS, and total RNA was isolated by the method of Chomczynski and Sacchi [17]. cDNA was synthesized from 10 μg of total RNA using a Stratagene RT–PCR kit as instructed. PCR was performed using the primer sequences and method of Dohr *et al.* [15] except that 27 cycles were used in the presence of 1.5 μCi of [^{32}P]dATP. PCR of glyceraldehyde 3-phosphate dehydrogenase was carried out using primers from Clontech for 19 cycles as instructed. Following PCR, 5 μL of high-density sample buffer was added to the samples, and they were subjected to electrophoresis on a 10% TBE gel in 1× TBE running buffer. The gel was dried, and the results were visualized and quantified on a Bio-Rad GS-363 Molecular Imaging System.

EMSA

Confluent cultures of MCF-7 cells were treated with 1 μM of DMBA and/or 5 μM of curcumin in growth medium for the times indicated in the graphs. Nuclear protein was isolated and EMSA was performed by the method of Denison *et al.* [18]. Synthetic DNA oligonucleotides containing the AhR-binding site of the dioxin-responsive element [19] were labeled with [^{32}P]CTP. The binding reactions were carried out for 30 min and contained 5 μg of nuclear protein, 1 μg of poly(dI-dC), 500 ng of salmon sperm DNA, and ~50,000 cpm of labeled probe in a final volume of 20 μL of binding buffer (15 mM of Tris, pH 7.9, 50 mM of KCl, 1 mM of $MgCl_2$, 0.5 mM of DTT, 0.25 mM of EDTA, and 5% glycerol). To determine specificity of binding to the oligonucleotide, a 200-fold excess of specific unlabeled probe, nonspecific unlabeled probe to the transcription factor AP-2, or 0.20 μg of anti-AhR antibody were added to the binding reaction and incubated on ice for 15 min prior to addition of the probe. DNA–protein complexes were separated under non-denaturing conditions on a 6% polyacrylamide gel using 0.5× TBE (45 mM of Tris borate, 45 mM of boric acid, 2 mM of EDTA) as a running buffer. The gels were dried, and the DNA–protein complexes were visualized on a Bio-Rad GS-363 Molecular Imaging System.

AhR Ligand Binding Assay

Specific binding to the AhR was measured by hydroxyapatite absorption chromatography by a modification of the

method of Poellinger *et al.* [20]. MCF-7 cells were grown to confluence in 175 cm^2 flasks. The cells were washed once in PBS, harvested by trypsinization, and pelleted by centrifugation at 800 *g* for 10 min at 4°. The pellet was washed once in cold PBS, repelleted as above, and resuspended in cold HEDMG buffer (25 mM of HEPES, 1 mM of EDTA, 1 mM of DTT, 20 mM of sodium molybdate, and 10% glycerol, pH 7.4), with protease inhibitors (100 μg/mL of phenylmethylsulfonyl fluoride, 300 μg/mL of EDTA, 0.5 μg/mL of leupeptin, 0.5 μg/mL of aprotinin, and 0.7 μg/mL of pepstatin A). The cells were homogenized by 30 strokes with a Dounce glass homogenizer on ice, and the homogenate was centrifuged at 100,000 *g* for 60 min at 4°. The supernatant (cytosol) was removed and protein was assayed by the Bradford method [21]. The cytosol was divided into aliquots and stored at −80°. Cytosolic protein (1.5 mg) was incubated with 10 nM of [^{3}H]TCDD in the presence of DMSO (control), 10 μM of α-naphthoflavone (positive control), or 10 μM of curcumin in a total volume of 500 μL of HEDGM for 3 hr at 4°. The sample was applied to a hydroxyapatite column, bed volume 1 mL, equilibrated in HEDGM without protease inhibitors. AhR with bound [^{3}H]TCDD was eluted with a step gradient (2 mL per step) of sodium phosphate in HEDGM every 25 mM from 0 to 300 mM. The radioactivity of the fractions was counted by liquid scintillation. Specific binding eluted in three fractions: 100, 125, and 150 mM of phosphate.

CYP1A1 Activity in MCF-7 Cells

MCF-7 cells in 24-well plates were treated with curcumin at the concentrations indicated in the figures with or without 1 μM of DMBA in growth medium for 24, 48, or 72 hr. At the end of the incubation, the medium was removed and the wells were washed two times with fresh medium. The EROD activity of CYP1A1 was determined as described in Kennedy and Jones [22] using 5 μM of ethoxyresorufin in growth medium as a substrate, in the presence of 1.5 mM of salicylamide to inhibit conjugating enzymes. Fluorescence was measured every 10 min for 60 min in a CytoFlor II multiwell fluorescence plate reader, with excitation at 530 nm and emission at 590 nm. A standard curve was constructed using resorufin.

Microsomal CYP1A1 Activity

MCF-7 cells were treated with 1 μM of DMBA for 24 hr to induce CYP1A1 activity. Microsomes were isolated as follows: the cells were washed once with PBS, trypsinized, and pelleted by centrifugation at 800 *g* for 10 min at 4°. The pellet was washed in PBS and repelleted. The pellet was resuspended in 0.25 M of sucrose, 10 mM of Tris–HCl, pH 7.5, with protease inhibitors as above. The cells were sonicated for 30 sec on ice using a Branson Sonifier at setting 2. The sonicate was centrifuged at 10,000 *g* for 10 min at 4°, and the supernatant was subjected to centrifugation at 500,000 *g* for 15 min at 4°. The resulting microsomal pellet was resuspended in the above buffer and the protein assayed as above. Aliquots of microsomes were stored at −80°. EROD activity in 10 μg of microsomes was assayed using the indicated concentrations of ethoxyresorufin in 100 μL of PBS, pH 7.2, in a 96-well plate, in a CytoFlor II plate reader as described above.

Measurement of DMBA–DNA Adduct Formation

Confluent cultures of MCF-7 cells in 75 cm^2 flasks were exposed to 0.1 μg/mL of [^{3}H]DMBA in the presence of curcumin for 6 hr. Cells were washed twice with cold PBS, trypsinized, and pelleted. Nuclei were isolated by incubating the cells for 10 min on ice in 10 mM of Tris–HCl, pH 7.5, with 320 mM of sucrose, 5.0 mM of $MgCl_2$, and 1% Triton X-100. The nuclei were pelleted by centrifugation at 800 *g* for 10 min at 4°. Genomic DNA was then isolated from the nuclei by the method of Miller *et al.* [23]. The amount of DNA was measured by spectrophotometry, and the adducts were quantified by liquid scintillation counting in Aquasol (Beckman).

Measurement of Cell Growth

MCF-7 cells were plated in 24-well plates at 25,000 cells per well and allowed to attach for 24 hr. DMBA at the indicated concentrations was added with or without 2.5 μM of curcumin in fresh medium. Total cell growth was assayed after 3 days, using sulforhodamine [24].

RESULTS

Effect of Curcumin on CYP1A1 mRNA

We measured CYP1A1 mRNA in MCF-7 cells treated with DMBA in the presence or absence of curcumin using RT–PCR. Treatment of cells with 1 μM of DMBA for 24 hr caused a 15-fold increase in CYP1A1 mRNA accumulation (Fig. 1). Curcumin at 5 μM of partially inhibited the DMBA-induced increase. Surprisingly, treatment with curcumin alone caused an approximately six-fold increase in CYP1A1 mRNA. We therefore examined the effect of curcumin in the absence of other treatment on CYP1A1 mRNA. Treatment with curcumin for 24 hr resulted in an increase in CYP1A1 mRNA accumulation in a concentration-dependent manner from 1 to 10 μM (Fig. 2A). A 5 μM concentration of curcumin induced a time-dependent increase in CYP1A1 mRNA (Fig. 2B) that was maximal after 9 hr of incubation but was still elevated after 24 hr of incubation. Actinomycin D (5 μg/mL, added 1 hr prior to the addition of curcumin) completely blocked curcumin-induced CYP1A1 mRNA accumulation (data not shown).

Activation of the AhR DNA-Binding Activity by Curcumin

To examine the effect of curcumin on the nuclear accumulation of activated AhR induced by DMBA, we performed

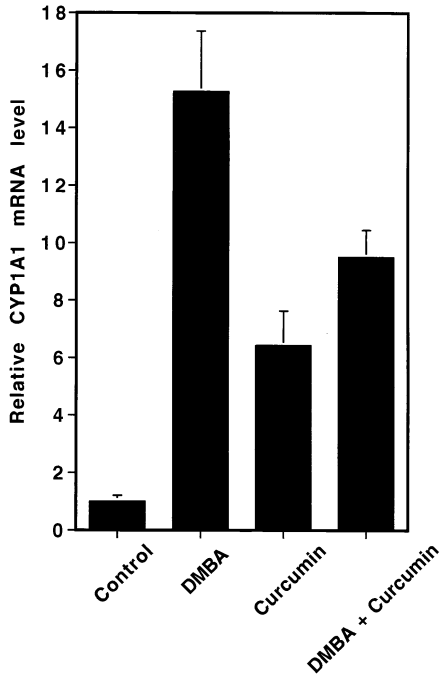

FIG. 1. Effect of DMBA on CYP1A1 mRNA in the presence or absence of curcumin. MCF-7 cells were treated with DMSO (control), 1 μM of DMBA, 5 μM of curcumin, or 1 μM of DMBA and 5 μM of curcumin for 24 hr. Total RNA was isolated, and RT–PCR for CYP1A1 mRNA was performed as indicated in Materials and Methods. Results were analyzed by denaturing gel electrophoresis, visualized, and quantified by phosphorimaging. The level of CYP1A1 mRNA in Figs. 1 and 2 was normalized to mRNA of glyceraldehyde 3-phosphate dehydrogenase. Bars represent the means ± SEM of 3 determinations.

EMSAs on nuclear extracts of MCF-7 human breast epithelial carcinoma cells that had been treated with curcumin in the presence or absence of DMBA (Fig. 3). DMBA at 1 μM caused an increase in the DNA-binding ability of AhR for an oligonucleotide containing the XRE (lanes 5 and 6) compared with controls (lanes 1 and 2). In the presence of 5 μM of curcumin, this increase was partially inhibited (lanes 3 and 4). Curcumin at 5 μM by itself caused an increase in activated AhR (lanes 7 and 8). Based on this result, we performed another EMSA on nuclear extracts of MCF-7 cells treated with 5 μM of curcumin at different time points in the absence of other treatment (Fig. 4). Curcumin induced a rapid increase in nuclear accumulation of activated AhR compared with the control (lanes 1 and 2) that was maximal at 3 hr (lanes 3 and 4) and declined thereafter. Specificity of this band as an AhR-mediated gel shift was confirmed by competition of nuclear extract from cells treated with 5 μM of curcumin for 6 hr with unlabeled XRE probe, by partial competition with a polyclonal antibody to AhR, and by lack of competition with the nonspecific probe to AP-2 (lanes 9–11, respectively; compare with lanes 5 and 6). In another gel-shift experiment, we were able to completely compete away the curcumin-induced band shift, using a higher concentration of anti-AhR antibody (data not shown).

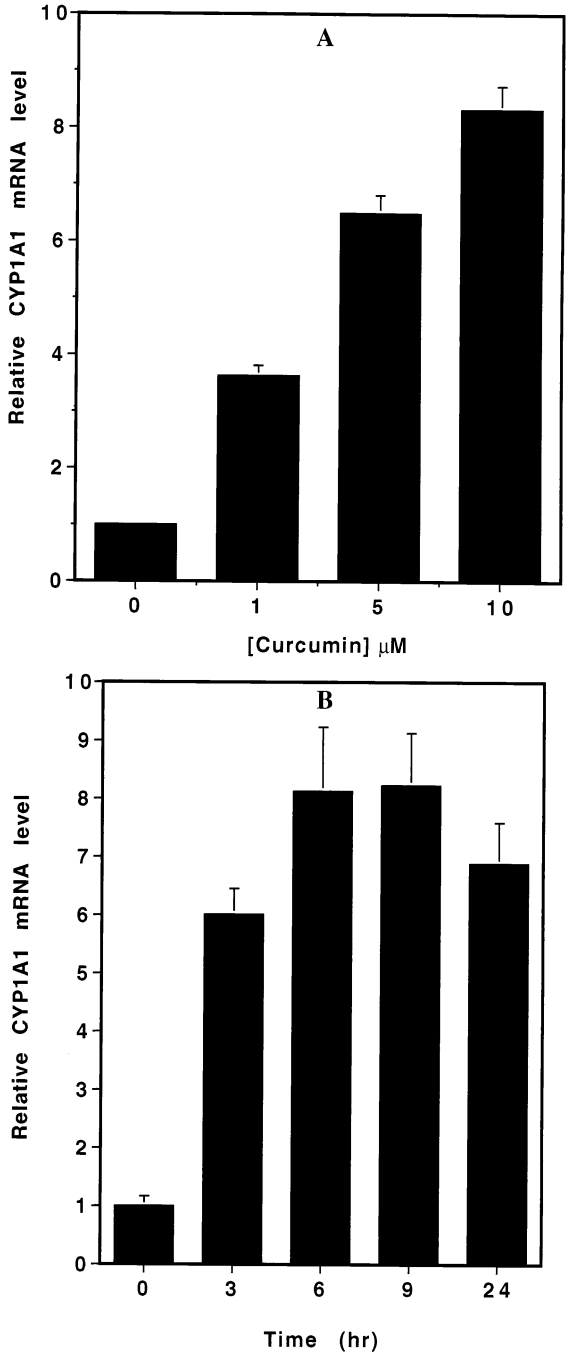

FIG. 2. Concentration–response (A) and time-course (B) of CYP1A1 mRNA to curcumin. MCF-7 cells were treated for 24 hr with the concentrations of curcumin indicated in (A), or with 5 μM of curcumin for the times indicated in (B). Bars represent the means ± SEM of 3 determinations.

Increase in CYP1A1 Activity in MCF-7 Cells Treated with Curcumin

CYP1A1 encodes the enzyme CYP1A1, which catalyzes the metabolic activation of DMBA. Based on our observations of the accumulation of CYP1A1 mRNA and the increased DNA-binding activity of AhR induced by curcumin in the absence of any other treatment, we measured the enzymatic

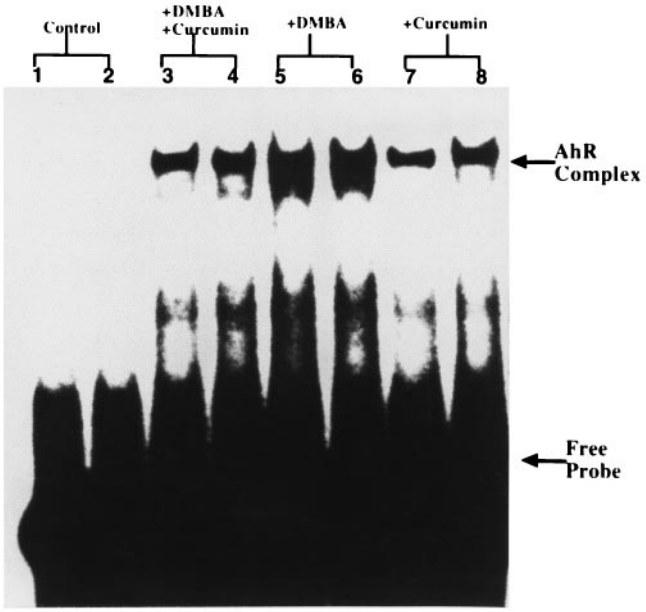

FIG. 3. XRE-binding activity of the nuclear AhR following treatment with DMBA with or without curcumin. Confluent MCF-7 cells were treated with DMSO (lanes 1 and 2), 1 μM of DMBA with 5 μM of curcumin (lanes 3 and 4), 1 μM of DMBA (lanes 5 and 6), or 5 μM of curcumin (lanes 7 and 8) for 6 hr at 37°. Five micrograms of nuclear protein was incubated with ^{32}P-labeled XRE sequence, and the DNA–protein complexes were resolved by EMSA and visualized by phosphorimaging.

activity of CYP1A1 in MCF-7 cells treated with curcumin using the EROD assay, which is specific for CYP1A1/2 monoxygenase activity. Twenty-four hours of curcumin treatment resulted in a biphasic response of CYP1A1 EROD activity. There was a concentration-dependent increase in activity up to 5 μM of curcumin, but an absence of measurable activity in cells treated with 10 μM (Fig. 5). After 48 hr, however, there was a linear, concentration-dependent increase in activity from 0.5 to 10 μM of curcumin. By 72 hr, enzyme activity increased in a linear fashion but was much reduced compared with the earlier time points. The protein synthesis inhibitor cyclohexamide blocked any increase in EROD activity in curcumin-treated cells (data not shown).

Effect of Curcumin on Binding of TCDD to AhR

The EROD data suggest that curcumin is a ligand of the AhR. To further demonstrate this, we examined the ability of curcumin to compete with the prototypical AhR ligand TCDD for binding to the AhR in the cytosolic fraction isolated from MCF-7 cells. Using hydroxyapatite chromatography to separate specific from nonspecific binding, we found that curcumin at 1000-fold molar excess reduced [^{3}H]TCDD binding by 45%, while the known AhR ligand α-naphthoflavone inhibited binding by 55% (Fig. 6). This indicates that curcumin is able to directly compete with TCDD for binding to the AhR.

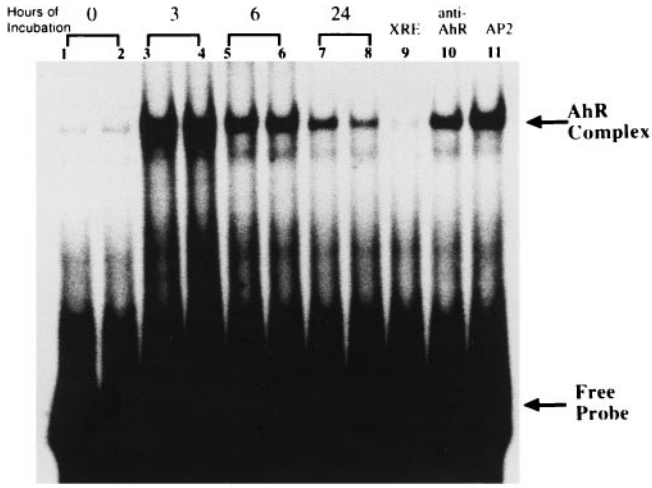

FIG. 4. Time-course of activation of XRE-binding activity and specificity of band-shift of the nuclear AhR following treatment with curcumin. MCF-7 cells were treated with 5 μM of curcumin for 0 (lanes 1 and 2), 3 (lanes 3 and 4), 6 (lanes 5 and 6) or 24 (lanes 7 and 8) hr. For competition, a 200-fold excess of unlabeled XRE (lane 9), 0.20 μg of polyclonal antibody to AhR (lane 10), or 200-fold excess of nonspecific unlabeled probe containing the AP-2 binding site (lane 11) was preincubated for 15 min on ice with 5 μg of nuclear protein from cells treated with curcumin for 3 hr (compare with lanes 3 and 4) prior to the addition of labeled probe.

Effect of Curcumin on DMBA-induced CYP1A1 Activity

When MCF-7 cells were treated with 1 μM of DMBA for 24 hr, there was an increase in CYP1A1 enzyme activity as measured by EROD assay (data not shown). Curcumin treatment of the cells during exposure to DMBA resulted in a concentration-dependent inhibition of DMBA-induced enzymatic activity (Fig. 7A). We also found that the CYP1A1 activity of microsomes isolated from DMBA-treated cells was inhibited in a concentration-dependent manner (Fig. 7B). The concentration at which enzyme activity was inhibited by 50% was approximately 1 μM. In the presence of increasing amounts of curcumin, the substrate concentration for half-maximal enzyme activity (K_m) shifted from approximately 200 nM in the absence of curcumin to 1200 nM in the presence of 2 μM of curcumin (Fig. 7C). No changes in maximal enzyme velocity were observed. Therefore, the inhibitory effect of curcumin on CYP1A1 activity is competitive in nature.

Effect of Curcumin on Metabolic Activation of DMBA

Metabolic activation of DMBA by CYP1A1 leads to metabolites that specifically react with DNA. Based on the above data, we hypothesized that the ability of curcumin to inhibit CYP1A1 enzymatic activity would lead to a decrease in the metabolic activation of DMBA, although curcumin treatment itself causes an increase of CYP1A1 activity. We measured DMBA activation using two indirect methods: first, we measured the formation of DMBA–DNA

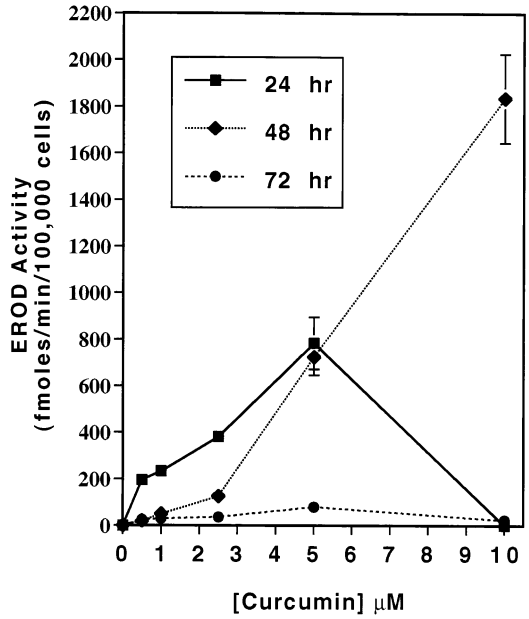

FIG. 5. Effect of curcumin treatment on the EROD activity of CYP1A1. MCF-7 cells were treated with DMSO or curcumin at the concentration indicated for 24, 48, or 72 hr. The EROD activity of CYP1A1 was measured with 5 μM of ethoxyresorufin as described in Materials and Methods. Points represent the means ± SEM of 4 determinations.

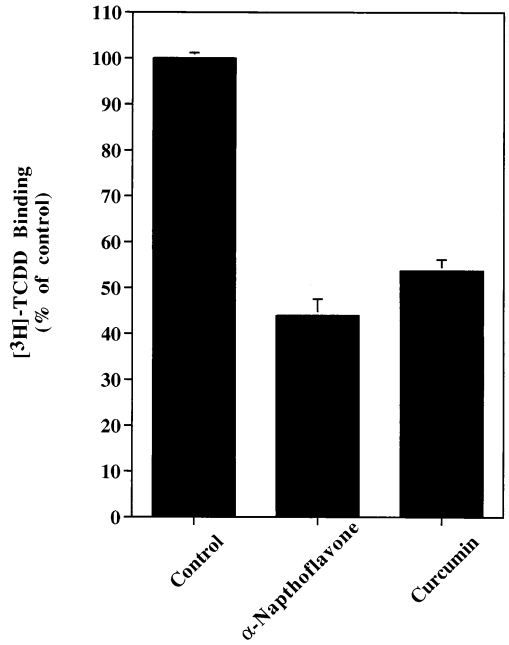

FIG. 6. Effect of α-naphthoflavone or curcumin on binding of [^{3}H]TCDD to AhR in MCF-7 cytosol. Cytosol was isolated from MCF-7 cells as described. Cytosolic protein (1.5 mg) was incubated with 10 nM of [^{3}H]TCDD in the presence of DMSO (control), 10 μM of α-naphthoflavone, or 10 μM of curcumin for 3 hr at 4°, and specific binding was separated from nonspecific binding using hydroxyapatite chromatography. [^{3}H]TCDD bound in controls was 2890 ± 400 dpm per 1.5 mg of protein. Bars represent the means ± SEM of 3 determinations.

adducts, and second, since the cytotoxicity of DMBA is due primarily to adduct formation, we measured DMBA-induced inhibition of cell growth. We found that curcumin caused a decrease in adduct formation in MCF-7 cells exposed to DMBA in a concentration-dependent manner (Fig. 8A). We also measured the effect of 2.5 μM of curcumin on DMBA-induced inhibition of cell growth. Curcumin substantially decreased the cytotoxicity of DMBA, shifting the IC_{50} approximately three-fold, from 300 nM to greater than 900 nM (Fig. 8B).

DISCUSSION

We examined the effect of curcumin on the AhR and the major carcinogen-activating enzyme in MCF-7 cells, CYP1A1 [25]. Using RT–PCR, we found that the mammary carcinogen DMBA caused an increase in CYP1A1 mRNA in MCF-7 cells that was partially antagonized by simultaneous treatment of the cells with curcumin (Fig. 1). It has been shown previously that the ability of the liganded AhR to induce transcription of genes depends on its ability to bind enhancer sequences, called the XRE, flanking the 5′-promoter region of *CYP1A1* [26]. Using EMSA to measure the nuclear accumulation of activated AhR, we showed that curcumin could partially inhibit the activation of the AhR by DMBA (Fig. 3). This decreased activation of AhR accounts for the decrease in DMBA-induced CYP1A1 mRNA seen in Fig. 1. The product of *CYP1A1*, the enzyme CYP1A1, catalyzes the first step in the metabolic activation of DMBA. DMBA induced CYP1A1 activity in a concentration-dependent fashion as measured by the EROD assay (data not shown). Treatment of the cells with curcumin during DMBA exposure resulted in a concentration-dependent inhibition of CYP1A1 activity (Fig. 7A). Based on these findings, we could not be certain whether this inhibition was due to direct inhibition of enzyme activity or the result of inhibition of AhR-mediated signal transduction. Accordingly, we isolated microsomes from cells treated with DMBA. The microsomes exhibited EROD activity that was inhibited by curcumin in a concentration-dependent fashion, with an IC_{50} of approximately 1 μM (Fig. 7B). This inhibition was competitive in nature, indicating that curcumin interacts with CYP1A1 at the active site (Fig. 7C). Inhibition of the AhR-mediated response of *CYP1A1* to DMBA and direct inhibition of CYP1A1 activity by curcumin would be expected to reduce the metabolic activation of DMBA. This was confirmed using two indirect measurements of DMBA metabolism. The formation of adducts between DNA and the reactive metabolites of DMBA, which result from CYP1A1 activity, were reduced substantially in curcumin-treated MCF-7 cells (Fig. 8A). Furthermore, the cytotoxicity of DMBA, as measured by inhibition of cell growth, was also reduced in the presence of curcumin (Fig. 8B). Metabolic activation of aryl hydrocarbons in MCF-7 cells under conditions of AhR stimulation is primarily, though not exclusively, due to the 1A1 isozyme [25]. Whether curcumin also inhibits the

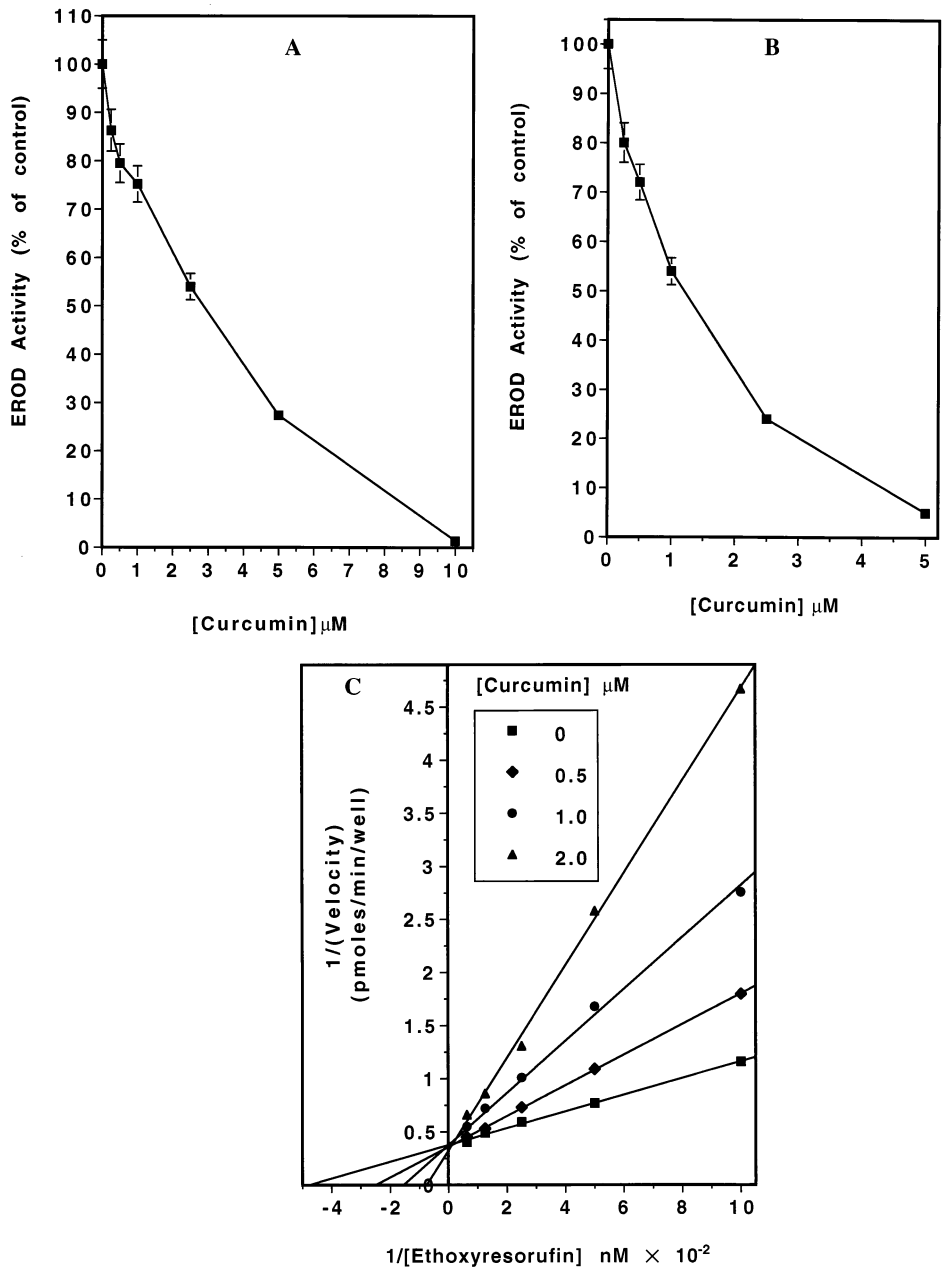

FIG. 7. Effect of curcumin on DMBA-induced CYP1A1 EROD activity. (A) Confluent MCF-7 cells were treated with 1 μM of DMBA in the presence of DMSO (control) or curcumin as indicated for 24 hr, and the EROD activity of CYP1A1 was measured. Activity in controls was 8.50 ± 0.64 pmol/min/100,000 cells. (B) Microsomes were isolated from MCF-7 cells treated with 1 μM of DMBA for 24 hr. The EROD activity of 10 μg of microsomal protein treated with DMSO (control) or curcumin at the indicated concentrations was measured. Activity in controls was 2.71 ± pmol/min/10 μg. For A and B, values are means ± SEM, N = 4. (C) Ten micrograms of microsomes from DMBA-treated MCF-7 cells was incubated with the indicated amounts of ethoxyresorufin in the presence or absence of curcumin at the concentrations indicated. EROD activity was measured, and a Lineweaver–Burk plot was generated. Points represent the averages of 4 determinations.

activity of CYP1B1, which is also expressed in these cells, is not known.

The chemopreventive effect of curcumin has been variously ascribed to the antioxidant properties of curcumin [27], inhibition of cyclooxygenase activity [28], suppression of activator protein-1-mediated transcription [29], and induction of apoptosis of tumor cells [30]. Based on our results, curcumin also appears to decrease carcinogen activation and inhibit carcinogen–DNA adduct formation by direct inhibition of CYP1A1 activity, and by partially inhibiting the AhR-mediated response to DMBA. Thus, the chemopreventive effect of curcumin may also be due to the multiple inhibitory effects of curcumin on the AhR pathway.

To our surprise, curcumin treatment of MCF-7 cells in the absence of DMBA caused an increase in the accumu-

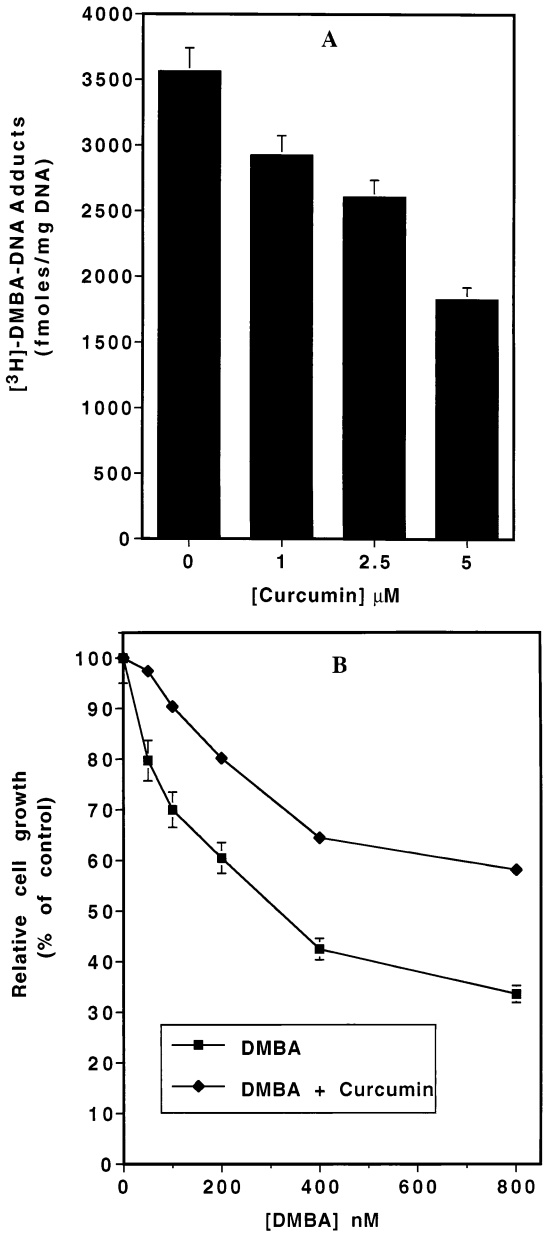

FIG. 8. Effect of curcumin on the formation of DMBA–DNA adducts (A) and DMBA-induced cytotoxicity (B) in MCF-7 cells. (A) MCF-7 cells were incubated with DMSO or curcumin at the concentrations indicated in the presence of 0.1 μg/mL of [^{3}H]DMBA for 6 hr. Genomic DNA was isolated, and the adducts were quantified. Bars represent the means ± SEM of 4 determinations. (B) MCF-7 cells were plated at 25,000 cells/well in 24-well plates and allowed to attach for 24 hr. DMBA was added at the concentrations indicated in the presence of DMSO (control) or 2.5 μM of curcumin. Four days later, the amount of cell proliferation was measured with sulforhodamine. Points represent the means ± SEM of 4 determinations.

lation of CYP1A1 mRNA (Fig. 1). This result was confirmed by RT–PCR in panels A and B of Fig. 2, which demonstrate that curcumin induced CYP1A1 mRNA in a concentration- and time-dependent manner. The RNA polymerase inhibitor actinomycin D blocked CYP1A1 mRNA accumulation caused by curcumin, indicating that *de novo* RNA synthesis via transcriptional activation is required (data not shown). Curcumin treatment alone also activated the DNA-binding capacity of the AhR (Fig. 3), as measured by EMSA. This was confirmed in Fig. 4, which demonstrates that curcumin caused a rapid but transient activation of the AhR. That the band shift caused by curcumin was due to activation of the AhR was demonstrated in Fig. 4 by the ability of unlabeled probe containing the XRE of *CYP1A1* or a polyclonal antibody to the AhR to compete away the band-shift. CYP1A1 activity, as measured by the EROD assay, increased in a time- and concentration-dependent fashion in cells treated with curcumin alone (Fig. 5). The direct inhibition of CYP1A1 EROD activity by curcumin seen in Fig. 7 leads us to suspect that the increase in EROD activity in curcumin-treated cells may be more profound than demonstrated in Fig. 5, and provides an explanation for the biphasic nature of the EROD response to curcumin at 24 hr of incubation. Although exogenous curcumin is removed before assay, intracellular curcumin remains, which would inhibit enzyme activity. We surmise that in cells treated with a 5 μM concentration or less of curcumin the increase in CYP1A1 EROD activity is suppressed only partially by enzyme inhibition, while at higher concentrations there is enough intracellular curcumin present to inhibit activity completely. That CYP1A1 mRNA is greater in cells treated with 10 μM of curcumin compared with lesser concentrations, while enzyme activity is absent at this concentration, seems to confirm this. The linear increase in CYP1A1 activity seen at 48 hr may be due to a reduction in cellular curcumin concentration, possibly as a result of oxidative catabolism of curcumin by CYP1A1. By 72 hr, there remained only a small increase in EROD activity compared with control levels.

Curcumin, therefore, activates the AhR, induces CYP1A1 mRNA accumulation, and induces CYP1A1 enzymatic activity. These results suggest but do not prove conclusively that curcumin is a ligand of the AhR. It has been demonstrated recently, that the gastric drug omeprazole induces *CYP1A1* transcription and increases EROD activity in human hepatocytes in culture, yet does not bind to the AhR [31]. Omeprazole either undergoes metabolic alteration in the cells, generating AhR ligands, or it releases unknown endogenous ligands. To determine whether curcumin acts similarly or interacts directly with the AhR, a ligand-binding assay was performed. Cytosol isolated from MCF-7 cells was incubated with radiolabeled TCDD in the presence of curcumin or the known AhR ligand α-naphthoflavone, a positive control. Specific binding was separated from nonspecific binding by hydroxyapatite chromatography. As shown in Fig. 6, curcumin, at 1000-fold excess, partially inhibited TCDD binding to the AhR. This indicates that curcumin interacts directly with the AhR, and, along with α-naphthoflavone, is of much lower affinity for the receptor than TCDD. It was not possible to separate specific from nonspecific binding in this assay using radiolabeled DMBA (data not shown), but it seems likely that

the inhibition of DMBA-induced AhR activation by curcumin demonstrated in the EMSA in Fig. 3 may be the result of such competition between AhR ligands. Thus, curcumin appears to be an antagonist of the AhR in the presence of other AhR ligands such as DMBA or TCDD. Curcumin also failed to induce CYP1A1 mRNA or EROD activity in an AhR-deficient clonal line of MCF-7 cells* (data not shown). These results confirm that curcumin is a ligand of the AhR.

With two exceptions (indolo[3,2-*b*]-carbazole [4], a constituent of some vegetables, and the marine neurotoxin brevetoxin-6 [32]), the known ligands of the AhR are man-made, either industrial byproducts such as TCDD, environmental contaminants such as benzo[*a*]pyrene, or synthetic compounds such as α-naphthoflavone or the drug omeprazole. Based on our current results, curcumin appears to be a natural, dietary ligand of the AhR. The fact that curcumin induces *CYP1A1* via the AhR, and is a competitive inhibitor of CYP1A1 activity, indicates that it may be a natural substrate for this enzyme. The decrease in receptor activation over time (Fig. 2) and the decrease in curcumin-stimulated enzyme activity after 72 hr of incubation indicate that curcumin is being metabolized by the cells, perhaps by CYP1A1, a possibility that we are currently investigating. Whether curcumin also stimulates the transcription of Phase II enzymes associated with the AhR is also under investigation. Thus, the chemopreventive qualities of curcumin may be the fortuitous result of sharing the same metabolic pathway as several important carcinogens.

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