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Short communication

Dietary curcumin modulates phenobarbital- and acetone-induced CYP450 2B1 and 2E1 isozymes in mice

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Abstract

Curcumin pretreatment has been shown to inhibit benzo(a)pyrene-induced cytochrome P450 1A1/1A2 isozymes in liver and lungs of mice, via its modulatory effect on their transcriptional regulator, aryl hydrocarbon receptor. However, its effects on the activities of other CYP450 isozymes induced by carcinogen/promoter/solvent *in vivo* remain unknown. In the present study, we investigated the effect of curcumin on phenobarbital (PB)- and acetone-induced CYP2B1 and 2E1 isozymes respectively, *in vivo*. Dietary pretreatment of mice with chemopreventive doses of curcumin showed significant inhibition of PB-induced enzyme activity, protein and mRNA levels of CYP2B1 in mouse liver. Similarly, mice fed with curcumin in diet showed significant inhibition in the acetone-induced enzyme activity and protein levels of CYP2E1 in liver. However, no differences in the mRNA expression of CYP2E1 were observed in any of the treatment groups under consideration. Dietary curcumin alone also led to an increase in the activity of CYP2B1 and CYP2E1 though, it was marginal when compared to the effects induced by phenobarbital and acetone respectively. Overall, the results point towards a curcumin-mediated transcriptional regulation of PB-induced CYP2B1 and a posttranslational mechanism of regulation for acetone-induced CYP2E1 isozyme *in vivo*.

Keywords: Curcumin; Acetone; Phenobarbital; *In vivo*; Cytochrome P450.

Introduction

Turmeric and/or its main coloring component, curcumin (diferuloylmethane) have been shown to prevent chemical-induced tumors in several experimental systems [1 -8]. Besides, the chemopreventive efficacy of turmeric/curcumin has been established at both initiation and promotion stages of carcinogenesis [8-14]. It has also been shown that turmeric/curcumin pre-treatment led to significant decreases in the levels of B(a)P/DMBA-derived DNA adducts in target and/or non-target tissues [11,15,16]. On the other hand, dietary treatment of mice with curcumin subsequent to B(a)P exposure was found to mediate disappearance of B(a)P-derived DNA adducts in both liver and lung tissues of mice [17]. Studies from our laboratory have demonstrated that dietary curcumin pre-treatment decreases B(a)P-induced cytochrome P450 1A1/1A2 (CYP1A1/1A2) via its modulatory effects on transcription regulator, aryl hydrocarbon receptor (AhR) [13,18]. However, it is yet not known if curcumin can also similarly modulate, *in vivo*, the carcinogen/promoter/solvent-induced activities of other members of CYP450 family of isozymes. The present study therefore aims to investigate the effect(s) of curcumin on phenobarbital (PB)- and acetone-induced CYP2B1 and 2E1 isozymes, respectively in mice. Our findings demonstrate that dietary curcumin pre-treatment decreased the PB-induced enzyme activity, protein as well as mRNA expression of CYP2B1 in mouse liver. Moreover, we also show that curcumin pre-treatment decreased the acetone-induced activity and protein levels of CYP2E1 but did not alter its mRNA expression.

Methods

Materials

Curcumin, pentoxyresorufin and primers were obtained from Sigma Chemical Company (St Louis, MO). Antibodies for CYP2B1 and CYP2E1 were purchased from Novus Biologicals (Littleton, CO). ECL chemiluminescence detection kit was purchased form Santa Cruz Biotechnology (Santa Cruz, CA). Acetone (ultra pure analytical grade) was obtained from Merck Limited (Mumbai, India). Methanol was procured form S.D.Fine Chemicals Company (Mumbai, India).

Animal treatment

Inbred male Swiss albino mice (6-8 week old) were randomized into various treatment groups. Animals belonging to the 1st experimental set received drinking water ad libitum and were fed with either powdered standard laboratory diet (control diet) or experimental diet (0.01%/0.05% curcumin in standard laboratory diet), throughout the experimental duration of 18 days (Chart 1). Mice were given 10 mg/kg phenobarbital (PB) in 0.01% DMSO, i.p.

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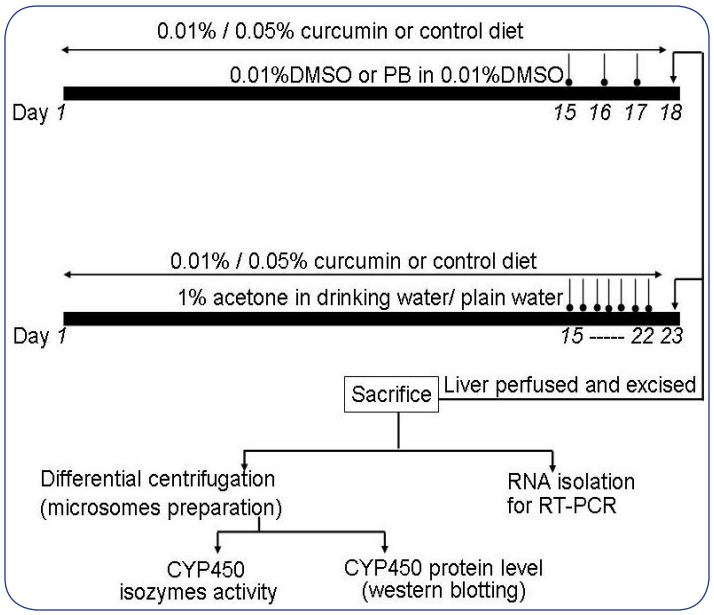
or 0.01% DMSO alone as vehicle, once a day during 15th-17th day of experiment. Amongst the various doses tried (10-80 mg/kg), relatively lower dose i.e. 10 mg/kg was selected for the induction of CYP2B1. Animals belonging to the 2nd set of experiment received control or experimental diet (0.01%/0.05% curcumin in diet) for the entire experimental period of 23 days and were given distilled water (vehicle control) or 1% acetone (v/v, made in distilled water) as the sole source of drinking water, during 15th-22nd day as reported [19]. Animals were sacrificed 24 h after the last PB/acetone treatment; their liver lobes were perfused, excised, and stored at -80°C until further use (Chart 1). Animals were weighed once in a week, their initial and final body weights and liver to body weight ratio were also recorded.



CYP2B1 Enzyme Activity

The activity of CYP2B1 isozyme was measured using specific probe drug as described [20]. Briefly, 1 mg microsomal protein was incubated for 5 min at 37°C in a 0.1 M sodium phosphate buffer, pH 7.4 with 5 µM pentoxyresorufin, 6.25 mM MgSO4, 60 µM EDTA in a final volume of 1 ml. Reaction was initiated by adding NADPH (250 µM), incubated for 5 min at 37°C and subsequently terminated by the addition of 2 ml ice-cold methanol. After centrifugation (4000 g, 5 min) to pellet the precipitated microsomal protein, the metabolite resorufin was measured by spectrofluorometry (Ex= 550 nm and Em= 585 nm). Reaction mixture without NADPH served as the blank. Resorufin formed in the test samples was calculated from the standard curve plotted with 0-5 µM of resorufin under identical experimental conditions. Results were expressed as nmoles resorufin formed /min/mg protein.

CYP2E1 Enzyme Activity

The CYP2E1 activity was measured in liver microsomes by HPLC determination of the hydroxylation of p-nitrophenol as described with some modifications [21]. Briefly, 1 mg microsomal protein was incubated in 50 mM Tris buffer pH 7.4, 5 mM MgCl2, 1.5 mM NADPH in a final volume of 500 ml at 37°C for 5 min. Reaction was initiated by the addition of p-nitrophenol (100 µM), incubated for an additional 20 min at 37°C before termination with 25 µl trifluoroacetic acid (99% pure). The amount of p-nitrocatechol formed was measured in the supernatants using Waters HPLC system consisting of dual pumps and a UV-Vis detector, at 345 nm. Samples were analyzed with a C18 reverse-phase column (250 x 4.6 mm) and eluted at a flow rate of 1.5 ml/min, with mobile phase comprising of 52.8% acetonitrile, 1% tri-fluoroacetic acid in distilled water, pH 2.5. A standard curve prepared with 0.025-0.5 ng/ml p-nitrocatechol was used for quantitating p-nitrocatechol in the sample, as the measure of CYP2E1 activity. Results were expressed as nmol p-nitrocatechol formed/min/mg protein.

Protein Immunoblotting

50 µg microsomal proteins were separated on a 12% SDS polyacrylamide gel as described earlier [13] and immunoblotted using polyclonal antibodies for CYP2B1/2E1 (1:10,000 dilutions). In the absence of a well accepted standard internal control for microsomal proteins, quantitative analysis was done by normalizing the CYP2B1/2E1 immunoblot with a prominent band visualized on membrane after Ponceau-S staining, as has also been done before [13].

RNA Isolation and Semiquantitative RT-PCR

Total RNA from mouse liver tissue was isolated and reverse transcribed to cDNA as previously described [13]. The resulting cDNAs were subjected to PCR using specific primer sequences for CYP2B9 (mouse ortholog of rat CYP2B1), CYP2E1, and GAPDH (house keeping gene), and amplification conditions as detailed in Table 1 and 2. PCR products were then, electrophorized on 1.8% agarose gel and visualized after ethidium bromide staining.

Table 1. Oligonucleotide sequences used in RT-PCR:

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Product (bp)
CYP2B9	TGAAGCTTTTCTGCCCTTCT	GTGTGAGCAGCTACCAATGG	311
CYP2E1	CTGATTGGCTGCGCACCCTGC	GAACAGGTCGGCCAAAGTCAC	381
GAPDH	CCTCTGGAAAGCTGTGGCGT	*TTGGAGGCCATGTAGGCCAT*	431

Table 2. Amplification conditions employed in RT-PCR analysis:

Gene	Annealing temperature, time	No. of cycles	*Reference*
CYP2B9	53°C, 30 sec	30	Damon et al., 1996
CYP2E1	67°C, 60 sec	35	Wahl et al., 2007

Results

Based on the body weight (Table 3 and 4), liver weight, or liver to body weight ratio, no toxicity was observed in animals belonging to the various treatment groups. However, mild histological changes in liver tissue architecture (focal necrosis, spongy appearance) were observed in all animals.

Table 3. Body weights of mice belonging to phenobarbital experimental set 1

Groups (1st set)	Initial body weights in grams (g) (Mean ± SE)	Final body weights in grams (g) (Mean ± SE)
VC	21.83 ± 3.92	24.12 ± 3.03
PB	22.45 ± 2.68	25.03 ± 2.65
0.01%C + PB	22.49 ± 3.8	24.94 ± 4.6
0.05%C	24.01 ± 3.64	26.83 ± 4.47
0.05%C + PB	23.83 ± 3.88	26.07 ± 2.57

Table 4. Body weights of mice belonging to acetone experimental set 2

Groups (2nd set)	Initial body weights in gram (g) (Mean ± SE)	Final body weights in grams (g) (Mean ±SE)
VC	23.78 ± 3.87	26.09 ± 3.92
Ace	24.87 ± 4.01	27.17 ± 2.76
0.01%C + Ace	24.45 ± 3.76	27.87 ± 4.12
0.05%C	23.56 ± 3.78	26.31 ± 3.76
0.05%C + Ace	23.96 ± 4.76	26.71 ± 4.75

Curcumin Pre-treatment Decreases PB/acetone-induced Activity of CYP2B1/2E1

It was observed that mice fed with control diet and receiving 10 mg/kg phenobarbital (PB), i.p., showed significant induction of CYP2B1 enzyme activity (Figure 1A) and those that received 1% acetone in drinking water showed significant induction of CYP2E1 enzyme activity in liver tissue (Figure 1B) as compared to the basal activity observed in respective vehicle treated control animals. 0.05% dietary curcumin alone also significantly increased CYP2B1 and CYP2E1 activity though; it was significantly less when compared to the induction mediated by phenobarbital/acetone treatment. However, dietary curcumin (0.01%/0.05%) pre-treatment significantly decreased the PB-induced CYP2B1 activity (26-32%) measured as the dealkylation of PR (Figure 1A) as well as that of the acetone-induced CYP2E1 activity (12-39%, Figure 1B), measured as the hydroxylation of p-nitrophenol (Figure 2).

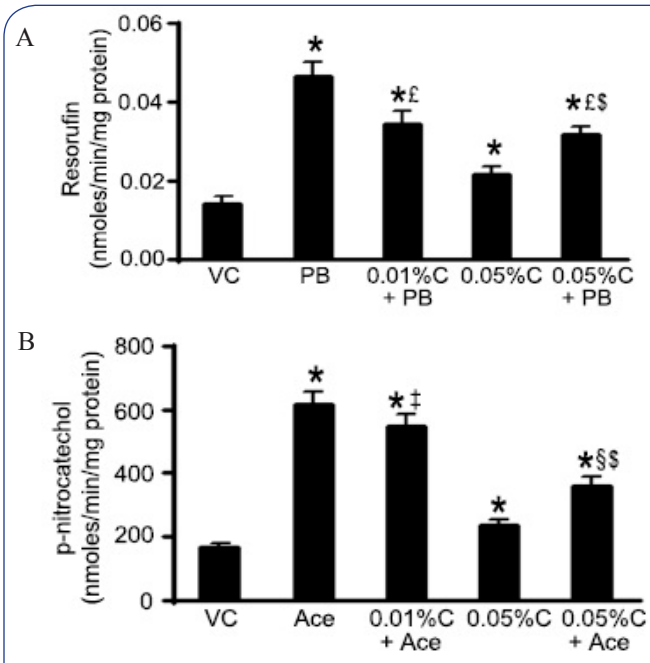
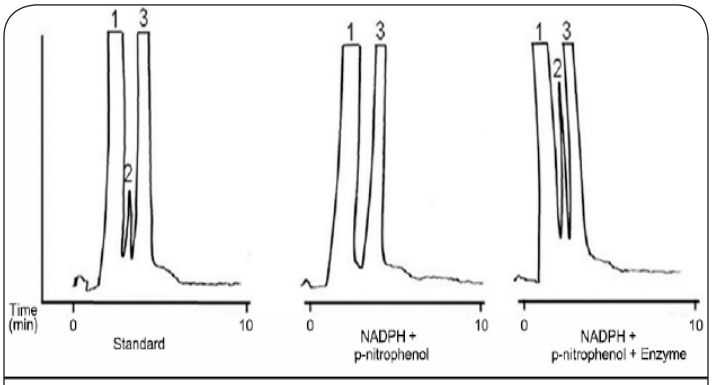


Figure 1. (A) Effect of curcumin pre-treatment on phenobarbital-induced CYP2B1 enzyme activity in mouse liver microsomes. Enzyme activity was expressed as nmol of resorufin formed/min/mg protein. Data represent mean ± SE of six observations. * = significantly different from VC; £ = significantly different from PB; $ = significantly different from 0.05%C ($p \leq 0.05$, ANOVA followed by Bonferroni Test). (B) Effect of curcumin pre-treatment on acetone-induced CYP2E1 enzyme activity in mouse liver microsomes. Enzyme activity was expressed as nmol of p-nitrocatechol formed/min/mg protein. Data represent mean ± SE of six observations. * = significantly different from VC; § = significantly different from Ace; $ = significantly different from 0.05% C; ‡ = significantly different from 0.05% C + BP ($p \leq 0.05$, ANOVA followed by Bonferroni Test).



Peak 1: NADPH (RT= 1.94); 2: p-nitrocatechol (RT= 2.96); 3: p-nitrophenol (RT= 3.55)

Figure 2. Typical HPLC chromatogram showing the separation of p-nitophenol, p-nitrocatechol and NADPH.

Curcumin Pre-treatment Decreases PB/acetone-induced protein levels of CYP2B1/2E1

Next, it was determined if the observed curcumin-mediated decrease in PB-induced CYP2B1 activity and acetone-induced CYP2E1 activity was due to the altered levels of proteins. Basal protein

expression of CYP2B1/2E1 was detected in liver of vehicle treated control animals. As compared to the respective vehicle treated control animals, CYP2B1 protein expression was significantly increased in PB treated animals (Figure 3A) and that of CYP2E1 in acetone treated animals (Figure 3B). 0.05% dietary curcumin alone also significantly increased the CYP2B1 and CYP2E1 protein levels. Notably, as observed with the enzyme activity, pre-treatment with dietary curcumin (0.01%/0.05%) significantly decreased the PB-induced CYP2B1 (21-32%) and acetone-induced CYP2E1 (11-40%) protein expression in mouse liver (Figure 3A and B).

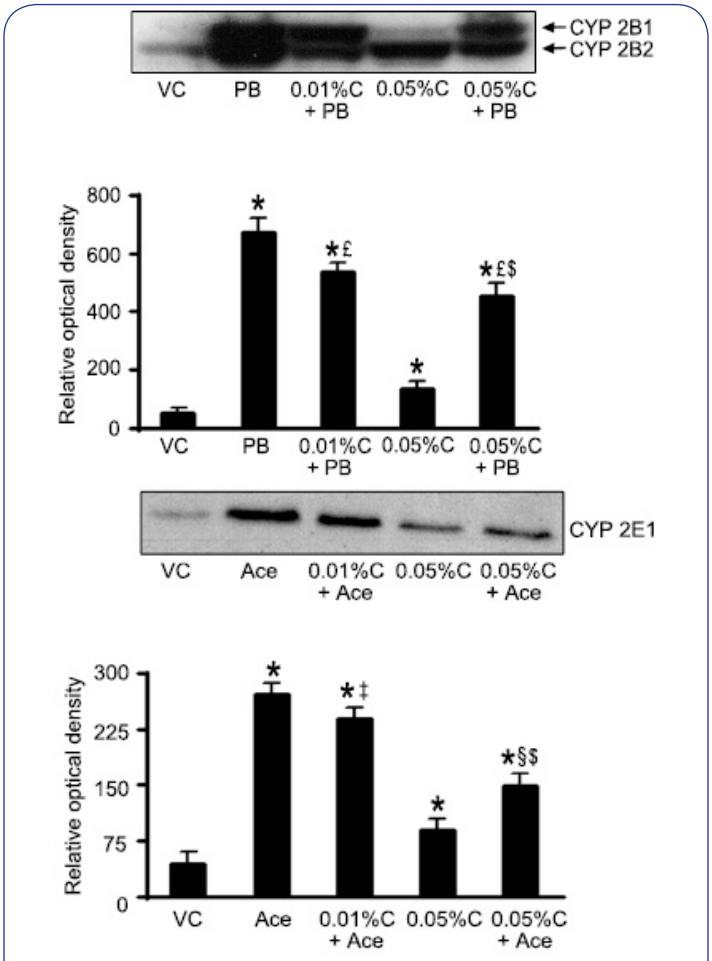


Figure 3. (A) Effect of curcumin pre-treatment on phenobarbital-induced CYP2B1 protein levels analyzed by immunoblotting. Representative blot and quantitation data presented as mean ± SE of six observations. * = significantly different from VC; £ = significantly different from PB; $ = significantly different from 0.05%C ($p \leq 0.05$, ANOVA followed by Bonferroni Test). (B) Effect of curcumin pre-treatment on acetone-induced CYP2E1 protein levels analyzed by immunoblotting. Representative blot and quantitation data presented as mean ± SE of six observations. Quantitative analysis was done by normalising the CYP2B1/2E1 immunoblot with a prominent band visualized on membrane after Ponceau-S staining. * = significantly different from VC; § = significantly different from Ace; $ = significantly different from 0.05% C; ‡ = significantly different from 0.05% C + Ace ($p \leq 0.05$, ANOVA followed by Bonferroni Test).

Curcumin Pre-treatment Decreases PB-induced mRNA levels of CYP2B1 but did not Affect CYP2E1 mRNA Expression

Furthermore, the analysis of mRNA profile revealed that PB treatment significantly increased the mRNA expression of CYP2B9 (mouse ortholog of rat CYP2B1) compared to vehicle treated control animals (Figure 4A). Of note, dietary curcumin (0.01%/0.05%) pre-treatment significantly reduced the PB-induced CYP2B9 (33-62%) mRNA level. However, the mRNA level of CYP2E1 remained unaltered in animals belonging to the various treatment groups (Figure 4B).

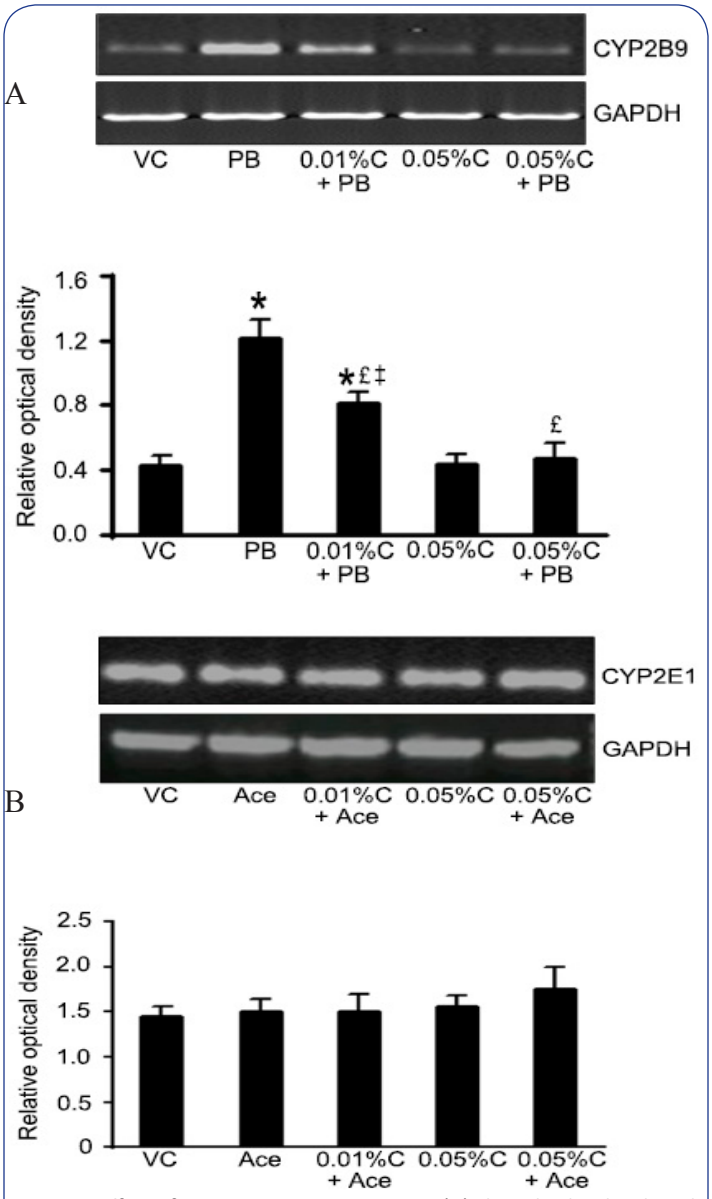


Figure 4. Effect of curcumin pre-treatment on (A) phenobarbital-induced CYP2B1 and (B) acetone-induced CYP2E1 mRNA levels analyzed by RT-PCR. Representative gel pictures and quantitation data presented as mean ± SE of six observations. * = significantly different from VC; £ = significantly different from PB; ‡ = significantly different from 0.05% C + PB ($p \leq 0.05$, ANOVA followed by Bonferroni Test).

Discussion

Earlier observations have demonstrated that curcumin regulates B(a)P-induced CYP1A1/1A2 via their transcriptional regulator, AhR [13,22]. In the present study, we demonstrate that dietary curcumin decreased PB-induced CYP2B1 enzyme activity in mouse liver as reported in rats by 1% turmeric [18]. Additionally, the study demonstrates that the observed decrease in activity is due to curcumin-mediated decrease in PB-induced protein as well as mRNA levels of CYP2B1 *in vivo*. These observations thus indicate towards a transcriptional regulation of PB-induced CYP2B1 by curcumin *in vivo*. The induction of CYP2B1 by phenobarbital (PB) and PB-like inducers (e.g. chlorpromazine, phenytoin, clofibric acid and polychlorinated biphenyls) has been shown to be mediated by the orphan nuclear receptor, constitutive androstane receptor (CAR) [23-25] and for optimal expression, requires steroid co-activator 1 (SRC-1) and transcription factor, SP1 [26]. It will be interesting to determine if curcumin could modulate CAR to exert its effect on PB-induced CYP2B1 and warrants further investigation.

Our demonstration of increase in CYP2E1 protein content and enzyme activity upon acetone treatment is consistent with other reports [19,27]. Of note, we report here for the first time that dietary curcumin treatment decreases acetone-mediated induction of CYP2E1 at both activity and protein level in mouse liver. However, these alterations in CYP2E1 activity and protein level were not reflected in the mRNA expression. Acetone has been documented to induce CYP2E1 activity and protein content without altering mRNA levels [27,28]. Our results thus clearly suggest that curcumin regulates CYP2E1 via a posttranslational mechanism in mouse liver. However, further investigation into the mechanism is needed to establish if the observed curcumin-mediated changes in acetone-induced CYP2E1 protein are due to the effects on protein synthesis or protein stabilization.

It is to be noted that dietary curcumin (0.05%) alone although did not alter the basal activity/protein expression of CYP1A1/1A2 [13,18], it increased the CYP2B1/2E1 activity and protein level in mouse liver. It is therefore also desirable to understand the modulatory effect of curcumin on the transcriptional/translational regulation of these CYP450 isozymes. In the present study, only higher dose of curcumin has been employed as curcumin control, inclusion of the lower (0.01%) dose would have been beneficial in ascertaining a dose-dependent and further protective effect of curcumin.

Studies from our lab and others have shown that dietary turmeric/curcumin inhibit nitrosodiethylamine-induced hepatocarcinogenesis in mice/rats [4,29]. The oxidative bio-activation of nitrosamines is known to be mediated by CYP2B1/2E1 isozymes. Thus, the observed modulatory effects of curcumin on CYP2B1/2E1 isozymes is likely to play an important role in decreasing the generation of DNA reactive species in N-nitrosamines associated hepatocellular carcinogenesis.

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