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Effects of Curcumin on Cytochrome P450 and Glutathione S-Transferase Activities in Rat Liver

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ABSTRACT. The stability of curcumin, as well as the interactions between curcumin and cytochrome P450s (P450s) and glutathione S-transferases (GSTs) in rat liver, were studied. Curcumin is relatively unstable in phosphate buffer at pH 7.4. The stability of curcumin was strongly improved by lowering the pH or by adding glutathione (GSH), N-acetyl L-cysteine (NAC), ascorbic acid, rat liver microsomes, or rat liver cytosol. Curcumin was found to be a potent inhibitor of rat liver P450 1A1/1A2 measured as ethoxyresorufin deethylation (EROD) activity in β-naphthoflavone (βNF)-induced microsomes, a less potent inhibitor of P450 2B1/2B2, measured as pentoxyresorufin depentylation (PROD) activity in phenobarbital (PB)-induced microsomes and a weak inhibitor of P450 2E1, measured as p-nitrophenol (PNP) hydroxylation activity in pyrazole-induced microsomes. K_i values were 0.14 and 76.02 μM for the EROD- and PROD-activities, respectively, and 30 μM of curcumin inhibited only 9% of PNP-hydroxylation activity. In ethoxyresorufin deethylation (EROD) and pentoxyresorufin depentylation (PROD) experiments, curcumin showed a competitive type of inhibition. Curcumin was also a potent inhibitor of glutathione S-transferase (GST) activity in cytosol from liver of rats treated with phenobarbital (PB), β-naphthoflavone (βNF) and pyrazole (Pyr), when measured towards 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. In liver cytosol from rats treated with phenobarbital (PB), curcumin inhibited GST activity in a mixed-type manner with a K_i of 5.75 μM and K_i of 12.5 μM. In liver cytosol from rats treated with pyrazole (Pyr) or β-naphthoflavone (βNF), curcumin demonstrated a competitive type of inhibition with K_i values of 1.79 μM and 2.29 μM, respectively. It is concluded that these strong inhibitory properties of curcumin towards P450s and GSTs, in addition to its well-known antioxidant activity, may help explain the previously observed anticarcinogenic, antimutagenic, and cytoprotective effects of this important natural compound and food constituent. BIOCHEM PHARMACOL 51;1:39–45, 1996.

KEY WORDS. curcumin; inhibitory potency; rat liver; cytochromes P450; glutathione S-transferases

Turmeric, a yellow product in the rhizomes of *Curcuma longa* L. and others containing curcumin, is reported to possess protein kinase C inhibition activity [1] and anti-inflammatory activity [2–4]. The mechanism of the anti-inflammatory action of curcumin involves inhibition of 5-lipoxygenase, 12-lipoxygenase, and cyclooxygenase [5, 6]. Curcumin also inhibits the microsomal-mediated mutagenicity of B[a]P§, capsaicin, chili extract, and cigarette smoke condensate [7, 8]. Topical application of curcumin before B[a]P has been shown to inhibit the covalent binding of polycyclic hydrocarbon metabolites to epidermal DNA and to prevent tumor initiation by B[a]P [9, 10]. Moreover, curcumin has strong antioxidant properties, which were shown to play a role in the protection against drug-induced lipid peroxidation (e.g. of paracetamol [11].

Curcumin has structural similarities to ferulic acid, which is also known as an alkaline degradation product of curcumin [12]. Plant phenols, such as ellagic acid, ferulic acid, caffeic acid, and chlorogenic acid, were shown to possess inhibitory properties towards GSTs with CDNB as substrate [13]. As yet, no data are available concerning possible inducing or inhibitory properties of curcumin towards GSTs nor towards P450s, two of the most important enzyme systems involved in the bioactivation and bioinactivation of xenobiotic compounds [14].

In this report, the effects of curcumin on P450 and GST activities were studied. The effect of curcumin on EROD and

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§ *Abbreviations*–P450, cytochrome P450; GST, glutathione S-transferase; GSH, glutathione; NAC, N-acetyl L-cysteine; EROD, ethoxyresorufin deethylation; PROD, pentoxyresorufin depentylation; PNP, p-nitrophenol; PB, naphthoflavone-phenobarbital; 3NF, β-naphthoflavone; Pyr, pyrazole; CDNB, 1-chloro-2,4-dinitrobenzene; LPO, lipid peroxidation; BSA, serum albumin; B[a]P, benzo[a]pyrene.
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PROD and PNP hydroxylation activities of rat liver microsomal P450 and CDNB GSH-conjugation activity of rat liver cytosolic GSTs were investigated. The stability of curcumin was also investigated.

MATERIALS AND METHODS

Materials

Curcumin was synthesized by condensation of vanillin and acetylaceton according to [15]. ^{1}H-NMR and HPLC measurements demonstrated that the synthetical curcumin had a purity >99%. Resorufin was purchased from Aldrich Chemie (Beerse, Belgium), ethoxyresorufin, pentoxyresorufin, GSH, CDNB, βNF, PB and Pyr from Sigma Chemical Co. (St Louis, MO), PNP from JT Baker (Deventer, The Netherlands), paranitrocatechol from Janssen Chimica (Beerse, Belgium), NADPH and BSA from Boehringer Mannheim BV (Almere, The Netherlands), and ascorbic acid from E. Merck (Darmstadt, Germany).

Animals, Pretreatment and Preparation of Subcellular Fractions

Male Wistar rats (200–220 g, Harlan Olac CPB, Zeist, The Netherlands) were housed in an environmentally (t = 25°C and air humidity-60%) controlled room, kept on a standard laboratory diet (RMH TM-10, Hope Farms, Woerden, The Netherlands) and starved overnight before use. Animals were pretreated with either βNF, once by i.p. injection of 80 mg/kg/day in arachidis oil, 0.1% (w/v) PB in the drinking water *ad libitum* for 7 days, or Pyr, 200 mg/kg/day in physiological saline by i.p. injection, once a day for 2 days. Livers were isolated 24 hr after the last treatment. Rat liver microsomal and cytosolic fractions were prepared by ultracentrifugation according to Lake [16] and Lundgren *et al.* [17], respectively, and stored at −80°C until use. Protein concentrations were determined by the method of Bradford [18] with BSA as a standard.

Stability Experiments with Curcumin

Solutions of curcumin in phosphate buffer, pH 7.4 and pH 6.5 (final concentration 25 μM), were scanned every 5 min between 200–600 nm using a Hitachi 150-20 spectrophotometer. To similar solutions of curcumin, GSH (final concentration 1 mM), NAC (50 μM), ascorbic acid C (25 μM), rat liver microsomes (0.03 mg/mL protein), or cytosol (0.01 mg/mL protein) were added and solutions subsequently scanned between 200–600 nm every 5 min.

7-Ethoxy- and 7-Pentoxyresorufin O-Dealkylation

Cytochrome P450-mediated O-dealkylation of EROD in βNF-microsomes of rat liver and of PROD in PB-microsomes were measured according to Burke and Mayer [19]. The reaction mixtures, prepared in a fluorimeter cuvette, contained 1.94 mL of phosphate buffer (0.1 M, pH 7.8), 25 μL of a suspension of βNF-microsomes (0.27 mg/mL of protein) or PB-microsomes (2.2 mg/mL of protein), 25 μL of curcumin stock solution in methanol (80, 240, 800, and 2400 μM for IC_{50} determination and 0, 2.4, 8, 24, and 80 μM for the determination of K_i and type of inhibition) and 10 μL of ethoxy- or pentoxyresorufin (10 mM in DMSO). Fluorescence was recorded at an excitation wavelength of 530 nm and an emission wavelength of 586 nm with a Perkin Elmer Model 3000 fluorescence spectrometer. A 100-μL aliquot of NADPH (5 mM in phosphate buffer pH 7.8) was added to the incubation mixtures to start the dealkylation reactions. Resorufin formation was followed spectrofluorometrically for 2 minutes. To ensure the stability of curcumin in these mixtures, 25 μL of a GSH solution (1 mM, final concentration) was added before adding curcumin. All EROD and PROD measurements were done in duplicate.

p-Nitrophenol Hydroxylation

Hydroxylation of PNP in pyrazole microsomes of rat liver was determined as described by Koop [20]. Reaction mixtures consisting of 1.68 mL of phosphate buffer (0.1 M, pH 6.8), 40 μL of PNP (100 mM in aquadest), 150 μL of a microsomal suspension (1.5 mg/mL), and 25 μL of a curcumin stock solution (80, 240, 800, and 2400 μM in methanol) were preincubated at 37°C for 2 min and the reaction started by adding 100 μL NADPH (10 mM in phosphate buffer pH 6.8). After 10 min, the reactions were terminated by the addition of 0.5 mL trichloroacetic acid (0.5 M in aquadest). Proteins were precipitated by centrifugation (2500 g, 15 min), and 1.0 mL of the resulting supernatant was mixed with 0.1 mL 10 N NaOH for the measurement of 4-nitrocatechol at 511 nm with a Novaspec II Pharmacia LKB spectrophotometer. All PNP hydroxylation measurements were done in duplicate.

GSH Conjugation of CDNB

GST activities towards CDNB were measured spectrophotometrically according to the method of Habig *et al.* [21]. The reaction mixtures contained either 25 μL of liver cytosol (0.2–0.3 mg/mL of protein) of rats pretreated with βNF, with PB, or with Pyr, 50 μL of GSH (10 mM in aquadest), and 10 μL of varying concentrations of CDNB (10, 12.5, 16.5, 25, and 50 mM in ethanol) in a solution of phosphate buffer (50 mM, pH 6.5, final volume 500 μL). In inhibition studies, 5-μL samples of solutions of curcumin in methanol were preincubated for 4 min and protected from light before GSH and CDNB were added. Methanol in the incubation mixtures (2% v/v) had no effect on the enzyme activities. The absorption differences (Δ abs/min) were recorded on a Philips PU 8720 UV-VIS scanning spectrophotometer at 340 nm.

Type of Inhibition

For the ethoxyresorufin O-deethylation (βNF induction) and pentoxyresorufin O-dependentylation (PB induction) reactions in the final incubation mixtures, the concentrations of ethoxy-

and pentoxyresorufin were 10, 5, 2.5, 1.25, and 0.63 μM, and those of curcumin 0, 0.03, 0.1, 0.3, and 1.0 μM (βNF-induction) and 0 and 3 μM (PB induction). In GSH conjugation reactions, the final concentrations of CDNB were 1.0, 0.5, 0.33, 0.25, and 0.20 mM, and those of curcumin 0, 7.5 and 15 μM (PB induction), 0, 3.5, and 15 μM (Pyr induction) and 0, 10, and 20 μM (βNF induction).

Analysis of Data

Apparent K_i constants were calculated using the following equations: (i) for competitive inhibition

$$K_i = K_m[I]/(K_m^* - K_m);$$

(ii) for noncompetitive inhibition,

$$K_i = V_{max}^*[I]/(V_{max} - V_{max}^*);$$

and (iii) for mixed-competitive inhibition,

$$K_i = V_{max}^* K_m[I]/(V_{max}K_m^* - V_{max}^*K_m)$$

and

$$K_i' = V_{max}^*[I]/(V_{max} - V_{max}^*),$$

where

$$K_m, V_{max}, K_m^*, V_{max}^*$$

were obtained in the absence and in the presence of inhibitor (I). [I] is the concentration of the inhibitor (Cai *et al.* [22]. The concentration of inhibitor resulting in 50% inhibition (IC_{50}) was determined from the plots of remaining activity *vs* the (varied) inhibitor concentration at a fixed concentration of the substrate.

RESULTS AND DISCUSSION

The primary aim of this study was to investigate the inhibitory properties of curcumin towards microsomal cytochrome P450s and cytosolic GSTs. EROD and PROD dealkylation and PNP hydroxylation reactions were chosen for study of the inhibition of differential P450 activities and GSH conjugation of CDNB for the inhibition of GST activities.

Solubility and Stability of Curcumin

The experimental conditions were carefully considered because of the low solubility and suspected instability of curcumin at higher pH values (Tonnesen and Karlsen [12]). Most experiments in aqueous solutions were, therefore, performed at concentrations <50 μM. The stability of curcumin was measured spectrophotometrically (200–600 nm) in phosphate buffers. At pH 7.4, curcumin (25 μM) degraded rapidly (Fig. 1). The absorbance at 426 nm decreased to approximately 50% after 5 min, and after 10 min the remaining absorbance was only about 10%. Two new absorptions appeared at 210 nm and 262 nm. The final solution was colorless, indicating that a yellow conjugated system no longer existed in the degradation products of curcumin. Phosphate buffer pH 6.5 resulted in significantly increased stability of curcumin. No changes in the spectral pattern occurred up to 30 min after preparing the curcumin solution (Fig. 1). In phosphate buffer pH 7.4, the instability of curcumin was completely prevented by adding GSH (1 mM), NAC (50 μM), ascorbic acid (25 μM), and also

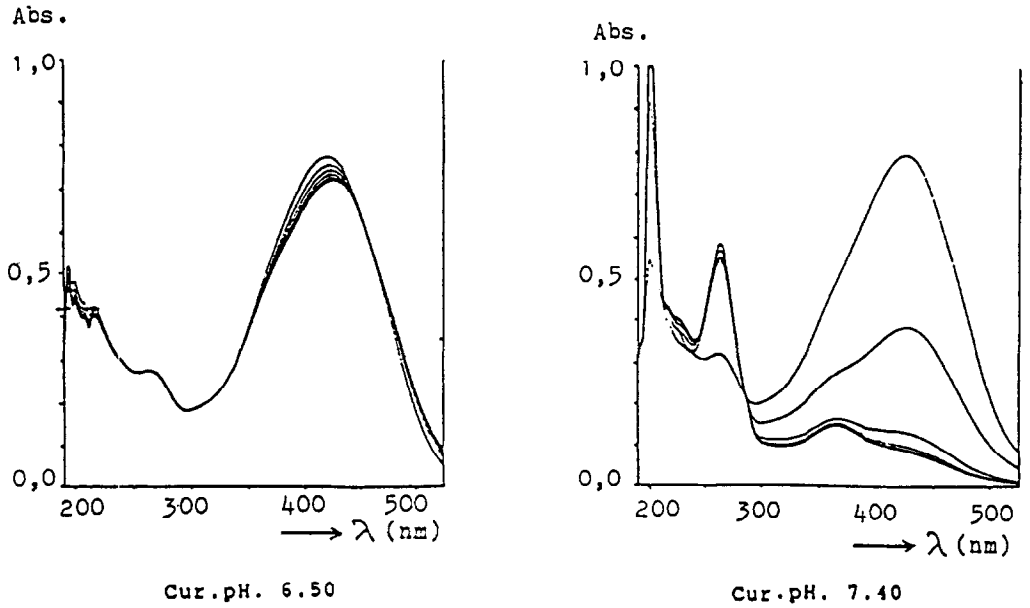

FIG. 1. Overlay of UV-VIS spectra of a 25 μM solution of curcumin in 50 mM phosphate buffer pH 7.4 (right) and pH 6.5 (left) every 5 min (200–600 nm; upper line t = o).

by adding protein from microsomal (0.03 mg protein/mL) and cytosolic fractions (0.01 mg/mL) of rat liver (data not shown). Apparently, the stability of curcumin in aqueous solutions is strongly increased by the presence of thiol- and non thiol-containing antioxidants.

Effects on P450 Activities

At concentrations of 1, 3, 10, and 30 μM, curcumin inhibited the EROD activity in βNF-induced liver microsomes by 36 up to 93%, the PROD activity in PB-induced microsomes by 13 up to 88%, and the PNP hydroxylation activity in Pyr-induced microsomes only by 0.5 up to 9%, respectively. IC_{50} values were calculated to be 2 μM for the EROD and 14 μM for the PROD activities (Table 1). The presence or absence of GSH (1 mM) in the incubation mixtures did not influence the inhibitory effects of curcumin on the PROD and EROD activities (data not shown). The results clearly demonstrate that curcumin is a potent inhibitor of P450 1A1/1A2 measured as EROD activity, a much weaker inhibitor of P450 2B1/2B2 measured as PROD activity, and causes no significant inhibition of P450 2E1 measured as PNP hydroxylation activity. Ferulic acid (one of the degradation products of curcumin) showed no significant inhibition on EROD activities in βNF-induced rat liver microsomes at concentrations of 1, 3, 10, and 30 μM (data not shown).

In liver microsomes from rats pretreated with βNF and PB, the V_{max} values of the EROD and PROD activities were 33.9 and 1.3 nmol/min/mg protein, respectively. In the presence of curcumin, these P450 activities in liver microsomes of PB-pretreated rats were only weakly inhibited, but in liver microsomes of βNF-pretreated rats a strong inhibition was observed. In both cases, the V_{max} values did not increase significantly when compared to the controls (Table 1). In the presence of curcumin, the K_m value was increased from 0.99 to 10.05 μM in βNF-microsomes. With increasing curcumin concentrations, the apparent K_m values of both βNF- and PB-induced liver microsomes were increased when compared to the controls of curcumin. The average K_i values of curcumin were 0.14 μM for the EROD activity and 76.02 μM for the PROD activity in βNF- and PB-induced microsomes, respectively (Table 1). Lineweaver-Burk plots indicated a competitive type of inhibition for both P450 activities (Table 1).

Recently, topical application of curcumin was reported to inhibit the formation of B[a]P-DNA adducts and to protect against tumorigenic activities of B[a]P and DMBA in the epidermis of female CD-1 mice (Huang *et al.*, [10]). Mukundan *et al.* [23] found that 0.03% curcumin in the diet for 4 weeks also significantly reduced the levels of B[a]P-DNA adducts in the liver of rats. Soudamini and Kuttan [24] suggested that the mechanism of action of curcumin as inhibitor of chemical carcinogenesis involved scavenging of peroxides and superoxides as a result of its antioxidant capacity. The carcinogen B[a]P, however, requires oxidative bioactivation to B[a]P-7,8-dihydrodiol-9,10-epoxide, the ultimate carcinogen known to bind to DNA [25]. B[a]P is bioactivated by P450 1A1 [26]. In the present study, we found that curcumin is an extremely potent competitive inhibitor of P450 1A1/1A2, measured as EROD activity in βNF-induced microsomes (Fig. 2). Our results suggest that, apart from its antioxidant activity [11], curcumin could also act as an anticarcinogen because of its strong and specific inhibitory activity towards P450 1A1/1A2.

Rao *et al.* [27] suggested that the inhibition of azoxymethane (AOM)-induced colon carcinogenesis by curcumin was mediated through modulation of P450 2E1-dependent AOM metabolism by curcumin. However, this explanation seems unlikely because, in the present study, we found that curcumin

TABLE 1. Enzyme kinetic parameters of the effects of curcumin on P450-dependent EROD and PROD activities in microsomal rat liver fractions from differentially induced rats

Microsomes	Curcumin added (μM)	V_{max} (nmol/min/mg)	K_m (μM)	K_i (μM)	IC_{50} (μM)	Type of inhibition
βNF-microsomes/EROD activity	0.00	33.96	0.99			
	0.03	31.45	1.19	0.15		Competitive
	0.10	32.37	1.60	0.16		Competitive
	0.30	37.06	2.94	0.15		Competitive
	1.00	36.38	10.05	0.11	2	Competitive
PB-microsomes/PROD activity	0.00	1.27	6.41	—	—	
	1.00	1.10	6.52	76.30		Competitive
	3.00	0.94	6.66	76.02		Competitive
	10.00	1.05	6.95	75.10	14	Competitive
Pyr-microsomes/PNP hydroxylation activity			% inhibition			
	1		0.5			
	3		4			
	10		8			
	30		9		nd	nd

The values of V_{max}, K_m, K_i, and K_i' are the average from two experiments. Mixed, mixed type of inhibition; competitive, competitive type of inhibition.

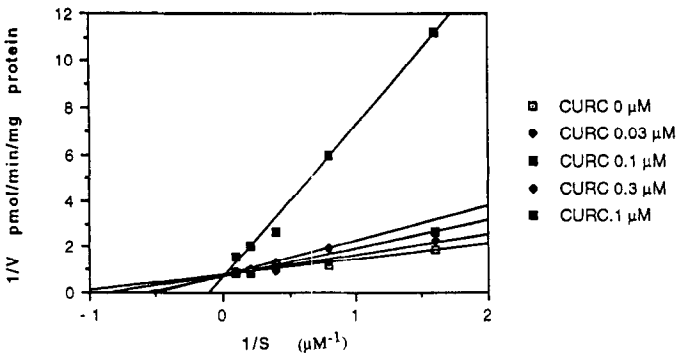

FIG. 2. Lineweaver-Burk plot showing the inhibition of cytochrome P450-mediated EROD activity in liver microsomes from βNF-induced rats, by curcumin at concentrations of 0, 0.03, 0.1, 0.3, and 1 μM.

showed no significant inhibition of P450 2E1 measured as PNP hydroxylation activity in pyrazole-induced microsomes (Table 1).

Effects on GST Activities

On the CDNB-GSH conjugation activity in liver cytosolic fractions from PB-induced rats, concentrations between 3 and 12 μM of curcumin gave from 35 up to 56% of inhibition, respectively. In Pyr-induced liver cytosolic fractions, curcumin concentrations between 5 and 15 μM gave from 38 up to 56% inhibition of the CDNB GSH conjugation activity. In βNF-induced rat liver cytosol, curcumin concentrations between 9 and 30 μM gave from 39 up to 58% inhibition of the CDNB GSH conjugation activity. The respective IC_{50} values were calculated to be 10 μM (PB-induction), 12 μM (Pyr-induction), and 21 μM (βNF-induction) (Table 2).

In liver cytosol from rats pretreated with βNF, PB, or Pyr, the apparent V_{max} values for the GST activity towards CDNB as substrate were 1.24, 4.14, and 1.32 μmol/min/mg protein, respectively. In the presence of curcumin, the V_{max} values of the CDNB-conjugation activities of GST decreased in the case of cytosol from rats pretreated with PB and Pyr (Table 2). In cytosol of βNF-pretreated rats, however, V_{max} values did not decrease significantly in the presence of curcumin when compared to controls. In all three types of cytosols, the apparent K_m values increased upon addition of curcumin (Table 2). In cytosol of rats pretreated with PB, the type of inhibition was mixed, and in the case of Pyr-cytosol the type of inhibition was competitive at the lower concentrations of curcumin but mixed at higher concentrations (Table 2). Lineweaver-Burk plots indicated a competitive type of inhibition in rat liver cytosol pretreated with βNF (Table 2, Fig. 3).

Plant phenols, such as ellagic acid, ferrulic acid, caffeic acid, and chlorogenic acid, have been reported to be *in vitro* inhibitors of GST activity from rat liver (Das *et al.*, [13]). However, Susan *et al.* [28] found that a semi chronically administered high oral dose of curcumin given to mice, daily for 15 days, increased hepatic GST activity towards CDNB by 1.8-fold compared to control animals. Moreover, Nijhoff *et al.* [29] recently reported that dietary curcumin significantly increased GST activity in the small intestine, but not in the liver of rats. Mathews *et al.* [30] found that curcumin interacts with GSH spontaneously and enzymatically in the presence of GSTs from a mouse liver postmichondrial fraction. In our study, we did not see a reaction between GSH and curcumin, neither in the presence nor in the absence of GSTs from cytosolic liver fractions of differentially induced rats. We found that the GST activity measured with CDNB as substrate was strongly inhibited by curcumin. Given the strong inhibition of GST activity in differentially induced rats (approx. 60%) and the approximately equal distribution of both class alpha (subunits 1 and 2) and class mu isoenzymes (subunits 3 and 4), as well as the absence of class pi-isoenzymes (Foliot and Beaune [31]), curcumin is not expected to demonstrate a high GST-isoenzyme selectivity.

Based on the present data on liver fractions of rats pretreated with βNF, curcumin appeared to show an extremely potent inhibitory effect towards P450 1A1/1A2 (K_i = 0.14 μM) and a 20-fold lower, but still potent, inhibitory effect on GST activity towards CDNB (K_i = 2.29 μM). In liver fractions of rats pretreated with PB, however, a reversed ratio of inhibitory effects of curcumin on P450 2B1/2B2 and GST was observed, albeit at a 50–100-fold higher concentration level (K_i = 76 μM for P450 2B1/2B2 *versus* K_i = 6 μM for GST). These strong, differential, and (iso)enzyme-selective inhibitory ef-

TABLE 2. Enzyme kinetic parameters of the effects of curcumin on GST activities towards CDNB in cytosol from differentially induced rats

Kind of cytosol	Curcumin added (μM)	V_{max} (μmol/min/mg)	K_m (μM)	K_i (μM)	K_i' (μM)	IC_{50} (μM)	Type of inhibition
βNF-cytosol	0	1.24	0.21				
	10	1.60	1.14	2.29	—		competitive
	20	1.53	1.26	4.06	—	21	competitive
PB-cytosol	0	4.14	0.39				
	7.5	2.59	0.56	5.75	12.50		mixed
	15	2.22	0.64	7.18	17.33	10	mixed
Pyr-cytosol	0	1.32	0.10				
	3.5	1.30	0.28	1.79	—		competitive
	15	1.09	0.44	3.29	71.28	12	mixed

The values of V_{max}, K_m, K_i, and K_i' are the average from two experiments. Mixed, mixed type of inhibition; competitive, competitive type of inhibition.

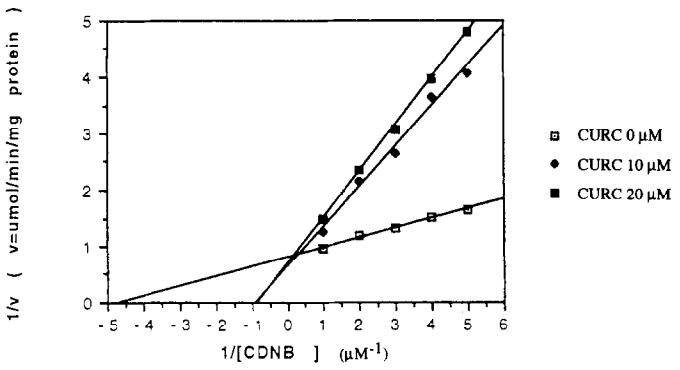

FIG. 3. Lineweaver-Burk plot showing the inhibition of GST-mediated CDNB GSH-conjugation activity in liver cytosol from βNF-induced rats by curcumin at concentrations of 0.1 and 20 μM.

fects of curcumin on cytochrome P450 and GSTs may have important physiological and toxicological consequences, because at relatively low concentrations of curcumin the balance between the bioinactivation and the bioactivation of drugs and other xenobiotic chemicals may be strongly influenced [14, 26].

In summary, we have shown that curcumin is relatively unstable in phosphate buffer at pH 7.4. The stability of curcumin can be improved by lowering the pH and by adding GSH, NAC, ascorbic acid, or microsomal and cytosolic proteins of rat liver. Curcumin is an extremely potent inhibitor of P450 1A1/1A2, a slightly less potent inhibitor of P450 2B1/2B2, and a weak inhibitor of P450 2E1. However, curcumin is also a potent inhibitor of GSTs in liver cytosol from rats pretreated with PB, Pyr and βNF. The observed isoenzyme-selective P450 inhibition properties as well as the GST-inhibition properties of curcumin might help explain previously observed anticarcinogenic, antimutagenic, and cytoprotective effects of curcumin. Given the strong inhibitory properties of curcumin, this compound may also be useful in studying P450- and GST-dependent biotransformations of exogenous and endogenous compounds.

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