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Mechanism of inhibition of benzo[*a*]pyrene-induced forestomach cancer in mice by dietary curcumin

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Curcumin (diferuloylmethane), the major yellow pigment in turmeric, has been shown to inhibit benzo[*a*]pyrene (BaP)-induced forestomach cancer in mice through mechanism(s) not fully understood. It is well known that while cytochrome P4501A1 (CYP1A1) and epoxide hydrolase (EH) are important in the conversion of BaP to its activated form, (+)-*anti*-7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(+)-*anti*-BaPDE], the detoxification of (+)-*anti*-BaPDE is accomplished by glutathione (GSH) *S*-transferases (GST). Therefore, it seems reasonable to postulate that curcumin may exert anti-carcinogenic activity either by inhibiting activation of BaP or (and) by enhancing the detoxification of (+)-*anti*-BaPDE. Administration p.o. of 2% curcumin in the diet to female A/J mice for 14 days, which has been shown to cause a significant inhibition in BaP-induced forestomach tumorigenesis, resulted in a modest but statistically significant reduction in hepatic ethoxyresorufin *O*-deethylase (EROD) activity, a reaction preferentially catalyzed by CYP1A1. While EROD activity could not be detected in the forestomach of either control or treated mice, curcumin feeding caused a statistically significant increase (~2.3-fold) in hepatic EH and GST activities. Hepatic and forestomach GSH levels, and forestomach EH and GST activities were not affected by curcumin treatment. Even though the levels of various hepatic GST isoenzymes were significantly increased upon curcumin feeding, maximum induction was noticed for the pi class isoenzyme (mGSTP1-1), which among murine hepatic GSTs is highly efficient in the detoxification of (+)-*anti*-BaPDE. In conclusion, the results of the present study suggest that curcumin may inhibit BaP-induced forestomach cancer in mice by affecting both activation as well as inactivation pathways of BaP metabolism in the liver.

Introduction

Curcumin (diferuloylmethane), the major yellow pigment in turmeric, curry etc., is known to possess anti-oxidative and anti-inflammatory properties (1,2). In addition, curcumin has been shown to prevent chemically induced cancers in several different animal tumor bioassay systems, including inhibition of benzo[*a*]pyrene (BaP*)-induced forestomach tumorigenesis in A/J mice, *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine-induced duodenal tumorigenesis in C57BL/6 mice and azoxymethane-induced colon carcinogenesis in CF-1 mice (3–5). For example, feeding 2% commercial grade curcumin in the diet has been shown to reduce the number of BaP-induced forestomach tumors per mouse by ~51 and 67% when administered during the initiation and post-initiation periods, respectively (4). However, the mechanism(s) by which dietary curcumin prevents BaP-induced forestomach tumorigenesis in mice is not clear.

It is well established that BaP requires metabolic activation, mediated by cytochrome P450 (CYP) dependent monooxygenases, for the generation of its ultimate carcinogen, (+)-*anti*-7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(+)-*anti*-BaPDE] (6–10). Covalent interaction of (+)-*anti*-BaPDE with nucleophilic sites in DNA is a critical event in BaP-induced tumorigenesis (11). Several different mechanisms exist that can convert (+)-*anti*-BaPDE to less harmful species, and thus protect DNA (12–17). These mechanisms include spontaneous hydrolysis to tetrols and keto diols, non-enzymatic as well as enzymatic metabolism to triols and triol epoxides, hydration by epoxide hydrolase (EH) and glutathione (GSH) *S*-transferase (GST)-catalyzed conjugation with GSH (12–17). In addition, certain plant phenols, such as ellagic acid, have been shown to inhibit mutagenic and carcinogenic effects of *anti*-BaPDE through direct interaction with this diol epoxide (18,19). Since BaPDE is a poor substrate for EH (20), the most important mechanism of BaPDE inactivation seems to be its conjugation with GSH. Thus, it has been shown that, in the presence of GSH, both rat liver cytosol and purified rat liver GST isoenzymes reduce the binding of BaPDE to DNA (21).

It seems reasonable to hypothesize that curcumin may inhibit BaP-induced forestomach tumorigenesis in mice either by inhibiting the activation of BaP or (and) by enhancing the detoxification of (+)-*anti*-BaPDE in the liver (the main detoxification site) and/or in the target organ (forestomach). In the present study, we have tested this hypothesis by determining the effects of curcumin feeding on the activities of the enzymes of BaP activation/inactivation pathways in the liver and forestomach of female A/J mice. The results of the present study suggest that dietary curcumin may inhibit BaP-induced forestomach tumorigenesis in mice by reducing the activation of BaP as well as by increasing the detoxification of (+)-*anti*-BaPDE in the liver.

Materials and methods

Materials

Female A/J mice (8 weeks old) were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD. Commercial grade curcumin was procured from Kalsec (Kalamazoo, MI). AIN-76 semi-purified diet was obtained from ICN Biomedicals (Aurora, OH). All other reagents were of highest purity available.

Animal treatment

Previous studies have shown that administration p.o. of 2% commercial grade curcumin in the diet for 14 days causes a significant reduction in the number

***Abbreviations:** BaP, benzo[*a*]pyrene; BaPDE, 7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; CDNB, 1-chloro-2,4-dinitrobenzene; CYP1A1, cytochrome P4501A1; EH, epoxide hydrolase; EROD, ethoxyresorufin *O*-deethylase; GSH, glutathione; GST, glutathione *S*-transferase.

of BaP-induced forestomach tumors in mice (4). In the present study, a similar protocol was used for curcumin administration to elucidate the mechanism of inhibition of BaP-induced tumorigenesis in mice by this phytochemical. Briefly, the mice were fed AIN-76 diet for 5 days and then divided into two groups. The experimental group of mice were given 2% commercial grade curcumin in the AIN-76 diet and water *ad libitum*, whereas the control mice were given AIN-76 diet without curcumin supplementation and water *ad libitum*. The mice were killed 2 weeks after the start of curcumin feeding. The liver and forestomach tissues were removed, washed thoroughly with ice-cold phosphate buffered saline and stored at –80°C until used.

Preparation of microsomal and cytosolic fractions

The tissue samples were homogenized in ice-cold 0.1 M potassium phosphate buffer, pH 7.4, using a Polytron. The homogenate was centrifuged at 500 *g* for 10 min to remove nuclear debris, and the supernatant fraction was centrifuged at 10 000 g for 15 min to remove mitochondria. The supernatant thus obtained was centrifuged at 100 000 *g* for 1 h. The microsomal pellet was resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 10 mM DTT, 10 mM EDTA and 20% (v/v) glycerol (buffer A) and centrifuged again for 1 h at 100 000 *g*. The microsomal pellet was resuspended in buffer A and used for the measurement of EROD and EH activities. The supernatant fraction was used for the determination of GSH levels and GST activity.

Enzyme assays

The EROD activity was determined by monitoring the formation of resorufin from 7-ethoxyresorufin in the presence of microsomal protein according to the method described by Pohl and Fouts (22) with some modifications. Briefly, the reaction mixture in a total volume of 1.25 ml contained 0.1 M Hepes buffer (pH 7.8), 5 mM glucose 6-phosphate, 1 U of glucose 6-phosphate dehydrogenase, 5 mM $MgSO_4$, 1.6 mg/ml bovine serum albumin, 2 μM 7-ethoxyresorufin and 100 μg of microsomal protein. The reaction mixture was pre-incubated for 5 min at 37°C, and the reaction was initiated by addition of 2 μM NADPH. The reaction mixture was incubated for 15 min at 37°C. The EROD activity was determined as a function of varying microsomal protein concentration and incubation time to optimize the assay conditions. Blanks without NADPH were incubated similarly. The reaction was terminated by mixing with 2.5 ml of methanol. The mixture was centrifuged at 12 000 *g* for 15 min, and the fluorescence of the supernatant was measured at 586 nm in a Shimadzu RF 5000U spectrofluorimeter using an excitation wavelength of 522 nm. A standard curve of resorufin (0.02–0.4 nmol) was generated to quantitate its formation from 7-ethoxyresorufin in the presence of microsomal protein. The microsomal EH was assayed according to the method described by Guenthner *et al*. (23). Briefly, the reaction mixture in a final volume of 0.5 ml contained 50 mM Tris–HCl, pH 9.0, 75 nmol [^{3}H]BaP-4,5-oxide (sp. act. 3.1 μCi/μmol) and 200 μg of the microsomal protein. The samples were incubated for 5 min at 37°C, and the reaction mixture was extracted with 3.5 ml light petroleum and 0.5 ml dimethylsulfoxide. The EH activity was determined as a function of varying microsomal protein concentration and incubation time to optimize the assay conditions. The samples were extracted twice more with 3.5 ml light petroleum. The diol product in the aqueous phase was extracted into 1 ml ethyl acetate. The ethyl acetate layer, containing tritiated diol product, was assayed for radioactivity by liquid scintillation counting. GSH level and GST activity were determined by the methods of Beutler *et al*. (24) and Habig *et al*. (25), respectively. Protein was determined by the Bradford method (26). Statistical significance of differences in enzyme activities between control and treated groups was assessed by Student's *t*-test.

Effects of curcumin feeding on the levels of hepatic and forestomach GSTs

The effects of curcumin feeding on the levels of hepatic and forestomach GSTs were determined by using a protocol described by us previously (27), which involves GSH-affinity chromatography followed by reverse-phase HPLC. Briefly, equal amounts (0.25 g) of liver or forestomach tissues from control and curcumin-fed mice were homogenized in 10 mM potassium phosphate buffer, pH 7.0, containing 1.4 mM 2-mercaptoethanol. The homogenate was centrifuged at 14 000 *g* for 40 min, and the supernatant fraction was dialyzed against 22 mM potassium phosphate buffer, pH 7.0, containing 1.4 mM 2-mercaptoethanol. The dialyzed supernatant was subjected to GSH-linked to epoxy activated Sepharose 6B affinity chromatography to isolate total GST. GSH-affinity chromatography was performed by the method of Simons and Vander Jagt (28), with some modifications described by us previously (29). The GSTs retained on the affinity column were eluted by using equal volumes of the elution buffer (5 mM GSH in 50 mM Tris–HCl, pH 9.6, containing 1.4 mM 2-mercaptoethanol). Individual GST isoenzymes were separated and quantitated by reverse-phase HPLC analyses of equal volumes of affinity purified GST preparations from tissues of control and curcumin-fed mice. A Waters Delta-Pak C_{18} reverse-phase column (150× 3.9 mm) was used for HPLC analysis. The column was pre-equilibrated with 61% solvent I (5% acetonitrile/0.1% trifluoroacetic acid) and 39% solvent II (90% acetonitrile/0.1% trifluoroacetic acid). The GST isoenzymes were eluted with a 30-min linear gradient of 39–69% solvent II at a column flow rate of 1 ml/min. The GST subunits were identified on the basis of their elution profiles during reverse-phase HPLC analysis (27). Each GST subunit was quantitated by using standard curve for the respective subunit (27).

Table I. Effect of curcumin feeding on hepatic and forestomach ethoxyresorufin *O*-deethylase and epoxide hydrolase activities

Enzyme	Group	Enzyme activity (nmol/min/mg protein)	
		Liver	Forestomach
Ethoxyresorufin *O*-deethylase	Control	0.04 ± 0.01[a]	ND[b]
	Curcumin-fed	0.03 ± 0.01[c]	ND
Epoxide hydrolase	Control	1.9 ± 0.17	0.47 ± 0.10
	Curcumin-fed	4.4 ± 0.56[c]	0.47 ± 0.05

[a]Data represent means ± SD of values from the liver or forestomach tissues of four mice.
[b]ND, not detected, limit of detection 0.02 nmol.
[c]Significantly different from control, $P < 0.05$.

Table II. Effect of curcumin feeding on hepatic and forestomach glutathione level and glutathione *S*-transferase activity toward CDNB

Group	Glutathione level (nmol/mg protein)		Glutathione *S*-transferase activity (nmol/min/mg protein)	
	Liver	Forestomach	Liver	Forestomach
Control	87 ± 11[a]	19 ± 5	470 ± 22	470 ± 120
Curcumin-fed	102 ± 9	26 ± 5	1100 ± 180[b]	450 ± 81

[a]Data represent means ± SD of values from the liver or forestomach tissues of four mice.
[b]Significantly different from control, $P < 0.05$.

Results and discussion

Cytochrome P450, in particular 1A1 isoform (CYP1A1), is believed to play an important role in the conversion of BaP to its ultimate carcinogenic metabolite, (+)-*anti*-BaPDE (6–8). In addition, CYP1A1 is important in the metabolism of BaP to less reactive/inactive phenolic metabolites, such as 3-hydroxy-BaP (6–8). The effects of curcumin feeding on CYP1A1 levels were assessed by determining EROD activity, a reaction preferentially catalyzed by CYP1A1, in the microsomes prepared from the tissues of control and curcumin-fed mice. As shown in Table I, the EROD activity could not be detected in the forestomach of either control or curcumin-fed mice. On the other hand, curcumin feeding caused a modest but statistically significant reduction (~25%, $P < 0.05$ control versus curcumin-fed) in hepatic EROD activity (Table I).

EH is another microsomal enzyme that plays an important role in the bioactivation of BaP (8,14). The effects of the dietary curcumin on EH levels were assessed by measuring the enzyme activity toward BaP-4,5-oxide in the microsomes prepared from the liver and forestomach tissues of control and curcumin-fed mice, and the results are summarized in Table I. Feeding curcumin did not alter forestomach EH activity. On the other hand, EH activity was increased by ~2.3-fold ($P < 0.05$) in the liver of curcumin-fed mice compared with the control.

The effects of curcumin feeding on hepatic and forestomach GSH levels and GST activities, which play an important role in cellular detoxification of (+)-*anti*-BaPDE (15–17), were also determined and the results are summarized in Table II.

While hepatic GST activity was increased significantly (~2.3-fold, $P < 0.05$) on curcumin administration, such a treatment did not alter GSH levels in the liver. On the other hand, forestomach GSH level and GST activity were not affected by curcumin feeding (Table II).

The cytosolic GST activity in mammalian tissues is because of multiple isoenzymes, which, on the basis of their structural and catalytic properties, can be grouped into four known major classes, alpha, mu, pi and theta (30–32). Previous studies from our laboratory have shown that >90% of total GST activity in the liver of female A/J mice is accounted for by four isoenzymes: mGSTA3-3 (alpha class), mGSTP1-1 (pi class), mGSTM1-1 (mu class) and mGSTA4-4 (alpha class) (27). Like liver, four isoenzymes, mGSTA1-2 (alpha class), mGSTP1-1, mGSTM1-1 and mGSTA4-4, constitute >90% of total GST activity in the forestomach of A/J mice (27). An interesting feature of GST system is that the members of alpha, mu and pi classes of isoenzymes exhibit overlapping yet distinct substrate specificities (30,31). For example, kinetic studies with purified human and rat GST isoenzymes have clearly shown that the pi class isoenzyme is relatively more efficient than other classes of GSTs in the GSH conjugation of *anti*-BaPDE (15,16). More recently, we have demonstrated that the pi class isoenzyme plays a major role in hepatic detoxification of (+)-*anti*-BaPDE in mice (33).

Table III summarizes the effects of curcumin feeding on the levels of various hepatic and forestomach GSTs. Even though curcumin feeding caused a statistically significant increase in the levels of each hepatic isoenzyme, maximum induction was observed for the pi class isoenzyme (mGSTP1-1). In agreement with the results of the GST activity determinations, the levels of forestomach GST isoenzymes were not altered by curcumin feeding (Table III).

The results of the present study indicate that administration p.o. of commercial grade curcumin in the diet causes a modest but statistically significant reduction in the hepatic EROD (CYP1A1) activity. Since CYP1A1 plays an important role in the bioactivation of BaP (6–8), it seems reasonable to postulate that curcumin may, at least in part, inhibit BaP-induced cancer in mice by reducing the conversion of BaP to (+)-*anti*-BaPDE, and subsequently inhibiting the formation of BaPDE–DNA adducts. On the other hand, curcumin-mediated reduction in hepatic EROD (CYP1A1) activity is likely to inhibit the metabolism of BaP to less reactive/inactive metabolites, which may also affect the extent of BaP–DNA adduction. In this regard, it is important to point out that topical application of curcumin to mice 5 min prior to the application of BaP has been shown to significantly inhibit the formation of [^{3}H]BaP–DNA adduct in the epidermis (5). However, whether or not dietary curcumin reduces the formation of BaP–DNA adduct(s) remains to be investigated. Studies are also needed to determine if reduction in hepatic EROD activity by curcumin feeding results from the decline in the level of CYP1A1 protein or is because of inhibition of the catalytic activity of this enzyme. In this regard, it is important to point out that curcumin has been shown to be a potent inhibitor *in vitro* of rat liver CYP1A1 (34).

Previous studies by Oetari *et al.* (34) have shown that curcumin is also a potent inhibitor *in vitro* of GST activity. Inhibition of GST activity by curcumin has also been observed by van Iersel *et al.* (35) in a human IGR-39 melanoma cell line. The results of the present study reveal that administration p.o. of curcumin causes a statistically significant increase, not reduction, in hepatic GST activity. Even though the reason for discrepancy between our results (present study) and those of previous studies (34,35) remains to be clarified, the observed differences may be linked to differential metabolism of curcumin between *in vitro* and *in vivo* systems. Since GSTs play an important role in the detoxification of (+)-*anti*-BaPDE (15–17,21), our results suggest that enhanced inactivation of the ultimate carcinogen of BaP may be another mechanism by which dietary curcumin inhibits BaP-induced cancer in mice.

Table III. Effects of curcumin feeding on the levels of predominant hepatic and forestomach glutathione *S*-transferase isoenzymes

Isoenzyme (class)	Glutathione *S*-transferase content (μg/g wet tissue)		
	Control	Curcumin-fed	Ratio[a]
Liver			
mGSTA3-3 (alpha)	224 ± 55[b]	312 ± 16[c]	1.4
mGSTP1-1 (pi)	83 ± 16	154 ± 9[c]	1.9
mGSTM1-1 (mu)	524 ± 81	700 ± 45[c]	1.3
mGSTA4-4 (alpha)	17 ± 4	23 ± 1[c]	1.4
Forestomach			
mGSTA1-2 (alpha)	8 ± 3	5 ± 2	0.6
mGSTP1-1 (pi)	112 ± 8	123 ± 8	1.1
mGSTM1-1 (mu)	117 ± 8	122 ± 13	1.0
mGSTA4-4 (alpha)	70 ± 3	75 ± 3	1.1

[a]Ratio of GST content in tissues of curcumin-fed mice/control mice.
[b]Data represent means ± SD of values from the liver of four mice.
[c]Significantly different from control, $P < 0.05$.

It is interesting to note that curcumin administration does not alter GST activity in the target organ (forestomach), and thus hepatic modulation of GST activity may be crucial in anti-neoplastic effect of this phytochemical against BaP-induced forestomach cancer in mice. In this regard, curcumin seems to behave differently from certain other naturally occurring inhibitors of BaP-induced cancer. For example, we have shown previously that the organosulfur compounds from garlic, which are potent inhibitors of BaP-induced forestomach cancer in mice (36), significantly increase GST activity not only in the liver but also in the forestomach of A/J mice (37,38).

Recent studies from our laboratory have shown that the GST isoenzymes of the liver and forestomach of female A/J mice significantly differ in their catalytic efficiency, and consequently relative contribution in the GSH conjugation of *anti*-BaPDE (17,33). Our studies reveal that while pi class isoenzyme mGSTP1-1 plays a key role in the hepatic GSH conjugation of *anti*-BaPDE, mGSTP1-1 as well as an alpha class isoenzyme (mGSTA1-2) are important in the detoxification of this diol epoxide in the forestomach of female A/J mice (17,33). For example, in liver, the contribution of pi class isoenzyme mGSTP1-1 in the GSH conjugation of *anti*-BaPDE (~79%) far exceeds the combined contribution of other classes of GST isoenzymes (33). In forestomach, however, the contribution of mGSTP1-1 in the detoxification of *anti*-BaPDE is more or less comparable with that of mGSTA1-2 (33). It is important to mention that an isoenzyme corresponding to mGSTA1-2 could not be detected in the liver of female A/J mouse (27). The results of the present study indicate that even though the levels of each hepatic GST isoenzyme are significantly increased upon curcumin feeding, maximum induction is observed for the pi class isoenzyme. On the other hand, none of the forestomach GST isoenzymes was significantly induced upon curcumin feeding.

In conclusion, our results suggest that curcumin may inhibit

BaP-induced forestomach cancer in mice, not only by inhibiting the activation of BaP through reduction of hepatic EROD activity, but also by enhancing the detoxification of *anti*-BaPDE through induction of hepatic GSTs, pi class isoenzyme in particular. However, the mechanism of the tissue specificity of the curcumin in modulating GST activity in mice remains to be clarified.

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