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Immunochemical determination of microsomal epoxide hydrolase (preneoplastic antigen) in extrahepatic tissue

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An increase in microsomal epoxide hydrolase (mEH, EC 3.3.2.3) activity is a common response to acute and chronic treatments by a number of diverse xenobiotics [1, 2], whereas only a few or no compounds have been reported to increase the cytosolic and cholesterol EHs respectively [3-8]. Many of these compounds are carcinogens [9-11], and after chronic treatment the increase in mEH activity is greater in hepatic nodules [12-14]. Furthermore, an antigen found specific for preneoplastic and neoplastic liver nodules in rats (preneoplastic antigen) has been identified as mEH [15]. Recently, this preneoplastic antigen has been found to increase in sera of humans [16] and rats [17] with hepatocellular carcinoma. For these reasons there has been speculation that increases in liver and serum mEH activity may be a suitable marker for, and related to, the neoplastic event.

An important consideration in the relationship between the increase in mEH activity and neoplasia is the mechanism of, and tissue localization of, the increase in mEH. For a few carcinogens and promoters the acute increase in mEH activity recently has been associated with an increase in enzyme content (i.e. induction) [9, 11]. These studies have been limited to hepatic tissue and confined to genotoxic carcinogens and established promoters of liver cancer. Clofibrate is one of a number of compounds which share the ability to cause proliferation of hepatic and renal peroxisomes and a decrease in serum lipids. All peroxisome proliferators so tested have been found to cause hepatic cancer [18], whereas none has shown any direct genotoxic action [18, 19]. It has been shown recently that clofibrate and other peroxisome proliferators increase mEH activity in liver and kidney microsomes of rats and mice [4, 6, 7, 20-22]. We have now used a new non-competitive double-sandwich enzyme-linked immunosorbent assay (ELISA) to test whether the increase in mEH activity arose from an increase in enzyme content by a nongenotoxic carcinogen. In addition, we have studied the content of mEH in extrahepatic tissue and tested whether these tissues are also responsive to induction.

Male mice (Swiss-Webster, 25-30 g, Bantin-Kingman, Fremont, CA) and rats (Sprague-Dawley, 180-200 g, Charles Rivers, Wilmington, MA) were housed as previously described [22], and treated with clofibrate and phenobarbital (see Table 1). Animals were killed, and liver, kidney and testis microsomes were prepared as previously described [22]. Blood for serum assays was drawn from anesthetized rats by cardiac puncture. The rate of hydrolysis of *cis*-stilbene oxide in microsomes was determined by

our previously described radiometric partition assay [23], and *cis*-stilbene oxide hydrolysis in sera and benzo[*a*]pyrene-4,5-oxide hydrolysis in microsomes were determined by previously described thin-layer chromatography methods [16, 20].

Immunochemical determination of mEH was carried out using a non-competitive double-sandwich ELISA as described in more detail in the legend to Fig. 1. Rat liver microsomes were solubilized by treatment with deoxycholate and centrifuged, and the resulting supernatant fractions were standardized by comparison with the original antigen and used as working standards. Using deoxycholate-solubilized microsomes, the ELISA demonstrated a sensitivity of 1-2 ng EH (Fig. 1). The sensitivity of this non-competitive ELISA was a slight improvement over the 2-5 ng reported previously for our competitive ELISA [24] with the additional advantages that less antigen is required and the final measurement is an increase from background rather than a decrease from 100% absorbance. Inclusion of 0.2% Lubrol PX, used to disperse microsomal samples, affected the slope of the standard (Fig. 1). Lubrol PX was therefore also used during dilution of the standard. The specificity of the ELISA was demonstrated by the negative reactions when cytosolic EH (Fig. 1), cytochrome *c*, ovalbumin, or bovine serum albumin (not shown) were used in place of microsomes and from the negative reaction when preimmune rabbit sera was used as the second antisera (Fig. 2).

Microsomes from control and clofibrate-treated rats and mice gave positive reactions in the ELISA with slopes characteristic of the purified mEH or solubilized microsomes used as standards (Fig. 2). In liver microsomes from control and treated animals, the slope for mouse tissue was consistently 75-85% of rat tissue. A similar difference in slope was also noted with the competitive ELISA [24] and may indicate the immunochemical difference between the rat and mouse mEH. These representative plots also demonstrate greater amounts of liver mEH in rats than in mice and that the quantity of mEH was elevated in both species after clofibrate treatment (Fig. 2). The values for mouse mEH were determined from rat standards and must, therefore, only be considered estimates which depend upon the immunochemical similarity of mEH in the two species. Control male mice had 4.4 μ g mEH/mg protein compared to 12.1 μ g mEH/mg protein in male rats. The amounts of mEH in rat and mouse liver microsomes are in close agreement with our own [24] and other [9, 11] previous immunochemical determinations of mEH content. In mice,

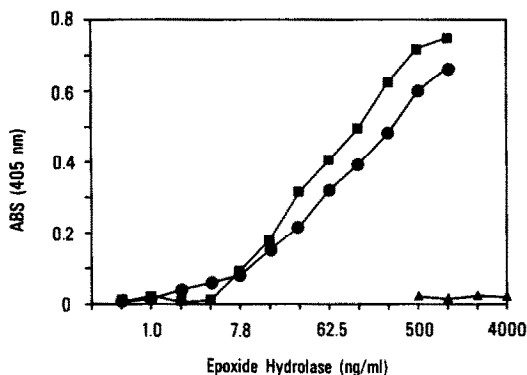


Fig. 1. ELISA for mEH: Increase in A_{405} with increasing amounts of deoxycholate-solubilized rat liver microsomes diluted in phosphate-buffered saline (pH 7.5) with 0.05% Tween 20 (PBST) with (■) or without (●) 0.2% Lubrol PX. Also shown is the lack of reaction when purified cytosolic EH (▲) was used in place of microsomes. Gilford EIA plates were activated with 0.2% glutaraldehyde in distilled water for 30 min, dried, and incubated overnight with goat anti-rat mEH at 1:10,000 in 0.05 M sodium carbonate (pH 9.8) at 4°. Test proteins were then diluted in PBST with 0.2% Lubrol PX, added in triplicate to sample wells and incubated for 2 hr. Subsequent additions included rabbit anti-rat mEH at 1:10,000 in PBST for 2 hr and goat anti-rabbit IgG conjugated to alkaline phosphatase (Miles-Yeda Ltd) at 1:2500 for 2 hr. Sample wells were emptied following each incubation and rinsed three times with PBST. The final step was incubation with 1 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Co.) in 10% diethylamine (pH 9.8). The reaction was terminated with 3 N NaOH and absorbance was read in a Gilford EIA manual reader. Rabbit and goat anti-mEH was prepared from animals inoculated with mEH isolated from *trans*-stilbene oxide treated male rats as previously described [24] and was provided by Franz Oesch and Tom Guenther. Cytosolic EH was purified from mouse liver as previously described [25], and the other control proteins were purchased from commercial sources.

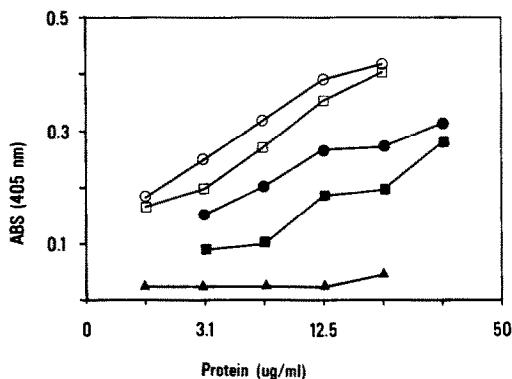


Fig. 2. Representative ELISAs of control rat (□), control mouse (■), clofibrate-fed rat (○) and clofibrate-fed mouse (●) liver microsomes. Use of normal rabbit sera (▲) in place of rabbit anti-rat mEH resulted in no reaction as shown with control rat liver microsomes as protein. Values are the mean of triplicate determinations. The three intermediate protein concentrations were used for subsequent immunochemical determination of mEH content. ELISAs were run as described in the legend to Fig. 1.

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treatments with clofibrate and phenobarbital resulted in quantities of mEH at 197 and 159% of controls respectively. Similar treatments to rats increased mEH content to 134 and 336% of controls after clofibrate and phenobarbital respectively (Table 1). Thomas *et al.* [9] reported increases of rat liver mEH to 211% of control and Kizer *et al.* [11] to 500% after 4 and 21 days of phenobarbital treatment respectively. In microsomes from rat kidneys and testis, 1.2 and 2.9 μg mEH/mg microsomal protein were found respectively (Table 1). Clofibrate treatment resulted in values that were 190 and 84% of control kidney and testis levels respectively. The ratio of enzymatic hydrolysis of *cis*-stilbene oxide and benzo[*a*]pyrene-4,5-oxide to mEH content was tissue specific. In control rats, nmoles of *cis*-stilbene oxide converted to diol per min per μg mEH were 1.4, 1.8, and 1.2 in liver, kidney, and testis respectively. For benzo[*a*]pyrene-4,5-oxide these ratios were 1.9, 13.5, and 2.3. The changes in quantity of mEH were accompanied by similar changes in the hydrolysis of two substrates selectively hydrated by mEH, *cis*-stilbene oxide and benzo[*a*]pyrene-4,5-oxide (Table 1). These results demonstrate that the increases in liver and kidney mEH activity arise from an accumulation of mEH as now shown for clofibrate treatment. In addition, the induction of liver mEH by phenobarbital has been demonstrated in the mouse and confirmed in the rat [9, 11].

An increase in serum mEH (preneoplastic antigen) has been found in humans and rats with hepatocellular carcinomas and hepatic necrosis. At this time we tested whether the serum levels of mEH would be altered during induction of this enzyme in liver. Control rat sera was found to contain 1.6 ng mEH/ml with 0.31 nmole *cis*-stilbene oxide converted to diol per min per μg mEH. Neither clofibrate nor phenobarbital treatment caused a significant change in the immunochemically determined content or enzymatic activity of serum mEH. These results suggest that induction of liver mEH without any concurrent neoplastic or necrotic events will not result in increased serum levels and, therefore, strengthen the hypothesis that serum mEH levels may be a suitable marker for hepatocellular carcinoma. The induction of mEH after acute treatment with a number of genotoxic and nongenotoxic carcinogens and promoters suggest that an increase in the quantity and activity of mEH is not a primary mechanism in neoplasia. The, as yet undefined, alteration in mEH which results in its increase in sera does, however, appear to be related to the neoplastic event and deserves further study.

In summary, immunochemical quantitation of mEH in rat kidney and testis has now been accomplished using an ELISA technique. The previously reported increase in mEH activity following clofibrate treatment has been found to arise from an accumulation of mEH protein. As studied in rats receiving acute treatment with clofibrate and phenobarbital, serum mEH-preneoplastic antigen levels were found not to increase along with induction in the liver, strengthening the hypothesis that serum mEH-preneoplastic antigen levels are a suitable marker for hepatocellular carcinoma.

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Table 1. Effects of acute clofibrate and phenobarbital treatment on mEH in mouse liver, rat liver and serum

Sample	Treatment	No.	Enzyme assay		
			ELISA ($\mu\text{g}/\text{mg}$ protein)	Substrate	
				CSO (nmoles/min/mg protein)	BPO (nmoles/min/mg protein)
Mouse liver	Control	8	4.4 \pm 0.3	3.6 \pm 0.2	4.3 \pm 0.3
	Clofibrate	4	8.7 \pm 1.3*	6.7 \pm 0.2*	6.4 \pm 0.3*
	Phenobarbital	4	7.0 \pm 1.0*	6.9 \pm 0.7*	8.1 \pm 0.4*
Rat liver	Control	8	12.1 \pm 1.5	17.0 \pm 1.0	23.1 \pm 0.7
	Clofibrate	4	16.2 \pm 2.7*	21.6 \pm 2.7*	28.8 \pm 2.0*
	Phenobarbital	4	33.6 \pm 10.7*	45.9 \pm 3.8*	39.2 \pm 0.8*
Rat kidney	Control	4	1.2 \pm 0.2	2.1 \pm 0.2	16.2 \pm 0.9
	Clofibrate	4	2.4 \pm 0.2*	3.6 \pm 0.3*	24.8 \pm 1.9*
Rat testis	Control	4	2.9 \pm 0.2	3.5 \pm 0.5	6.7 \pm 1.4
	Clofibrate	4	2.4 \pm 0.5	3.4 \pm 0.9	5.6 \pm 0.8
Rat serum	Control	4	1.62 \pm 0.36 (ng/ml)	0.50 \pm 0.03 (pmoles/min/ml)	ND
	Clofibrate	4	1.90 \pm 0.15	0.58 \pm 0.07	ND
	Phenobarbital	3	1.42 \pm 0.24	0.52 \pm 0.06	ND

Animals were fed diets containing 0.5% (w/w) clofibrate dissolved in 5% corn oil for 2 weeks or given three daily i.p. injections of 50 mg/kg phenobarbital. Controls were given the respective vehicles, and their values were pooled. Values from ELISA were determined from at least three protein concentrations each in triplicate as shown in the legend to Fig. 2. Enzymatic activities were determined in triplicate. Values are mean \pm SE. Abbreviations: CSO, *cis*-stilbene oxide; BPO, benzo[*a*]pyrene-4,5-oxide; and ND, not determined.

* Significantly different from controls, $P < 0.05$.

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