Carcinogenesis vol.25 no.9 pp.1671–1679, 2004
doi:10.1093/carcin/bgh165

Modulation of arachidonic acid metabolism by curcumin and related β-diketone derivatives: effects on cytosolic phospholipase A_2, cyclooxygenases and 5-lipoxygenase

Jungil Hong, Mousumi Bose, Jihyeung Ju, Jae-Ha Ryu, Xiaoxin Chen, Shengmin Sang, Mao-Jung Lee and Chung S.Yang[1]

Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA

[1]To whom correspondence should be addressed
Email: csyang@rci.rutger.edu

Aberrant arachidonic acid metabolism is involved in the inflammatory and carcinogenic processes. In this study, we investigated the effects of curcumin, a naturally occurring chemopreventive agent, and related β-diketone derivatives on the release of arachidonic acid and its metabolites in the murine macrophage RAW264.7 cells and in HT-29 human colon cancer cells. We also examined their effects on the catalytic activities and protein levels of related enzymes: cytosolic phospholipase A_2 ($cPLA_2$), cyclooxygenases (COX) as well as 5-lipoxygenase (5-LOX). At 10 μM, dibenzoylmethane (DBM), trimethoxydibenzoylmethane (TDM), tetrahydrocurcumin (THC) and curcumin effectively inhibited the release of arachidonic acid and its metabolites in lipopolysaccharide (LPS)-stimulated RAW cells and A23187-stimulated HT-29 cells. Inhibition of phosphorylation of $cPLA_2$, the activation process of this enzyme, rather than direct inhibition of $cPLA_2$ activity appears to be involved in the effect of curcumin. All the curcuminoids (10 μM) potently inhibited the formation of prostaglandin E_2 (PGE_2) in LPS-stimulated RAW cells. Curcumin (20 μM) significantly inhibited LPS-induced COX-2 expression; this effect, rather than the catalytic inhibition of COX, may contribute to the decreased PGE_2 formation. Without LPS-stimulation, however, curcumin increased the COX-2 level in the macrophage cells. Studies with isolated ovine COX-1 and COX-2 enzymes showed that the curcuminoids had significantly higher inhibitory effects on the peroxidase activity of COX-1 than that of COX-2. Curcumin and THC potently inhibited the activity of human recombinant 5-LOX, showing estimated IC_{50} values of 0.7 and 3 μM, respectively. The results suggest that curcumin affects arachidonic acid metabolism by blocking the phosphorylation of $cPLA_2$, decreasing the expression of COX-2 and inhibiting the catalytic activities of 5-LOX. These activities may contribute to the anti-inflammatory and anticarcinogenic actions of curcumin and its analogs.

Abbreviations: COX, cyclooxygenase; $cPLA_2$, cytosolic PLA_2; DBM, dibenzoylmethane; HETE, hydroxyeicosatetraenoic acid; HPLC, high performance liquid chromatography; LOX, lipoxygenase; LPS, lipopolysaccharides; LTB_4, leukotriene B_4; MAPK, mitogen activated protein kinase; MEK, MAPK kinase; PLA_2, phospholipase A_2; PG, prostaglandin; PGE_2, prostaglandin E_2; TDM, trimethoxydibenzoylmethane; THC, tetrahydrocurcumin.

Introduction

Curcumin (diferuloyl methane) is a major constituent found in the spice turmeric, which is a dried powder from the rhizomes of *Curcuma longa L.*. This spice has been used as a traditional medicine for treatment of inflammation, gastrointestinal disorders, hepatic disorders, diabetic wounds, skin wounds, rheumatism, sinusitis and other disorders (1). Curcumin is also a food coloring and flavoring agent approved by the Food and Drug Administration.

Studies using chemically induced animal carcinogenesis models demonstrated the chemopreventive effect of curcumin in the colon, duodenum, forestomach, mammary glands, oral cavity and skin (2–7). A variety of mechanisms have been suggested for the anticarcinogenic effect of curcumin, including antioxidative activities, modulation of the cell cycle, inhibition of the enzymes related to tumor promotion such as ornithine decarboxylase, protein kinase C and inducible nitric oxide synthase, inhibition of epidermal growth factor receptor tyrosine kinases, inhibition of activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), and inhibition of angiogenesis (8–12). The anticarcinogenic action of curcumin derivatives with β-diketone structure, such as tetrahydrocurcumin (THC) and dibenzoylmethane (DBM), has also been reported (6,13).

Many studies have demonstrated the involvement of aberrant arachidonic acid metabolism in carcinogenesis. Membrane phospholipids, the major source of arachidonic acid are hydrolyzed by phospholipase A_2 (PLA_2); the released arachidonic acid is further metabolized by three different types of oxygenases: cyclooxygenase (COX), lipoxygenase (LOX) and cytochromes P450. Modulation of arachidonic acid metabolism by inhibiting these enzymes has been considered as an effective mechanism for chemoprevention. Inhibition of arachidonic acid metabolism by curcumin has been suggested to be a key mechanism for its anticarcinogenic action (14–16). Curcumin has been reported to inhibit COX-2 expression in gastrointestinal cancer cells and mouse skin (17–19). Several previous studies have also indicated that curcumin affects the formation of COX- and LOX-dependent metabolites and decreases activities of PLA_2 and PLCγ1 (20,21). However, the precise mechanisms involved in the decreased enzyme activities and metabolite formation are not clear. The inhibition could be due to the direct inhibition of the enzyme activities, to the decrease of protein levels of these enzymes or to altered molecular regulation. In the present study, we analyzed the effect of curcumin and related β-diketone analogs on the release of arachidonic acid and its metabolites in the intact cells as well as catalytic activities and protein levels of related enzymes, cytosolic PLA_2 ($cPLA_2$), COX-1, COX-2 and 5-LOX, in the murine macrophage RAW264.7 cells and HT-29 human colon cancer cells. Our results suggest that curcumin and related β-diketone derivatives are potent modulators of arachidonic acid metabolism. The results also indicate that curcumin can affect arachidonic acid metabolism

Downloaded from http://carcin.oxfordjournals.org/ by guest on April 3, 2013

by inhibiting the phosphorylation of $cPLA_2$, expression of COX-2 and the enzyme activity of 5-LOX. Their inhibitory effect may be an important anti-inflammatory mechanism and may contribute to their anticarcinogenic actions.

Materials and methods

Chemicals and cell lines

[5,6,8,9,11,12,14,15-^{3}H](N) Arachidonic acid and 1-palmitoyl 2-[1-^{14}C] arachidonyl sn-glycero-3-phosphorylcholine were purchased from NEN Life Science (Boston, MA). Arachidonic acid metabolite standards, ovine COX-1 and COX-2, human recombinant 5-LOX and human COX-2 monoclonal antibody were from Cayman Chemical Company (Ann Arbor, MI). Phospho-$cPLA_2$ (Ser^{505}) and $cPLA_2$ antibody were from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-5-LOX antibody was obtained from Transduction Laboratories (Lexington, KY). Curcumin and related β-diketone derivatives were generously provided by Dr M.-T.Huang of our department. The purity of curcumin and DBM was determined to be >95% by high performance liquid chromatography (HPLC), whereas THC and trimethoxydibenzoylmethane (TDM) was ~70% pure. Structures of these compounds are shown in Figure 1. Murine macrophage RAW264.7 and the HT-29 human colon cancer cell line were obtained from American Type Culture Collection (Rockville, MD). RAW264.7 and HT-29 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine, glucose and sodium bicarbonate, and McCoy 5A medium, respectively, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin, at 37°C in 95% humidity and 5% CO_2. All other chemicals were purchased from Sigma Chemical (St Louis, MO).

Release of arachidonic acid and its metabolites in intact cell system

RAW264.7 and HT-29 cells were plated into a 24-well plate at ~3.5×10^5 and 2.0×10^5 cells/well, respectively, in the growth media. After 24 h, the media were removed and replaced with 1 ml of serum free DMEM for RAW cells or Ham's F-12 media for HT-29 cells containing 0.1 μCi/ml [5,6,8,9,11,12,14,15-^{3}H](N) arachidonic acid. The cells were incubated overnight, resulting in >90% of arachidonic acid incorporated into the cell membrane. The cells were washed twice with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin to remove unabsorbed arachidonic acid. RAW cells were stimulated with 2 μg/ml lipopolysaccharides (LPS) (from *Escherichia coli*, serotype 055:b5) for 1 h, and then incubated with fresh medium containing tested compounds or vehicle (final concentration, 0.1% DMSO) for 18 h. HT-29 cells were incubated with 10 μM A23187 for 20 min, and then were treated with tested compounds or vehicle in fresh medium for 90 min. After incubation, the culture medium was collected, and centrifuged for 10 min at 10 000 *g*. Radioactivity of the cell culture media was measured by a scintillation counter (Model LS3801, Beckman Coulter, Fullerton, CA). For analyzing prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) levels, RAW cells were treated in a similar manner as above without using radiolabeled arachidonic acid. After incubation, the culture medium was collected and the PGE_2 and LTB_4 levels were analyzed by an enzyme immunoassay (Cayman, Ann Arbor, MI).

Western blotting

RAW264.7 cells were plated into a 6-well plate at ~2×10^6 cells/well. After 24 h, the media were replaced with serum free DMEM for 24 h, and the cells were treated as described above. The cells were washed with ice cold PBS twice and lysed with cell lysis buffer (1 mM PMSF, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_2VO_4, in 20 mM Tris, pH 7.4). The cell lysate was sonicated and centrifuged at 10 000 *g* for 15 min at 4°C. The supernatant containing 30–50 μg protein was loaded onto 10% or 4–15% gradient sodium dodecyl sulfate (SDS)–polyacrylamide gel. After electrophoresis, the proteins were transferred onto PVDF membrane and probed with antibodies for $cPLA_2$, phospho-$cPLA_2$, COX-2 and 5-LOX. The western blot was visualized using an ECL detection kit (Amersham, Arlington Heights, IL) and the densitometry was quantified using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD). Protein concentration in the cell lysates was determined by the method of Bradford (Bio-Rad, Hercules, CA).

$cPLA_2$ assays

For preparing the substrate solution, 1-palmitoyl 2-[1-^{14}C]arachidonyl sn-glycero-3-phosphorylcholine (hot:cold, 1:3) and phosphoinositides were dried under a stream of N_2 (g). Triton X-100 (2 mM) in 100 mM Tris–HCl buffer, pH 7.4, was added to the dried lipids, and the substrate micelles were prepared by sonification in a bath-sonicator for 3 min. $cPLA_2$ activity was assayed at 37°C in a reaction mixture (100 μl) consisting of 20 μg microsomal protein from HT-29 cells (the enzyme source), 20 μM substrate, 5 μg/ml phosphoinositides, 100 μM $CaCl_2$ and 200 μM Triton X-100 in 100 mM Tris–HCl buffer, pH 7.4. The reaction was initiated by adding 10 μl of substrate solution after a 5 min pre-incubation, and terminated after 30 min of incubation by the addition of 10 μl of 0.5 N HCl. Modified Dole's method was used for the extraction of free arachidonic acid (22). In brief, 5 vol (550 μl) of Dole solution (2-propanol:heptane:1 N H_2SO_4, 78:20:2) was added to the reaction mixture. After 10 min, arachidonic acid was extracted by adding 2 vol (220 μl) of water and 3 vol (330 μl) of heptane. The upper heptane layer was collected, and contaminated phospholipids were removed from the organic layer by treatment of silicic acid (30 mg, 100–200 mesh) twice. After separation of the heptane layer from silicic acid by centrifugation, 200 μl of the heptane layer was collected and measured for radioactivity in 4 ml scintillation cocktail.

COX and LOX assays

COX-dependent activity was measured at 37°C for 30 min in a reaction mixture (100 μl) consisting of 50 μg of RAW cell lysates, 20 μM (0.1 mCi) [^{3}H]arachidonic acid, 1 mM reduced glutathione and 1 mM epinephrine in a 100 mM Tris–HCl buffer, pH 7.4. For the 5-LOX assay, the reaction mixture (100 μl), containing 0.5 U human recombinant 5-LOX, 20 μM (0.1 μCi) [^{3}H]arachidonic acid, 2 mM $CaCl_2$ and 1 mM ATP in a 100 mM Tris–HCl buffer, pH 7.4, was incubated at 37°C for 10 min with tested compounds. The reactions were terminated by the addition of 15 μl of 0.5 N HCl. An equal volume of ice cold acetonitrile was added to the reaction mixture and centrifuged at 10 000 *g* for 10 min. COX and 5-LOX metabolites in the supernatant were analyzed by a reverse phase HPLC system (23). For peroxidase assay with isolated ovine COX-1 and COX-2, each enzyme (25 U) was incubated at an ambient temperature in a reaction mixture (200 μl) consisting of 100 μM arachidonic acid, 170 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) and 1 μM bovine hemin in a 100 mM Tris–HCl buffer, pH 7.4 (24). The absorbance change, at 590 nm due to oxidation of TMPD during the initial 5 min reaction, was analyzed in a microplate reader.

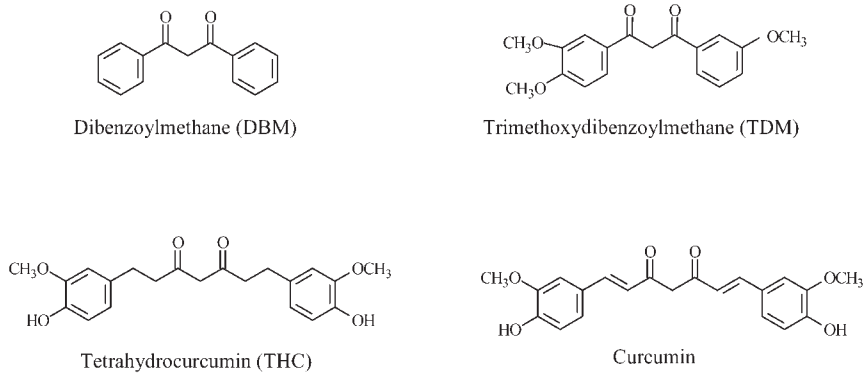

Fig. 1. Structures of curcumin and related β-diketone derivatives used in the present study.

Downloaded from http://carcin.oxfordjournals.org/ by guest on April 3, 2013

Data analysis

Statistical significance was evaluated using the Student's *t*-test.

Results

Effects of curcuminoids on the release of arachidonic acid and its metabolites in intact cells

The release of pre-labeled arachidonic acid and its metabolites from RAW264.7 murine macrophages to the culture media increased >2-fold after stimulation by LPS. The released radioactivity accounted for 3–5% of total labeled arachidonic acid during an 18-h incubation with the stimulated cells. HPLC profile showed that most of the released radioactivity was due to metabolites; arachidonic acid accounted for <3% of the total radioactivity in the media (data not shown). The time-dependent release of metabolites and its inhibition by curcumin are shown in Figure 2A. All the curcuminoids (at 10 μM) significantly decreased the release of arachidonic acid metabolites from RAW264.7 cells during the 18-h incubation (Figure 2B). TDM, THC and curcumin similarly inhibited the release by >50%, whereas DBM was slightly less effective. To study their effects on specific arachidonic acid metabolites, released PGE_2 and LTB_4 in the media from LPS-stimulated RAW cells were investigated. PGE_2 formation was markedly increased after stimulation with LPS, and all curcuminoids (10 μM) strongly inhibited the formation of PGE_2, with

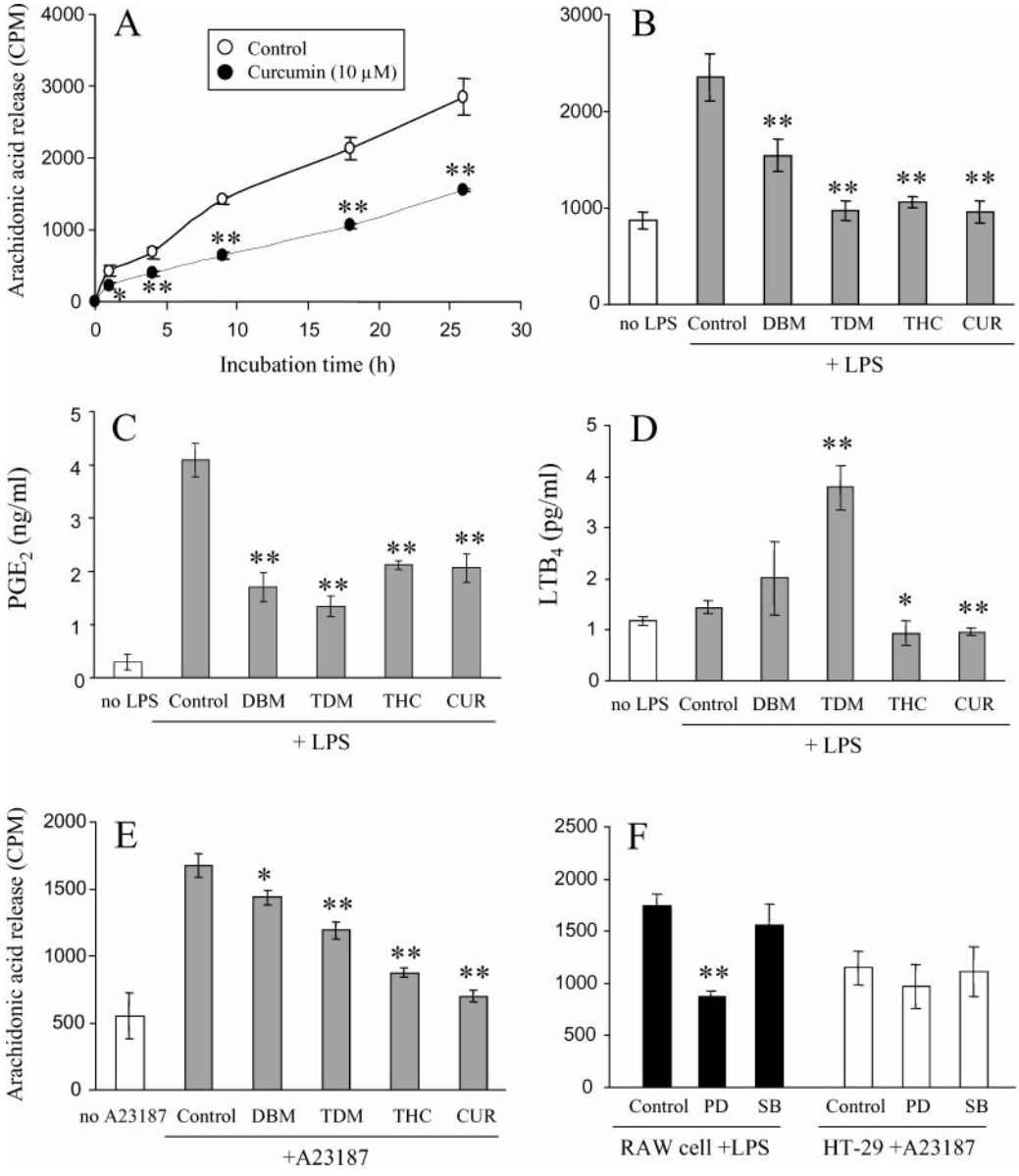

Fig. 2. Effects of curcuminoids on the release of arachidonic acid and its metabolites. RAW264.7 and HT-29 cells labeled with 0.1 μCi/ml [5,6,8,9,11,12,14,15-^{3}H](N) arachidonic acid were stimulated with 2 μg/ml LPS for 1 h and 10 μM A23187 for 20 min, respectively. Fresh medium containing 10 μM of curcuminoids or vehicle (final concentration, 0.1% DMSO) was then added. (**A**) Time-dependent release of arachidonic acid metabolites from RAW cells and its inhibition by curcumin. The radioactivity in culture medium was analyzed. (**B**) Inhibitory effects on the release of arachidonic acid and its metabolites by RAW264.7 cells after an incubation of 18 h. (**C** and **D**) Effects of curcuminoids on PGE_2 and LTB_4 levels in culture media after a 18-h incubation with RAW cells. The metabolites were analyzed by enzyme immunoassay. (**E**) Inhibitory effects of curcuminoids on A23187-stimulated arachidonic acid release from HT-29 cells. After a 90-min incubation with HT-29 cells, the radioactivity in the culture media was analyzed. (**F**) Effects of MAPK inhibitors (10 μM; PD, PD98059, and SB, SB203587) on the release of arachidonic acid and metabolites from RAW and HT-29 cells. Each bar represents the mean±SD ($n = 4$ in the case of A, B, E and F, $n = 8$ in the case of C and D). *,**Significantly different from control ($^{*}P < 0.05$; $^{**}P < 0.01$).

Downloaded from http://carcin.oxfordjournals.org/ by guest on April 3, 2013

TDM showing the most potent inhibitory effect (Figure 2C). There was no significant change in the level of LTB_4 after stimulation of RAW cells by LPS. Curcumin and THC also significantly decreased the level of LTB_4 in the culture medium; TDM, however, increased the LTB_4 level (Figure 2D).

A23187, a calcium ionophore, markedly increased arachidonic acid release in HT-29 cells, and all curcuminoids also significantly inhibited this event (Figure 2E). Curcumin, which showed the most potent inhibitory effect, inhibited the release in a concentration-dependent manner with an estimated IC_{50} of 4.4 μM, and the inhibition was significant even at a 1-μM concentration (by ~17%) in HT-29 cells (data not shown). Curcumin also significantly inhibited A23187-induced arachidonic acid release in other types of cancer cells and normal immortalized cells including HCT-116 human colon adenocarcinoma cells, KYSE-150 and KYSE-450 human esophageal squamous carcinoma cells, and IEC-6 immortalized rat intestinal cells to a similar extent (data not shown). To explore the involvement of mitogen activated protein kinases (MAPKs) in the release of arachidonic acid and metabolites from RAW or HT-29 cell system, the effects of PD98059, a MEK (MAPK kinase) inhibitor, or SB203587, a p38 and c-jun N-terminal kinase (JNK) inhibitor, were investigated. PD98059 significantly inhibited the release from LPS-stimulated RAW264.7 cells, but SB203587 was not effective (Figure 2F). Both inhibitors did not show any appreciable inhibition in A23187-stimulated HT-29 cells.

Effects on cPLA$_2$

Among several types of PLA_2, $cPLA_2$ has been reported to play a major role in releasing arachidonic acid from membrane phospholipids (25). In order to evaluate the potential of curcuminoids for the inhibition of catalytic activity of $cPLA_2$, the microsomal fraction from HT-29 cells was used as an enzyme source. The activity was almost exclusively due to $cPLA_2$, because (i) the microsomal fraction showed a 7.7-fold increase of PLA_2 activity in the presence of 5 μg/ml phosphoinositides and 100 μM of calcium (data not shown), and (ii) it was almost completely abolished by methyl arachidonyl fluorophosphate (a $cPLA_2$ inhibitor) and EGTA (a calcium chelator), but not significantly affected by bromoenol lactone (an $iPLA_2$ inhibitor) and dithiothreitol (a $sPLA_2$ inhibitor) (data not shown). Among the curcuminoids (50 μM), only THC inhibited $cPLA_2$ activity (by 36%), but curcumin slightly increased the activity (Figure 3A). To account for the strong inhibition of the release of arachidonic acid and its metabolites in intact cells, we investigated the effect of curcumin on the protein level and phosphorylation of $cPLA_2$. Treatment of RAW264.7 cells with

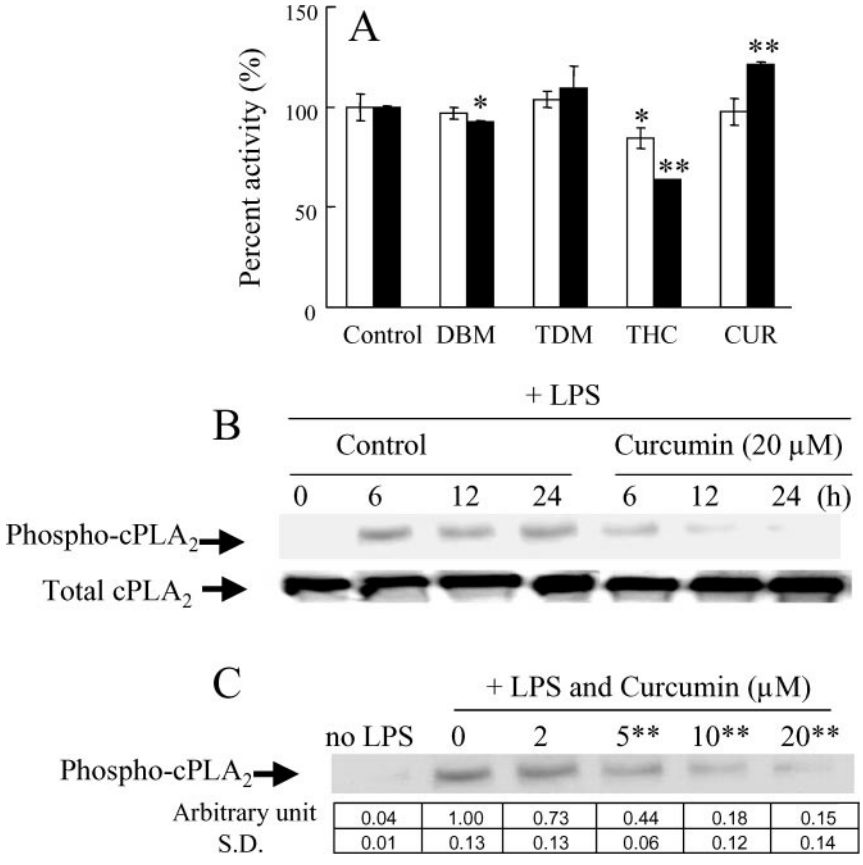

Fig. 3. Effects of curcuminoids on $cPLA_2$ activity from microsomal fraction of HT-29 cells and on the levels of $cPLA_2$ and phospho-$cPLA_2$ in LPS-stimulated RAW264.7 cells. (**A**) The reaction mixture (100 μl) consisted of 20 μg microsomal protein from HT-29 cells, 20 μM (0.0024 μCi) 1-palmitoyl 2-[1-^{14}C]arachidonyl sn-glycero-3-phosphorylcholine, 5 μg/ml phosphoinositides, 100 μM $CaCl_2$ in 100 mM Tris–HCl buffer, pH 7.4, with 5(hollow bar) or 50(filled bar) μM of curcuminoids. The reactions were carried out at 37°C for 30 min. The results are the mean ± SD ($n = 3$). (**B** and **C**) Time- and concentration-dependent effects of curcumin on levels of total $cPLA_2$ and phospho-$cPLA_2$ in LPS-stimulated RAW264.7 cells. Cells were incubated with 20 μM of curcumin or vehicle (DMSO) for different time periods or with different concentrations of curcumin for 18 h followed by treatment with 2 μg/ml LPS for 1 h. Western blot analysis was performed on cell lysates with antibodies against $cPLA_2$ or phospho-$cPLA_2$ (Ser^{505}). The results are representative of two (B) or three (C) independent experiments. Lower panel in (C) shows the densitometry quantification of phospho-$cPLA_2$ level normalized to each control (mean ± SD, $n = 3$). *,**Significantly different from control (*$P < 0.05$; **$P < 0.01$).

Downloaded from http://carcin.oxfordjournals.org/ by guest on April 3, 2013

LPS induced the phosphorylation of $cPLA_2$ at Ser^{505} without changing the protein level. Incubation of curcumin for different periods of time with LPS-stimulated cells decreased the level of phospho-$cPLA_2$ and the effect was concentration-dependent (Figure 3). Significant inhibition was observed even with 5 μM of curcumin.

Effects on COX

RAW264.7 cell lysates were used as an enzyme source for COX-dependent activity. Without LPS stimulation, the lysate did not have measurable COX-dependent activity. The lysate from LPS-stimulated RAW cells, however, actively catalyzed arachidonic acid metabolism (Figure 4). The formation of arachidonic acid metabolites was almost completely inhibited by 20 μM indomethacin, a COX inhibitor (Table I), whereas nordihydroguaiaretic acid, a LOX inhibitor, did not affect the reaction (data not shown). The western blot analysis showed that the COX-2 protein level was not clearly detected in non-stimulated RAW cells, and COX-2 was markedly increased by LPS-stimulation (Figure 4). The results indicate that the metabolites were produced mainly by COX-2. After a 30-min incubation of arachidonic acid with the lysates of LPS-stimulated RAW cell, several products were formed, including $PGF_2\alpha$, PGE_2, PGD_2, 2-hydroxyheptadecatrienoic acid (HHT) and hydroxyeicosatetraenoic acid (HETEs) (Figure 4B). All curcuminoids (50 μM) inhibited the COX-dependent arachidonic acid metabolism by 8–32% when the sum of the metabolites was compared. THC showed the most potent inhibitory effect, followed by curcumin, showing 32 and 23% inhibition, respectively; DBM and TDM showed only ~10% inhibition. Among the COX-dependent metabolites, inhibition of PGD_2 formation by curcumin was most pronounced, but PGE_2 formation was slightly increased (Table I).

In order to determine the selectivity of curcuminoids in inhibiting the two COX isoforms, their effects on the peroxidase activity of isolated ovine COX-1 and -2 were analyzed. All curcuminoids generally showed more potent inhibition against COX-1 than COX-2 (Figure 5). Curcumin showed the strongest inhibitory effect on the peroxidase activity of ovine COX-1 with an estimated IC_{50} of ~50 μM (Figure 5). Other curcuminoids (50 μM) all inhibited COX-1 by ~20%.

Stimulation of RAW264.7 cells with LPS increased the COX-2 protein level within 6 h, and the level was sustained until 24 h (Figure 6A). Curcumin (20 μM) significantly decreased the LPS-induced COX-2 expression in an 18-h treatment (Figure 6B). In another experiment, the time-dependent induction of COX-2 by LPS was seen, and at each time point marked inhibition by curcumin was observed (Figure 6C). Interestingly, curcumin induced COX-2 expression in RAW264.7 cells in the absence of LPS-stimulation, and

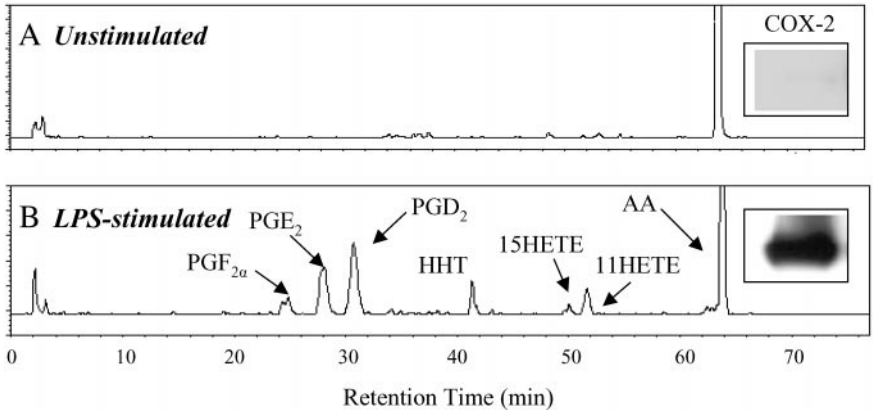

Fig. 4. COX-2 levels and chromatograms of [^{3}H]arachidonic acid metabolites in lysates from unstimulated RAW264.7 cells (**A**) and LPS-stimulated (18 h incubation) RAW264.7 cells (**B**). COX-2 levels were determined by western blot. The incubation mixture (100 μl) consisted of RAW264.7 cell lysates (50 μg protein), 20 μM (0.1 μCi) arachidonic acid, 1 mM glutathione and 1 mM epinephrine in 100 mM Tris–HCl buffer, pH 7.4. The reactions were carried out at 37°C for 30 min. (HHT, 2-hydroxyheptadecatrienoic acid; AA, arachidonic acid).

Table I. Effects of curcuminoids (50 μM) and indomethacin (20 μM) on COX-dependent arachidonic acid metabolites by LPS-stimulated RAW264.7 cell lysates

	Relative amount of product formed (%)						
	PGF_{2a}	PGE_2	PGD_2	HHT	15-HETE	11-HETE	Total
DBM	107.8	88.0	91.4	82.0	53.9	69.1	87.4
TDM	99.0	79.4	100.1	84.3	95.9	92.4	91.9
THC	77.8 ± 14.4	80.5 ± 4.2	62.8 ± 2.2	80.7 ± 21.3	29.1 ± 5.3	57.5 ± 2.3	68.9 ± 1.0
CUR	122.1 ± 8.2	116.5 ± 3.9	36.9 ± 2.9	103.3 ± 5.4	57.1 ± 27.0	70.3 ± 5.2	77.1 ± 1.8
Indo	1.6	0.2	0.8	2.3	1.5	1.4	0.9

The reaction mixture (100 μl) contained RAW264.7 cell lysates (50 μg protein), 20 μM (0.1 μCi) arachidonic acid, 1 mM glutathione and 1 mM epinephrine with or without 50 μM curcuminoids or indomethacin (20 μM) in 100 mM Tris–HCl buffer, pH 7.4. The reactions were carried out at 37°C for 30 min. The amounts of metabolites formed were analyzed by HPLC for PGF_{2a}, PGE_2, PGD_2, HHT, 15-HETE and 11-HETE. Values are the mean ± SD of three determinations (THC and CUR) or mean of duplicate experiments (DBM, TDM or indomethacin).

Downloaded from http://carcin.oxfordjournals.org/ by guest on April 3, 2013

the COX-2 protein level peaked at 14 h (Figure 6C), whereas resting cells did not show any appreciable COX-2 expression during the 24-h incubation time (data not shown).

Effects on 5-LOX

Since curcumin and THC significantly decreased LTB_4 level (Figure 2D), the effect of curcumin and THC on the 5-LOX pathway was investigated. When human recombinant 5-LOX was incubated with arachidonic acid, a major peak with the same retention time as 5-HETE (~57 min) and several unidentified peaks were produced (Figure 7). AA-861, a specific 5-LOX inhibitor, selectively inhibited the formation of the major peak. Significant inhibition of this metabolite by curcumin (2 μM) was also observed. Inhibitors for 5-LOX activating protein (MK-886) and LTA_4 hydrolase (bestatin) did not affect the reaction (data not shown).

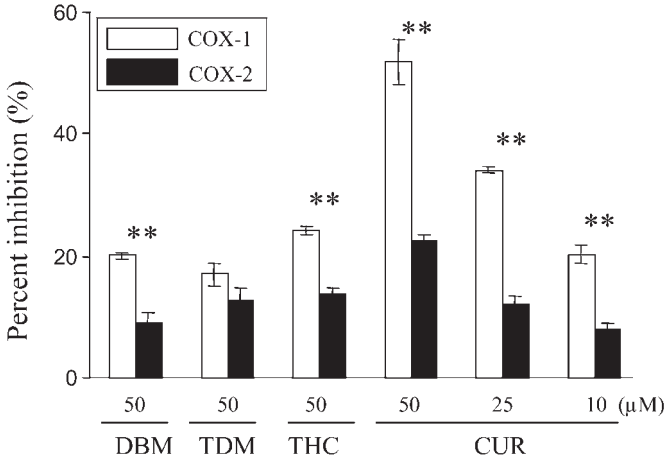

Fig. 5. Different effects of curcuminoids on ovine COX-1 and COX-2. The incubation mixture (200 μl) consisted of 25 U of ovine COX-1 or COX-2, 100 μM arachidonic acid, 1 μM hemin and 170 μM TMPD with curcumin in 100 mM Tris–HCl buffer, pH 7.4, with or without of curcuminoids. The reactions were carried out at an ambient temperature for 5 min. The peroxidase activity of COX was analyzed using microplate reader at 590 nm. **Significantly different between the effects on COX-1 and COX-2 ($P < 0.01$).

Curcumin and THC inhibited the formation of 5-HETE by human recombinant 5-LOX dose-dependently. The IC_{50} values of curcumin and THC were calculated to be 0.69 and 2.99 μM, respectively (Figure 7D). The effect of curcumin on the 5-LOX protein level in LPS-stimulated RAW cells was also investigated. The protein level of 5-LOX was not significantly changed after stimulation with LPS, and curcumin down-regulated 5-LOX protein at 20 μM (by 38%) after 18 h of treatment.

Discussion

Aberrant arachidonic acid metabolism occurs in inflammatory and carcinogenic processes, and modulation of arachidonic acid metabolism has been suggested to be an important strategy for cancer prevention (26–28). The present study demonstrates that curcumin and related β-diketone derivatives effectively inhibit the release of arachidonic acid and its metabolites from murine macrophage RAW264.7 cells and HT-29 cells. The release of arachidonic acid by PLA_2 from membrane phospholipids is usually the rate-limiting step for further arachidonic acid metabolism. Among several types of PLA_2, $cPLA_2$ plays a major role in catalyzing the release of arachidonic acid in most tissues (25,29). Our observation that curcuminoids are not effective in inhibiting the catalytic activity of $cPLA_2$ suggests that other mechanisms may be involved. Indeed, the results in Figure 3 demonstrate that curcumin inhibited the LPS-induced phosphorylation of $cPLA_2$ without affecting its protein level. Therefore, inhibition of $cPLA_2$ phosphorylation appears to be an important mechanism for decreasing arachidonic acid release by curcumin. This mechanism may also account for the observation by Rao *et al.* that oral administration of curcumin significantly decreased the PLA_2 activity by ~50% in the colonic mucosa and tumors of azoxymethane-treated rats (21).

Several previous studies indicated that LPS stimulates MAPKs, including extracellular signal-regulated kinases 1/2, p38 and JNK in cancer cells and inflammatory cells (30–32). The MAPKs also induce phosphorylation of $cPLA_2$ either directly or indirectly (33–36). Curcumin is reported to inhibit the MAPKs (19,37,38). In our RAW cell system, PD98059, an

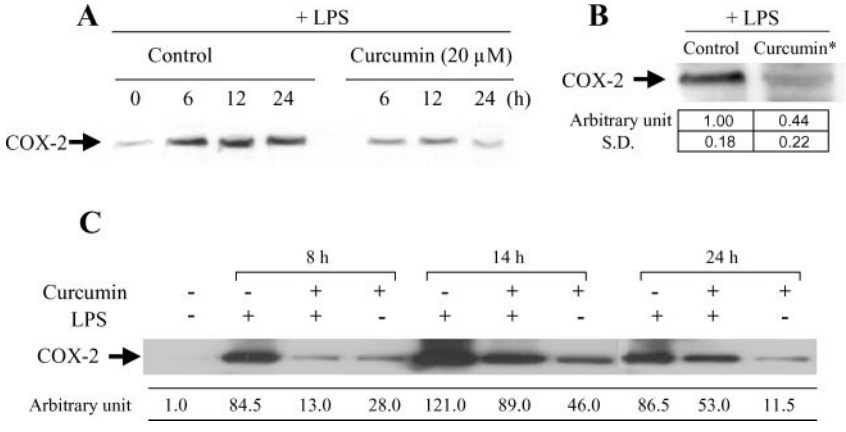

Fig. 6. Effects of curcumin on COX-2 expression in RAW264.7 cells. RAW264.7 cells were incubated with 20 μM of curcumin or vehicle (DMSO) for different time periods (**A**) or for 18 h (**B**), followed by treatment with 2 μg/ml LPS for 1 h. Western blot analysis was performed on cell lysates with antibodies against COX-2. (**C**) RAW264.7 cells were incubated with curcumin (30 μM), LPS or curcumin and LPS for the time periods indicated. Densitometry quantification of COX-2 level was normalized to each LPS control (mean ± SD) (B), or to unstimulated control (C). The results are representative of three (B) or two (A and C) independent experiments. *Significantly different from control ($P < 0.05$).

Downloaded from http://carcin.oxfordjournals.org/ by guest on April 3, 2013

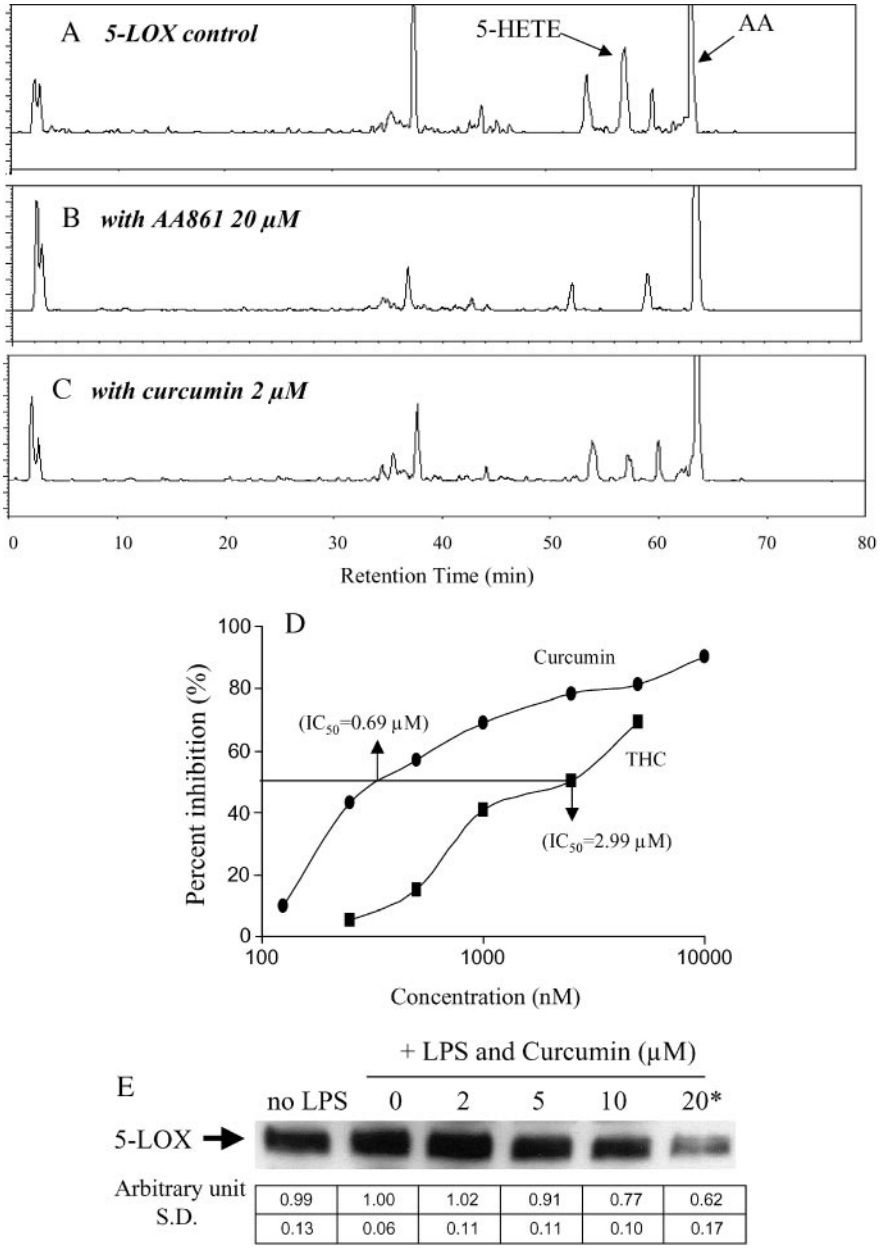

Fig. 7. Effects of curcumin and THC on 5-LOX activity. Chromatograms of [^{3}H]arachidonic acid metabolites by human recombinant 5-LOX preparation were shown from a reaction with 5-LOX (**A**), in the presence of 20 μM AA-861, a 5-LOX inhibitor (**B**) or in the presence of 2 μM curcumin (**C**). (**D**) Concentration-dependent inhibitory effect of curcumin (circle) and THC (square) on the formation of 5-HETE by human recombinant 5-LOX. The incubation mixture (100 μl) contained 0.5 U of 5-LOX, 20 μM (0.1 μCi) arachidonic acid, 2 mM $CaCl_2$ and 1 mM ATP with different concentrations of curcumin or THC in 100 mM Tris–HCl buffer, pH 7.4. The reactions were carried out at 37°C for 10 min, and the products were analyzed by HPLC. Each symbol represents the mean of duplicates. (**E**) Effects of curcumin on 5-LOX protein level in LPS-stimulated RAW264.7 cells. Cells were incubated with different concentrations of curcumin or vehicle (DMSO) for 18 h followed by treatment with 2 μg/ml LPS for 1 h. Western blot analysis was performed on cell lysates with an antibody against 5-LOX. Lower panel in (E) shows the quantification of 5-LOX level normalized to each control (mean ± SD, $n = 3$). *Significantly different from control ($P < 0.05$).

MEK inhibitor, significantly inhibited arachidonic acid release, whereas SB203587, a p38 and JNK inhibitor, did not show the inhibition. Therefore, it is probable that curcumin also inhibits a MEK-related pathway, resulting in the inhibition of $cPLA_2$ phosphorylation. Nevertheless, this mechanism may not apply to the effect of curcuminoids on A23187-induced arachidonic acid release in HT-29 cells, since inhibition of MAPKs had no effect in this system. Stimulation with A23187 is known to induce a sustained calcium mobilization, but only weak activation of MAPKs (36). The modulation of calcium mobilization by curcuminoids may also be important in the inhibition of arachidonic acid release, and this mechanism remains to be investigated.

In LPS-stimulated RAW cell lysates, the COX-dependent activity is believed to be due mainly to COX-2. The inhibition of THC and curcumin on COX activity appears to be higher than that of DBM and TDM (Table I), suggesting that the phenolic group of THC and curcumin is important for the inhibition. Considering the effect of curcuminoids on the catalytic activity of COX, it may not be a major mechanism for inhibition of PGE_2 formation in intact cells (Figure 2C). Their inhibition might be due mostly to modulation of expression of

Downloaded from http://carcin.oxfordjournals.org/ by guest on April 3, 2013

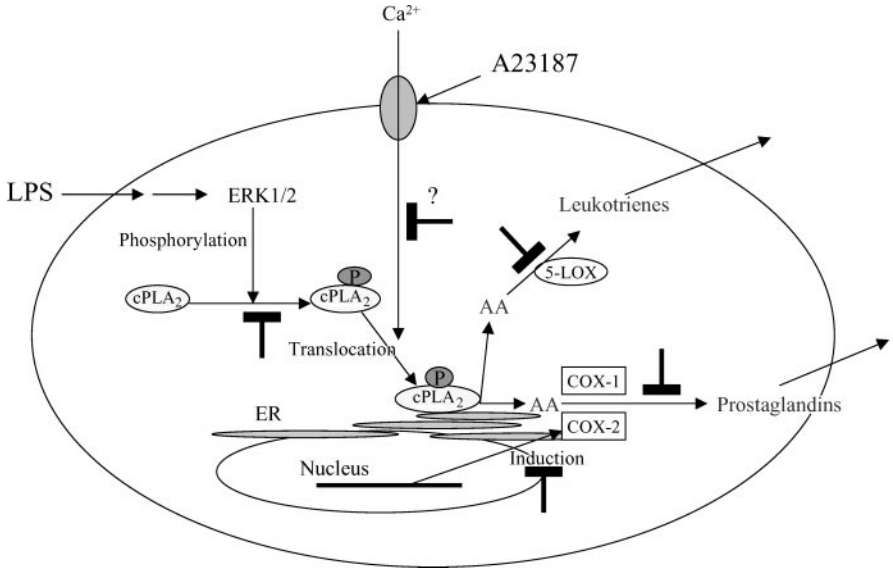

Fig. 8. Proposed targets for modulation of arachidonic acid metabolism by curcumin in cells. Curcumin modulates arachidonic acid metabolism at different stages by inhibiting phosphorylation of $cPLA_2$ (the activation process of the enzyme), inhibiting COX-2 protein expression and catalytic activity, and inhibiting 5-LOX activity (**T**, possible inhibitory targets of curcumin).

COX-2 as illustrated by the data in Figure 6. The possible effects of curcuminoids on the down-stream enzyme PGE synthase and others also remain to be further investigated.

Apparently, COX-2 is less susceptible to inhibition by curcuminoids than COX-1 (Figure 5). Huang *et al*. reported that curcumin (100 μM) almost completely inhibited the formation of COX-dependent metabolites by mouse epidermis (20). Our results are somewhat different, showing that curcumin (50 μM) inhibited the activity by <25%. It is possible that the major source of COX isoform from mouse epidermis in the previous study was COX-1. It was reported that curcumin also decreased the level of COX-dependent metabolites in colonic mucosa and tumors of rats (21). Since our results indicate that curcumin inhibits COX-1 and shows similar inhibitory effects on arachidonic acid release in both normal immortalized and cancer cells, there is no selective advantage in its action against cancer cells versus normal cells. In addition to the inhibition of COX activity by curcumin, the decrease of COX-2 protein level, as observed herein, could play an even more important role. The decrease of COX-2 expression at the protein or mRNA levels by curcumin has been observed in several gastrointestinal cells and mouse skin (17–19). The mechanism involved in the repression has been suggested to be due to its inhibitory effect of curcumin on AP-1 and NF-κB. Curcumin, however, increased the COX-2 protein level in RAW cells without LPS-stimulation. It is possible that curcumin may act as a stress factor to activate related signaling, including AP-1 and NF-κB in the cells under resting status.

Metabolites of 5-LOX are reported to be important regulators in the proliferation and apoptosis of cancer cell lines (39,40). They are believed to be involved in tumor development by affecting cell proliferation, inflammation, apoptosis and angiogenesis (27). We found that curcumin and THC showed potent inhibitory effects on the catalytic activity of 5-LOX. It was reported that curcumin can bind to the active site of the soybean LOX, inhibit the enzyme activity competitively and become oxygenated (41,42). Since curcumin has a potent inhibitory effect on 5-LOX, this activity may contribute significantly to its anticarcinogenic activities. Considering the effects of DBM and TDM on LTB_4 formation in RAW cells, the phenolic group and the size of the molecule appear to be more important than the β-diketone structure in the inhibition of 5-LOX.

The overall schemes of arachidonic acid metabolism modulated by curcumin are illustrated in Figure 8. Curcumin can modulate arachidonic acid metabolism at several targets, including inhibition of phosphorylation of $cPLA_2$, inhibition of COX-2 protein expression and catalytic activity (although weakly), and inhibition of 5-LOX activity. Dual inhibition of 5-LOX/COX has been suggested to be a desirable approach in the development of new drugs for anti-inflammation and chemoprevention (43,44). Curcumin and THC are demonstrated herein to fit in this category of agents. They can be utilized as chemopreventive agents or for therapeutic purposes if a high enough concentration of the agents can be delivered to the target sites.

References

1. Ammon,H.P. and Wahl,M.A. (1991) Pharmacology of *Curcuma longa*. *Planta Med*., **57**, 1–7.
2. Huang,M.T., Lou,Y.R., Ma,W., Newmark,H.L., Reuhl,K.R. and Conney,A.H. (1994) Inhibitory effects of dietary curcumin on forestomach, duodenal and colon carcinogenesis in mice. *Cancer Res*., **54**, 5841–5847.
3. Huang,M.T., Smart,R.C., Wong,C.Q. and Conney,A.H. (1988) Inhibitory effect of curcumin, chlorogenic acid, caffeic acid and ferulic acid on tumor promotion in mouse skin by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Res*., **48**, 5941–5946.
4. Chuang,S.E., Kuo,M.L., Hsu,C.H., Chen,C.R., Lin,J.K., Lai,G.M., Hsieh,C.Y. and Cheng,A.L. (2000) Curcumin-containing diet inhibits diethylnitrosamine-induced murine hepatocarcinogenesis. *Carcinogenesis*, **21**, 331–335.
5. Ushida,J., Sugie,S., Kawabata,K., Pham,Q.V., Tanaka,T., Fujii,K., Takeuchi,H., Ito,Y. and Mori,H. (2000) Chemopreventive effect of curcumin on N-nitrosomethylbenzylamine-induced esophageal carcinogenesis in rats. *Jpn. J. Cancer Res*., **91**, 893–898.
6. Inano,H., Onoda,M., Inafuku,N., Kubota,M., Kamada,Y., Osawa,T., Kobayashi,H. and Wakabayashi,K. (1999) Chemoprevention by curcumin

Downloaded from http://carcin.oxfordjournals.org/ by guest on April 3, 2013

during the promotion stage of tumorigenesis of mammary gland in rats irradiated with gamma-rays. *Carcinogenesis*, **20**, 1011–1018.
7. Li,N., Chen,X., Liao,J., Yang,G., Wang,S., Josephson,Y., Han,C., Chen,J., Huang,M.T. and Yang,C.S. (2002) Inhibition of 7,12-dimethylbenz[*a*] anthracene (DMBA)-induced oral carcinogenesis in hamsters by tea and curcumin. *Carcinogenesis*, **23**, 1307–1313.
8. Huang,T.S., Lee,S.C. and Lin,J.K. (1991) Suppression of c-Jun/AP-1 activation by an inhibitor of tumor promotion in mouse fibroblast cells. *Proc. Natl Acad. Sci. USA*, **88**, 5292–5296.
9. Plummer,S.M., Holloway,K.A., Manson,M.M., Munks,R.J., Kaptein,A., Farrow,S. and Howells,L. (1999) Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-κB activation via the NIK/IKK signalling complex. *Oncogene*, **18**, 6013–6020.
10. Arbiser,J.L., Klauber,N., Rohan,R., van Leeuwen,R., Huang,M.T., Fisher,C., Flynn,E. and Byers,H.R. (1998) Curcumin is an *in vivo* inhibitor of angiogenesis. *Mol. Med.*, **4**, 376–383.
11. Mohan,R., Sivak,J., Ashton,P., Russo,L.A., Pham,B.Q., Kasahara,N., Raizman,M.B. and Fini,M.E. (2000) Curcuminoids inhibit the angiogenic response stimulated by fibroblast growth factor-2, including expression of matrix metalloproteinase gelatinase B. *J. Biol. Chem.*, **275**, 10405–10412.
12. NCI (1996) Clinical development plan: curcumin. *J. Cell Biochem. Suppl.*, **26**, 72–85.
13. Lin,C.C., Lu,Y.P., Lou,Y.R., Ho,C.T., Newmark,H.H., MacDonald,C., Singletary,K.W. and Huang,M.T. (2001) Inhibition by dietary dibenzoylmethane of mammary gland proliferation, formation of DMBA-DNA adducts in mammary glands and mammary tumorigenesis in Sencar mice. *Cancer Lett.*, **168**, 125–132.
14. Cuendet,M. and Pezzuto,J.M. (2000) The role of cyclooxygenase and lipoxygenase in cancer chemoprevention. *Drug Metabol. Drug Interact.*, **17**, 109–157.
15. Huang,M.T., Newmark,H.L. and Frenkel,K. (1997) Inhibitory effects of curcumin on tumorigenesis in mice. *J. Cell Biochem. Suppl.*, **27**, 26–34.
16. Conney,A.H., Lysz,T., Ferraro,T., Abidi,T.F., Manchand,P.S., Laskin,J.D. and Huang,M.T. (1991) Inhibitory effect of curcumin and some related dietary compounds on tumor promotion and arachidonic acid metabolism in mouse skin. *Adv. Enzyme Regul.*, **31**, 385–396.
17. Goel,A., Boland,C.R. and Chauhan,D.P. (2001) Specific inhibition of cyclooxygenase-2 (COX-2) expression by dietary curcumin in HT-29 human colon cancer cells. *Cancer Lett.*, **172**, 111–118.
18. Zhang,F., Altorki,N.K., Mestre,J.R., Subbaramaiah,K. and Dannenberg,A.J. (1999) Curcumin inhibits cyclooxygenase-2 transcription in bile acid- and phorbol ester-treated human gastrointestinal epithelial cells. *Carcinogenesis*, **20**, 445–451.
19. Chun,K.S., Keum,Y.S., Han,S.S., Song,Y.S., Kim,S.H. and Surh,Y.J. (2003) Curcumin inhibits phorbol ester-induced expression of cyclooxygenase-2 in mouse skin through suppression of extracellular signal-regulated kinase activity and NF-κB activation. *Carcinogenesis*, **24**, 1515–1524.
20. Huang,M.T., Lysz,T., Ferraro,T., Abidi,T.F., Laskin,J.D. and Conney,A.H. (1991) Inhibitory effects of curcumin on *in vitro* lipoxygenase and cyclooxygenase activities in mouse epidermis. *Cancer Res.*, **51**, 813–819.
21. Rao,C.V., Rivenson,A., Simi,B. and Reddy,B.S. (1995) Chemoprevention of colon carcinogenesis by dietary curcumin, a naturally occurring plant phenolic compound. *Cancer Res.*, **55**, 259–266.
22. Dole,V.P. and Meinertz,H. (1960) Microdetermination of long-chain fatty acid in plasma and tissues. *J. Biol. Chem.*, **235**, 2595–2599.
23. Hong,J., Smith.,T.J., Ho,C.T., August,D.A. and Yang,C.S. (2001) Effects of purified green and black tea polyphenols on cyclooxygenase- and lipoxygenase-dependent metabolism of arachidonic acid in human colon mucosa and colon tumor tissues. *Biochem. Pharmacol.*, **62**, 1175–1183.
24. Gierse,J.K. and Koboldt,C.M. (1998) Cyclooxygenase assays. In Enna,S.J., Williams,M., Ferkany,J.W., Kenakin,T., Porsolt,R.D. and Sullivan,J.P. (eds) *Current Protocols in Pharmacology*. John Wiley & Sons, Inc., New York, vol. 1, pp. 3.1.1–16.
25. Leslie,C.C. (1997) Properties and regulation of cytosolic phospholipase A_2. *J. Biol. Chem.*, **272**, 16709–16712.
26. Romano,M. and Claria,J. (2003) Cyclooxygenase-2 and 5-lipoxygenase converging functions on cell proliferation and tumor angiogenesis: implications for cancer therapy. *FASEB J.*, **17**, 1986–1995.
27. Steele,V.E., Holmes,C.A., Hawk,E.T., Kopelovich,L., Lubet,R.A., Crowell,J.A., Sigman,C.C. and Kelloff,G.J. (1999) Lipoxygenase inhibitors as potential cancer chemopreventives. *Cancer Epidemiol. Biomarkers Prev.*, **8**, 467–483.
28. Subbaramaiah,K., Zakim,D., Weksler,B.B. and Dannenberg,A.J. (1997) Inhibition of cyclooxygenase: a novel approach to cancer prevention. *Proc. Soc. Exp. Biol. Med.*, **216**, 201–210.
29. Hirabayashi,T. and Shimizu,T. (2000) Localization and regulation of cytosolic phospholipase A_2. *Biochim. Biophys. Acta*, **1488**, 124–138.
30. Geppert,T.D., Whitehurst,C.E., Thompson,P. and Beutler,B. (1994) Lipopolysaccharide signals activation of tumor necrosis factor biosynthesis through the ras/raf-1/MEK/MAPK pathway. *Mol. Med.*, **1**, 93–103.
31. Hambleton,J., Weinstein,S.L., Lem,L. and DeFranco,A.L. (1996) Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. *Proc. Natl Acad. Sci. USA*, **93**, 2774–2778.
32. Schafer,P.H., Wang,L., Wadsworth,S.A., Davis,J.E. and Siekierka,J.J. (1999) T cell activation signals up-regulate p38 mitogen-activated protein kinase activity and induce TNF-alpha production in a manner distinct from LPS activation of monocytes. *J. Immunol.*, **162**, 659–668.
33. Lin,L.L., Wartmann,M., Lin,A.Y., Knopf,J.L., Seth,A. and Davis,R.J. (1993) $cPLA_2$ is phosphorylated and activated by MAP kinase. *Cell*, **72**, 269–278.
34. Kramer,R.M., Roberts,E.F., Um,S.L., Borsch-Haubold,A.G., Watson,S.P., Fisher,M.J. and Jakubowski,J.A. (1996) p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A_2 ($cPLA_2$) in thrombin-stimulated platelets. Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2. *J. Biol. Chem.*, **271**, 27723–27729.
35. Fouda,S.I., Molski,T.F., Ashour,M.S. and Sha'afi,R.I. (1995) Effect of lipopolysaccharide on mitogen-activated protein kinases and cytosolic phospholipase A_2. *Biochem. J.*, **308** (Pt 3), 815–822.
36. Gijon,M.A. and Leslie,C.C. (1999) Regulation of arachidonic acid release and cytosolic phospholipase A_2 activation. *J. Leukoc. Biol.*, **65**, 330–336.
37. Chen,Y.R. and Tan,T.H. (1998) Inhibition of the c-Jun N-terminal kinase (JNK) signaling pathway by curcumin. *Oncogene*, **17**, 173–178.
38. Jobin,C., Bradham,C.A., Russo,M.P., Juma,B., Narula,A.S., Brenner,D.A. and Sartor,R.B. (1999) Curcumin blocks cytokine-mediated NF-κB activation and proinflammatory gene expression by inhibiting inhibitory factor I-κB kinase activity. *J. Immunol.*, **163**, 3474–3483.
39. Ghosh,J. and Myers,C.E. (1998) Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. *Proc. Natl Acad. Sci. USA*, **95**, 13182–13187.
40. Ghosh,J. and Myers,C.E. (1997) Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. *Biochem. Biophys. Res. Commun.*, **235**, 418–423.
41. Skrzypczak-Jankun,E., McCabe,N.P., Selman,S.H. and Jankun,J. (2000) Curcumin inhibits lipoxygenase by binding to its central cavity: theoretical and X-ray evidence. *Int. J. Mol. Med.*, **6**, 521–526.
42. Began,G., Sudharshan,E. and Appu Rao,A.G. (1998) Inhibition of lipoxygenase-1 by phosphatidylcholine micelles-bound curcumin. *Lipids*, **33**, 1223–1228.
43. Fiorucci,S., Meli,R., Bucci,M. and Cirino,G. (2001) Dual inhibitors of cyclooxygenase and 5-lipoxygenase. A new avenue in anti-inflammatory therapy? *Biochem. Pharmacol.*, **62**, 1433–1438.
44. Leval,X., Julemont,F., Delarge,J., Pirotte,B. and Dogne,J.M. (2002) New trends in dual 5-LOX/COX inhibition. *Curr. Med. Chem.*, **9**, 941–962.

Received January 14, 2004; revised March 23, 2004;
accepted April 3, 2004

Downloaded from http://carcin.oxfordjournals.org/ by guest on April 3, 2013