Inhibition of Lipoxygenase 1 by Phosphatidylcholine Micelles-Bound Curcumin

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ABSTRACT: Curcumin (diferuloyl methane) from rhizomes of *Curcuma longa* L. binds to phosphatidylcholine (PC) micelles. The binding of curcumin with PC micelles was followed by fluorescence measurements. Curcumin emits at 490 nm with an excitation wavelength of 451 nm after binding to PC-mixed micelles stabilized with deoxycholate. Curcumin in aqueous solution does not inhibit dioxygenation of fatty acids by Lipoxygenase 1 (LOX1). But, when bound to PC micelles, it inhibits the oxidation of fatty acids. The present study has shown that 8.6 μM of curcumin bound to the PC micelles is required for 50% inhibition of linoleic acid peroxidation. Lineweaver-Burk plot analysis has indicated that curcumin is a competitive inhibitor of LOX1 with K_i of 1.7 μM for linoleic and 4.3 μM for arachidonic acids, respectively. Based on spectroscopic measurements, we conclude that the inhibition of LOX1 activity by curcumin can be due to binding to active center iron and curcumin after binding to the PC micelles acts as an inhibitor of LOX1.
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Some natural phenolic antioxidants have potential preventive activity in the initial stages of antioxidant-related diseases (1). Recently, curcumin, the major constituent of the spice turmeric, has received much attention because of both its antioxidant and antiinflammatory activities (2–4). It has been shown to be highly effective in inhibiting the stimulation of HIV type 1 virus (5). It inhibits neutrophil activation, mixed lymphocyte reaction, and proliferation of smooth muscle cells, and suppresses nitrogen-induced proliferation of blood mononuclear cells (6). Further, it is a potent scavenger of reactive oxygen species (7), protects hemoglobin from nitrate-induced oxidation, and inhibits lipid peroxidation (8,9). Huang *et al.* (10) reported that the antitumor-promotion activity of curcumin in mouse epidermis is linked with the suppression of arachidonic acid metabolism. This metabolic pathway is one of the lipid peroxidation events in living organisms catalyzed by lipoxygenases.

Arachidonic acid lipoxygenases are involved in the biosynthesis of various bioregulators that are closely related to the pathogenesis of some diseases, such as allergy, atherosclerosis, and cancer (11). Lipoxygenase catalyzes the initial step in the production of leukotrienes and lipoxins, which mediate allergic and inflammatory responses (12). Since the discovery of the role of lipoxgenases in the biosynthesis of effectors critical in animal and plant physiology, extensive research has been carried out to study their effective inhibitory activities. Most studies have used soybean lipoxygenase (LOX1), whose three-dimensional structure and catalytic mechanism have been characterized in detail (13,14). Earlier reports showed that curcumin is a potent inhibitor for nonenzymatic lipid peroxidation (15,16).

The results presented in this paper suggest that curcumin inhibits LOX1-catalyzed oxidation of linoleic and arachidonic acids and demonstrate that this natural antioxidant inhibits the enzyme by binding to the active center iron. The inhibitory activity of curcumin is potentiated in the presence of hydrophobic environment, which can be provided by phosphatidylcholine (PC) micelles.

MATERIALS AND METHODS

Materials. Soybean LOX1 was isolated according to the method of Axelrod *et al.* (17) with some modifications as described earlier (18). The specific activity was 200–240 μmoles/min/mg of protein. Linoleic and arachidonic acids were from Nu-Chek-Prep (Elysian, MN). Egg PC, curcumin, and sodium deoxycholate (DOC) were purchased from Sigma (St. Louis, MO). Stock solutions of fatty acids were prepared with a concentration of 10 mM in 10% ethyl alcohol containing 50 mM borate buffer pH 9.0

Preparation of the mixed micelles. Mixed micelles were prepared using the mixture of PC and DOC. After solubilizing the PC and DOC in chloroform/methanol (2:1) mixture, the solvent was evaporated by flash evaporator and dried in the presence of nitrogen gas. The resulting thin film was solubilized in 50 mM Tris HCl, pH 7.4, and then sonicated for 5 min using a bath-type sonicator.

Fluorescence microscopy. PC micelles (10 mM), after mixing with curcumin (60 mM) solubilized in ethyl alcohol (the final concentration of alcohol did not exceed 2 mM), were immediately examined under Leitz Diaplan (Wetzlar, Germany) Fluorescence microscope fitted with exciter filter BP 450-490 nm and suppression filter block of 520 nm.

Assay of lipoxygenase and inhibition by curcumin. The concentration of LOX1 was calculated from A_{280} by using the value E_{280} = 14.0 (18). Activities were determined by spec-

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Abbreviations: DOC, deoxycholic acid; LOX1, lipoxygenase 1; PC, phosphatidylcholine.

trophotometry. The formation of hydroperoxide was followed at 234 nm (ε = 25,000 $M^{-1}cm$) in a solution of 50 mM Tris HCl (pH 7.4) containing 100 μM PC micelles and 100 μM of fatty acid solubilized in ethyl alcohol (the final concentration of alcohol did not exceed 1 mM). In the inhibition experiments, curcumin was added to the assay mixture as an ethanolic solution (the final concentration of alcohol was 2 mM). The residual activity was independent of the sequence of reagents addition, when related to the activity in the absence of the inhibitor. Reversibility of inhibition was assessed by assaying residual activity after dilution of the enzyme/inhibitor complexes with 50 mM Tris HCl, pH 7.4 at 4°C.

Curcumin fluorescence studies. Fluorescence measurements were performed with a Shimadzu (Kyoto, Japan) spectrofluorophotometer RF 5000. Temperature was maintained at 25°C by circulating the water through the thermostated cuvette holder. Fluorescence titrations of curcumin and PC micelles were made according to the method of Azzi (19). Samples were excited at 451 nm, and emission was recorded at 490 nm. The dissociation constant (K_{cur}) and the number of binding sites on PC micelles were determined using the Scatchard plot method. The interaction of LOX1 and metal ion, Fe^{3+} ($FeCl_3$), was made by following the fluorescence quenching. The PC micelles were saturated with curcumin as reflected in maximum relative fluorescence intensity, F_{max}, at 490 nm. To this PC–curcumin complex, LOX1 or $FeCl_3$ was added gradually and the decrease in fluorescence intensity, F, was recorded. The dissociation constant (K_D) for the fluorescence quenching ligand (L) was determined using equation $F_{max}/F = (1 + K_{cur}/[cur])(1 + [L]/K_D)$ (20), where K_{cur} is the dissociation constant for PC–curcumin determined by Scatchard plot, [cur] is the concentration of curcumin, and [L] is the concentration of LOX1 or $FeCl_3$ added during the titration. The reciprocal of the dissociation constant was used as the equilibrium constant (K_{eq}).

The affinity of curcumin for PC micelles in the presence of fatty acid was calculated using the fluorometric titration of curcumin with PC micelles and fatty acid, and K_{cur} was calculated using the Scatchard plot method. The concentration of bound curcumin used in the inhibition studies was determined using the fluorometric titration of curcumin against 100 μM of PC micelles and 100 μM fatty acid (the concentration similar to activity measurements); the following equation was used to calculate the bound curcumin concentration: $C_B = C_T - (K_D/n)$ (RFI/RFI_{max} – RFI), (21) where C_T was the concentration of curcumin used for the inhibition, K_D was the dissociation constant of LOX1 or Fe^{3+} with PC micelles in the presence of fatty acid, RFI_{max} was maximum fluorescence intensity which was the intercept of the reciprocal plot of curcumin against PC micelles in the presence of fatty acid, and RFI was obtained from the reciprocal plot for the corresponding C_T of curcumin.

RESULTS

Figure 1 shows that curcumin in the presence of PC micelles has a characteristic fluorescence emission maximum of 490 nm at an excitation wavelength of 451 nm. This result indicates that the binding of curcumin with PC micelles could be due to the interaction between the hydrophobic group of curcumin and the hydrophobic regions of PC micelles. This observation was confirmed by fluorescence microscopy measurements which clearly show the binding of curcumin to the PC micelles and the influence of curcumin fluorescence in visualizing the size and shape of PC-micelles (Fig. 2). The luminous disks had diameters of 0.1 to 0.3 μm. Curcumin in aqueous solution and in a solution containing only DOC has a weak fluorescence, but its fluorescence intensity is very high in organic solvents, and the quantum yield increases with increase in the hydrophobicity of the solvent (22). It has been

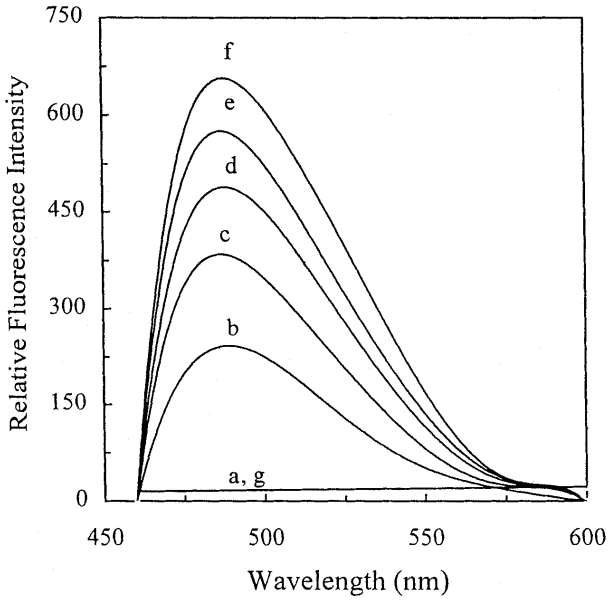

FIG. 1. Fluorescence emission spectra of curcumin with phosphatidylcholine (PC) micelles. To 50 μM PC micelles, various concentrations of curcumin were added in 50 mM Tris HCl buffer, pH 7.4 (a, 0; b, 1; c, 2; d, 3; e, 4; and f, 5 μM of curcumin; g, 5 μM curcumin in 100 μM dioxycholic acid).

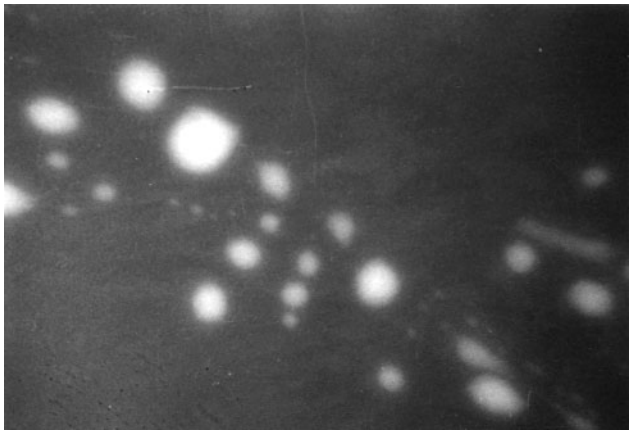

FIG. 2. Fluorescence photomicrograph of PC micelles–curcumin complex in 50 mm Tris HCl buffer, pH 7.4 (PC to curcumin molar ratio was 6.0). Magnification factor 40×. For abbreviation see Figure 1.

reported that curcumin in aqueous solvent is not stable in the alkaline pH and undergoes degradation at high alkaline pH. Therefore, all measurements were made at pH 7.4 in the present study.

LOX1 utilizes linoleic acid bound to the PC micelles as substrate, with a pH optimum of 7.4 (23). The LOX1-catalyzed rate of oxidation of linoleic and arachidonic acids inserted into PC micelles at pH 7.4 is given in Figure 3A. Only the fatty acids bound to the PC micelles are the substrates for LOX1, and the LOX 1 activity is maximum with equal concentrations of fatty acid and PC micelles. This method is very useful for LOX activity determination in the presence of curcumin, because measurements can be made at neutral pH. Although activity of LOX1 with linoleic and arachidonic acids solubilized in equal amounts of Tween 20 had a pH optimum around 9.0, the enzyme had around 50% of its activity at pH 7.4. The effect of different concentrations of curcumin in aqueous system on the LOX1 activity for the Tween 20-solubilized fatty acid substrate suggested that curcumin did not inhibit LOX1 activity (Fig. 3B). However, the inhibitory effect of curcumin on LOX1 activity was observed only with PC-bound fatty acid as substrate. These results clearly show that curcumin inhibits LOX1 in the presence of PC micelles, presumably by binding to the PC micelles, but not in aqueous solution.

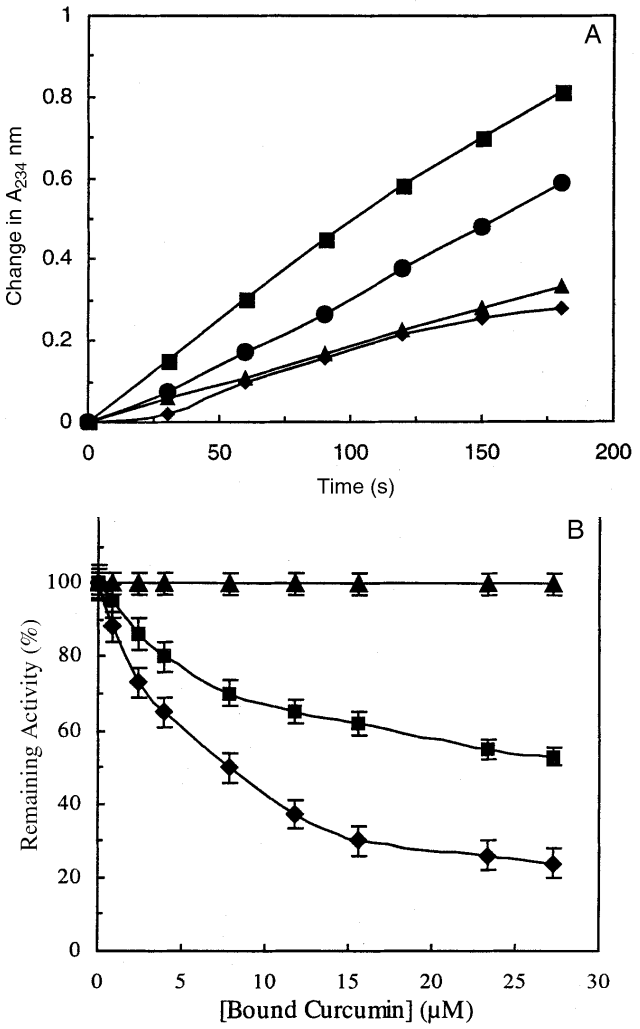

FIG. 3. (A) Soybean lipoxygenase 1 (LOX1)-catalyzed oxidation of fatty acids. Rates were measured in the presence of (i) 100 μM of fatty acid with 100 μM of micellar PC (arachidonic acid, ■; linoleic acid, ●) and (ii) 100 μM of fatty acid dispersed in Tween 20 (arachidonic acid, ▲; linoleic acid, ◆) at pH 7.4 (50 mM Tris HCl). (B) The concentration-dependence inhibition of curcumin on LOX1-dependent dioxygenation. The reaction mixture consisted of 100 μM fatty acid with (i) 100 μM PC micelles (linoleic acid, ◆; arachidonic acid, ■) and (ii) Tween 20 (arachidonic and linoleic acids, ▲) in 50 mM Tris HCl, pH 7.4, and various concentrations of ethanolic solution of curcumin. The reaction was initiated by adding LOX1. For other abbreviation see Figure 1. In Part B the error bars signify the percentage error in measurements.

LOX1 has optimal activity at 100 μM of fatty acid and 100 μM of micellar PC. Since only bound fatty acid is the substrate and the inhibitory effect by curcumin is also due to binding to PC micelles, the concentration of PC micelles is the limiting factor in the LOX1 activity and its inhibition by curcumin. As shown in Figure 3B, the incorporation of increasing amounts of curcumin into the PC micelles led to a successive inhibition of oxidation of fatty acid bound to the PC micelles. Curcumin inhibited dioxygenation of linoleic acid more effectively than arachidonic acid. The 50% inhibitory activity toward linoleic acid oxidation was observed with 11 μM curcumin, which corresponded to 8.6 μM bound curcumin.

When the effects of linoleic and arachidonic acids on lipoxygenase inhibition by curcumin were examined, the rate of oxidation became greater, as the concentration of substrate increased. The Lineweaver-Burk plots at a fixed curcumin concentration showed that K_m increased without changing the V_{max} of the reaction (Fig. 4), suggesting that curcumin is a competitive inhibitor of LOX1, K_i values being 1.7 μM and 4.3 μM for linoleic and arachidonic acid oxidation, respectively (Fig. 5).

Earlier reports on the antioxidant activity of curcumin suggested that curcumin can chelate the metal ion (24,25). Further, LOX1 contains iron as the cofactor. Therefore, studies were made to check whether the competitive inhibition of curcumin is due to binding to the active site iron. Fluorimetric titrations of curcumin with PC micelles with increasing concentrations of LOX1 and $FeCl_3$ suggest that curcumin inhibits LOX1 activity competitively by binding to the active site iron, as the LOX1 quenches the curcumin fluorescence in PC micelles. The equilibrium constant of curcumin bound to the PC micelles with LOX1 is 0.33×10^6 M^{-1}; this value is comparable with the affinity of curcumin with PC micelles, which is 0.37×10^6 M^{-1}. These data further confirm that curcumin can elicit its inhibitory activity only after binding to the PC micelles. The reversible inhibition of LOX1 by curcumin bound to the PC micelles was further confirmed by complete recovery of catalytic activity after dilution of enzyme/inhibitor complex with the buffer. Fe^{3+} also quenched curcumin fluorescence, which was used for determining the affinity of curcumin (PC bound) with Fe^{3+}. This had equilibrium constant of 0.23×10^6 M^{-1}. However, Fe^{2+} was more ef-

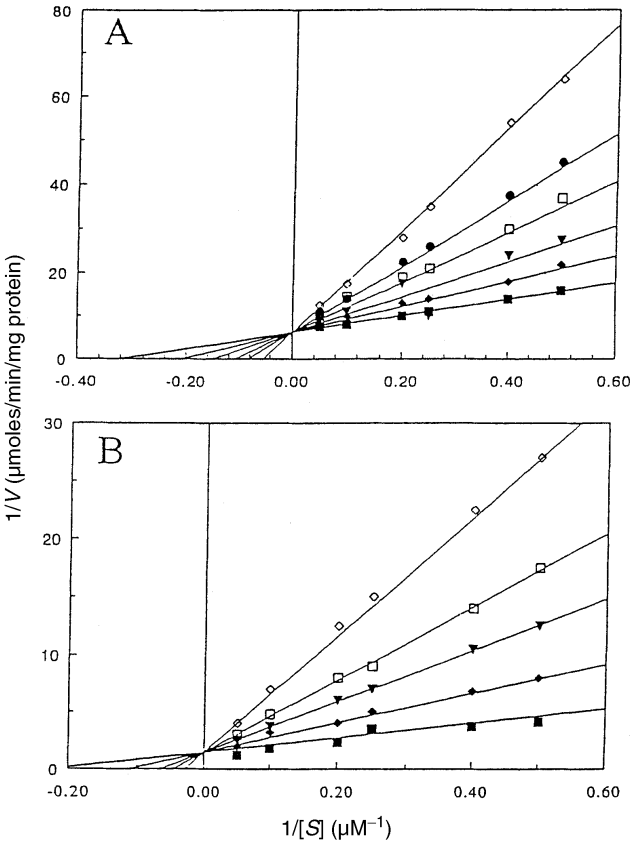

FIG. 4. Lineweaver-Burk plot analysis of the inhibitor of LOX1 by curcumin for the dioxygenation of (A) arachidonic acid and (B) linoleic acid. The concentrations of bound curcumin for arachidonic acid were 0 (■), 3.9 (◆), 7.8 (▼), 11.73 (□), 15.6 (●), and 24 μM (◇); and for linoleic acid 0 (■), 3.13 (◆), 6.25 (▼), 9.4 (□), and 15.6 μM (◇). The micellar PC concentration was 100 μM, and the fatty acid concentration was 100 μM. For abbreviations see Figures 1 and 3.

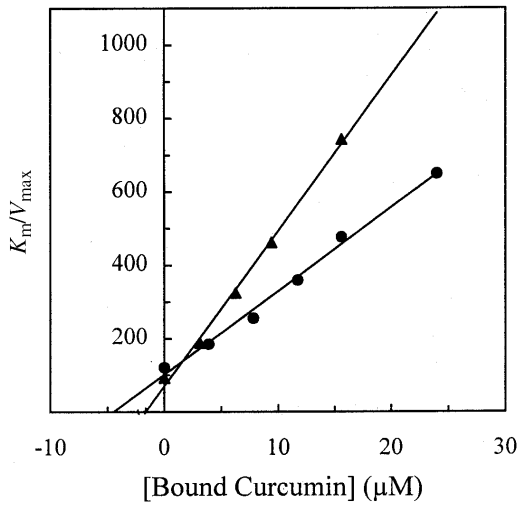

FIG. 5. The slope (K_m/V_{max}) of the lines described from the double reciprocal plot are plotted against the curcumin concentration (bound to PC micelles) in order to derive the K_i value for curcumin (linoleic acid, ◆; arachidonic acid, ●). For abbreviation see Figure 1.

Our earlier study demonstrated that LOX1 can oxidize fatty acids bound to the PC micelles at neutral pH (23). Therefore, this reaction system to monitor the LOX1 activity was used in the present study. Curcumin binds to PC micelles with a high affinity, exhibiting a characteristic fluorescence spectrum that could be attributed to the amphilic nature of curcumin. The binding of curcumin with PC micelles enhances its hydrophobicity. Further, from the equilibrium constants for curcumin–PC micelles and the LOX1–curcumin (bound to PC micelles), it can be inferred that enhancing the hydrophobicity of the curcumin makes it more effective in reaching the active site iron of LOX1. Similar observations

fective in quenching the curcumin fluorescence (data not shown). Comparison of equilibrium constant of PC micelles-bound curcumin with LOX1 (0.33×10^6 M^{-1}) and Fe^{3+} (0.23×10^6 M^{-1}) suggests that curcumin incorporated in PC micelles inhibits LOX 1 activity by binding to the active site iron (Fig. 6).

DISCUSSION

Several studies on the inhibition of LOX1 have shown that inhibitors can act through a number of mechanisms, for example, by reducing the catalytically active ferric enzyme to its inactive ferrous form through the formation of free-radical metabolites (26), or by preventing the formation of the activated Fe(III) form of LOX (27), or by binding to sites other than the active site of the enzyme molecule (28). In general, the inhibition of lipoxygenase reaction seems to be derived from inactivation of the active site of the enzyme or scavenging of free radical at the active site. Therefore, most antioxidants are inhibitors of LOX1. The results of the present study suggest that curcumin inhibits LOX1 by binding with the active site iron.

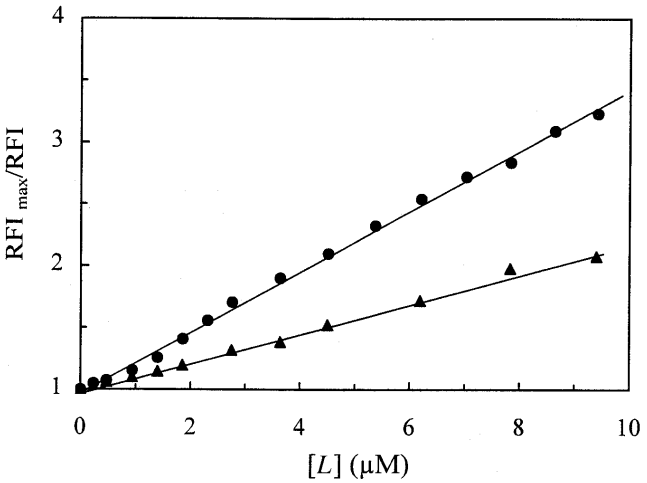

FIG. 6. Fluorescence titration of LOX1 (●) and $FeCl_3$ (▲) against curcumin bound to PC micelles. To 100 μM PC micelles, 100 μM fatty acid and 5 μM curcumin in 50 mM Tris HCl, pH 7.4, various concentrations of LOX1/$FeCl_3$ were added, and the fluorescence quenching was recorded. The plot was made as described in the Materials and Methods section. RFI_{max}, maximum fluorescence intensity, defined as the intercept of the reciprocal plot of curcumin against PC micelles in the presence of fatty acid. For other abbreviations see Figures 1 and 3.

have been made in the earlier study on the competitive inhibition of LOX1 by *n*-alcohols, where longer alkyl chains were shown to increase the affinity of these compounds for the enzyme (29). Similarly, 6-palmitoyl ascorbic acid inhibits more effectively than ascorbic acid (30). It has also been shown that some of the membrane-bound enzymes like protein kinase C and pp $60^{c\text{-}src}$, tyrosine kinase are inhibited by curcumin (31). Hence, it is conceivable that the inhibitory effect of curcumin could be potentiated after binding to the membranes.

The results of the present studies demonstrate that curcumin after binding to the PC micelles acts as competitive and reversible inhibitor of LOX1. Unlike other antioxidant inhibitors of LOX1, curcumin does not require radical formation during LOX inhibition. The dissociation constant of curcumin (bound to PC micelles) with LOX (3.03 μM) determined by fluorometric titrations is comparable with the K_i (1.72 μM) value of LOX inhibition by curcumin for linoleic acid oxidation, indicating clearly that curcumin inhibits LOX1 by binding to the active site iron, the binding is reversible, and the inhibition can be overcome by excess of fatty acid. On the other hand, the K_{eq} ($0.33 \times 10^6 M^{-1}$) for Fe^{3+} binding with PC-bound curcumin is comparable with the K_{eq} (0.23×10^6 M^{-1}) constant of LOX1 interaction with PC micelles-bound curcumin, suggesting that curcumin can bind the active Fe^{3+} form of LOX1. The present observations that PC micelle-bound curcumin binds to the Fe^{3+} can be explained using earlier reports, which suggested that the β-diketone moiety of curcumin could chelate the metal ion, and chelation affinity increased with the increase in hydrophobicity of the curcumin molecule. Hence, curcumin became more potent for inhibiting the LOX1 after its association with PC micelles.

The low K_i value of curcumin for linoleic acid oxidation (K_i = 1.72 μM) compared to arachidonic acid (K_i = 4.3 μM) implies that curcumin is more specific for inhibiting the LOX1 for linoleic acid peroxidation, although LOX 1 shows almost similar values of K_{cat} (4.53×10^7 $M^{-1}s^{-1}$) and K_m (9 μM) for arachidonic acid oxidation compared to linoleic acid oxidation ($K_{cat} = 3.6 \times 10^7$ $M^{-1}s^{-1}$ and K_m = 10 μM). However, some of the reported anti-inflammatory effects of curcumin could be due to inhibition of arachidonic acid peroxidation (10). It is also known that curcumin inhibits mammalian 5-lipoxygenase and cyclooxygenase (32).

These studies imply that curcumin, after binding to PC micelles, acts as a potent inhibitor of LOX1-dependent fatty acid peroxidation, although the micellar system in these experiments may not reflect directly the event of lipid peroxidation occurring *in vivo*. However, observations made in this study may have physiological significance, involving lipoxygenase in biomembrane alteration, both in animals and plants.

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