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EPOXIDE HYDROLYSIS IN THE CYTOSOL OF RAT LIVER, KIDNEY, AND TESTIS

MEASUREMENT IN THE PRESENCE OF GLUTATHIONE AND THE EFFECT OF DIETARY CLOFIBRATE

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Abstract—The hydrolysis of *trans*- and *cis*-stilbene oxide and benzo[*a*]pyrene-4,5-oxide was measured in cytosol and microsomes of liver, kidney, and testis of control and clofibrate-fed rats Significant levels of nonprotein sulfhydryls were detected in cytosol from liver (4.6 mM) and testis (1 5 mM) Glutathione was moderately stable in these fractions and interfered with the partition assays as conjugates were retained in the aqueous phase along with diols When the products were separated by thin-layer chromatography, significant amounts of glutathione-conjugates were found to have been formed in the cytosol of liver and testis Overnight dialysis or preincubation of cytosol with 0 5 mM diethylmaleate eliminated conjugate formation without affecting diol production In dialyzed cytosol from clofibrate-fed rats (0 5%, 14 days), the rates of hydrolysis of *trans*-stilbene oxide were 506, 171, and 96% of controls for liver, kidney, and testis, respectively, and 126% of controls in liver microsomes Rates of hydrolysis of *cis*-stilbene oxide were 149, 172, and 96% of controls in microsomes and 154, 124, and 91% of controls in cytosols from livers, kidneys, and testis of clofibrate-fed rats respectively Hydrolysis of benzo[*a*]pyrene-4,5-oxide was similar to that of *cis*-stilbene oxide Conjugation of the *cis*-stilbene oxide with glutathione was detected in cytosols from all three tissues with lesser amounts in the microsomes from liver and kidneys After clofibrate treatment, the rates of this activity were 200, 173, and 95% of controls in cytosol from liver, kidneys and testis, and 203 and 202% of controls in microsomes from liver and kidneys respectively These results indicate that epoxide hydrolysis and conjugation in rat liver and kidney are responsive to clofibrate treatment and support other evidence which suggests that hydrolysis of *cis*- and *trans*-stilbene oxides in cytosol is catalyzed, in part, by distinct enzymes

Hydrolysis of the oxirane (epoxide, arene oxide) moiety is an important degradative route of metabolism for this potentially toxic functional group. Three distinct epoxide hydrolases (EH†, EC 3.3 2.3, epoxide ether hydrolases) have now been described in mammalian tissue Two are predominantly located in the microsomal cell fraction and include a broad-range xenobiotic metabolizing EH referred to as microsomal EH (mEH; [1–5]), and a second enzyme which appears specific for delta-5-steroids [6, 7]. The third enzyme is also a broad-range xenobiotic metabolizing EH predominantly localized in the cell sap and referred to as cytosolic EH (cEH; [4, 5]). Preliminary evidence has been presented from a variety of sources [8–11] that another EH with some similarities to mEH exists within the cytosol of mammalian cells.

Besides hydrolysis, epoxides also can be degraded enzymatically by reduction and conjugation with glutathione (GSH) The former pathway has only been described for a limited number of epoxides. The latter activity is associated with a family of isozymes with broad substrate specificity [12] referred to as glutathione *S*-transferases (GST, EC 2 5 1.18). Recently radiometric partition [13–15] and continuous spectrophotometric [16] assays have been developed that can distinguish between hydrolysis and glutathione conjugation of the same epoxides in cytosolic fractions. When *trans*- (TSO) and *cis*-stilbene oxide (CSO) are used, cEH and mEH also can be distinguished by their respective substrate specificities [14, 15] As the majority of reduced GSH is present in the cytosolic cell fraction, it is conceivable that conjugation with GSH or potentially other nonprotein sulfhydryls (NPS) could compete with hydrolysis of epoxides as a degradative pathway within this cell fraction Tests for this competition have seldom been carried out [13, 16], but they may be of importance when conducting *in vitro* assays for EH, particularly if significant GSH levels are preserved in the cytosolic cell fraction in question This competition could be of particular importance when comparing EH activities in different tissues or in tissues from control versus treated animals

Several studies have shown that mEH and GST can be induced by treatment with several diverse xenobiotics, which in general also induce the microsomal polysubstrate–mixed-function-oxidase system

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† Abbreviations EH, epoxide hydrolase, cEH, cytosolic epoxide hydrolase, mEH, microsomal epoxide hydrolase, GST, glutathione *S*-transferase, CSO, *cis*-stilbene oxide, TSO, *trans*-stilbene oxide, BPO, benzo[*a*]pyrene-4,5-oxide, GSH, glutathione, NPS, nonprotein sulfhydryl, and DEM, diethylmaleate

[1–3, 12] Recently, we have shown that the activities of cEH along with mEH and at least one form of GST are increased in liver and kidney of mice treated with clofibrate, a peroxisome proliferator [17–19] cEH activity was not increased by several other xenobiotics, many of which did increase the activities of mEH and/or a GST [17] Increases in hepatic cEH have also been demonstrated in male mice treated with di-2-ethylhexylphthalate, 2-ethylhexanol [17], and nafenopen [20] These four compounds are all peroxisome proliferators [21–24] This class of xenobiotics is known to cause an increase in the collective volume of hepatic and proximal tubular peroxisomes and to lower serum lipids [25, 26] All peroxisome proliferators so tested have also been found to cause hepatocellular carcinomas through non-genotoxic mechanisms [25–29]. Peroxisome proliferators are, at this time, the only compounds known to increase cEH activity significantly

cEH activity has been detected in the livers of several species, but special attention has been given to the mouse which has one of the higher specific activities [30]. Measurement of the comparative levels of this enzymatic activity in different tissues has been conducted in the mouse but no data are available for extrahepatic tissue in the rat [30] At this time we have compared the activities for hydrolysis of TSO (cEH specific), CSO and benzo[*a*]pyrene-4,5-oxide (BPO) (mEH specific) [31, 32], and for conjugation of CSO with GSH in cytosol and microsomes from the livers, kidneys, and testis of male adult control and clofibrate-fed rats The finding of significant levels of NPS in the cytosol from rat liver and testis is described along with appropriate modifications to our previously described radiometric partition assay for EH that overcome this presence of NSP in the cytosol

MATERIALS AND METHODS

Materials Clofibrate (ethyl-α-p-chlorophenoxyisobutyrate) was provided by Ayerst Laboratories (New York, NY) TSO, *cis*-stilbene, isooctane (99%), and hexanol (98%) were purchased from the Aldrich Chemical Co (Milwaukee, WI). CSO and radiolabeled CSO and TSO were prepared as previously described [14] *meso*-1,2-Diphenyl-1,2-ethanediol was purchased from MCB-Schuchardt (Cincinnati, OH). Radiolabeled and unlabeled BPO was prepared by Midwest Research Institute (Kansas City, MO) and provided by Richard Armstrong (Department of Chemistry, University of Maryland) respectively Bovine serum albumin (fraction V) and GSH were purchased from the Sigma Chemical Co. (St Louis, MO) ASC scintillation fluid was purchased from Amersham (Arlington Heights, IL) LK5DF Linear-K silica gel thin-layer chromatography (TLC) plates (250 μ, 20 × 20 cm) were purchased from Whatman Chemical Separation Inc (Clifton, NJ), and Bio-rad protein reagents were purchased from Bio-Rad Laboratories (Richmond, CA)

Animals, treatment, and preparation of cell fractions Male Sprague–Dawley rats (180–200 g, CD strain, Charles Rivers, Wilmington, MA) and Swiss–Webster mice (25–30 g, Bantin–Kingman, Fremont, CA) were housed in steel cages with kiln-dried pine shavings as bedding in an environmentally controlled room (23 ± 1 5°, 14 10 hr light dark, constant humidity) Food (Purina laboratory chow) and water were provided *ad lib* for the first week after which control and treated animals received diets of ground chow containing 5% corn oil or 0 5% clofibrate (w/w) dissolved in a similar concentration of corn oil respectively Following 2 weeks on the diets animals were killed (mice by cervical dislocation, rats by exsanguination while under light ether anesthesia) between 9 00 and 10 00 a m Tissues were removed, and livers were perfused with 1 15% KCl, rinsed placed into buffer, minced and weighed Tissue homogenates were then prepared (20-sec burst with Polytron) from individual tissues and used for the preparation of cell fractions Crude microsomal and cytosolic fractions were prepared in 76 mM sodium phosphate (pH 7 4) as previously described [19] and used fresh or stored at −70° (no longer than 30 days) prior to assays

Enzyme assays cEH, mEH, and GST activities were routinely assayed using our previously described radiometric partition assay [14, 15] In brief, protein was incubated with radiolabeled substrate (cEH, TSO at pH 7 4, mEH, CSO at pH 9 0, and GST, CSO with GSH at pH 7 4) at 37° for 10–30 min and the epoxide, or epoxide and diol, was preferentially extracted with isooctane and hexanol respectively As described in more detail in the Results, the partition assay for cytosol was modified by either a 10-min preincubation of the cytosol with 0 5 mM diethylmaleate (DEM) at room temperature or overnight dialysis of the cytosol against 76 mM sodium phosphate (pH 7 4) to remove NPS groups As an alternative procedure, product formation was determined using a modification of our previously described TLC method [13, 33] After incubation of cytosol with tritiated substrate, the reaction was stopped by addition of 50 μl of methanol containing 10 mg/ml cold *meso*-1,2-diphenyl-1,2-ethanediol and TSO or CSO to the 100-μl assay solution Samples were then vortexed and spun briefly, and the supernatant fraction was added to a slot on a LK5DF Linear-K silica gel TLC plate Diols, epoxides, and GSH conjugates were separated using toluene–propanol (19:1). Products were identified by fluorescent quenching, and spots were scraped and placed in ACS scintillation fluid for counting in a LKB 1217 Rackbeta liquid scintillation counter Location of substrate and products on the plates was confirmed by autoradiography

Other assays Protein was determined using a previously described modification of the Bradford method which is appropriate for reading assays with a Gilford EIA reader [19] Bovine serum albumin was used as standard NPS was detected in supernatant fractions of tissue homogenates and cytosol precipitated with 10% trichloroacetic acid using a modification of Ellman's procedure [34] Aliquots of 100 μl of the supernatant fractions were added to individual wells in EIA plates and mixed with 0 5 ml of 0 6 mM 5,5′-dithiobis(2-nitrobenzoic acid), absorbance was read at 405 nm GSH in 5% trichloroacetic acid was used as standard

Calculations and statistics Enzyme activities

routinely were expressed relative to protein content. When treated samples were being compared to controls, activities also were calculated per gram tissue and as total organ content. These latter values are only mentioned when they demonstrate changes in enzyme activity that may be due to changes in recovery of cell fraction protein or organ weight Significant differences between 14-day clofibrate-treated samples and controls were determined using Student's t-test, with $P = 0.05$ considered significant. Significant differences between groups of three or more values were determined using a one-way ANOVA When nonequality at the 0.05 level was found, samples were ranked using Duncan's multiple range test [35] Correlations between rates of hydrolysis of epoxides in the three different tissues of control and treated rats were determined using previously described methods [36, 37] In brief, a correlation (e.g between TSO hydrolysis in cytosol and CSO hydrolysis in microsomes) was determined after matching the individual activities (nmoles/min/mg protein) from liver, kidney and testis of control and clofibrate-fed rats This procedure allowed a matching of thirty-six different samples with a considerable range of activity suitable for this analysis. Correlation coefficients, 95% confidence intervals, and significant differences were calculated as previously described [36].

RESULTS AND DISCUSSION

Epoxides are commonly metabolized by hydrolysis or conjugation with GSH. The latter pathway usually can be excluded in assays for EH by omission of GSH. In crude cytosols used for enzyme assays, NPS content was not consistently similar to values for rapidly processed fresh tissue (Table 1, see also Refs. 34 and 38). In particular, cytosolic levels of NPS in mouse liver and rat kidney were much lower, testis slightly lower, and rat liver slightly higher than values for fresh whole tissue homogenates (Table 1). This tissue-dependent decrease in NPS levels could not be explained by variation in the pH of the tissue fraction, as cytosols were consistently at a pH of 7 0 to 7 4 (data not shown). Retention of buffer pH throughout tissue fractionation and storage precludes significant formation of additional GSH from non-enzymatic reduction of glutathione-disulfide which may occur with low pH buffers or tissues prepared in non-buffered sucrose

When cytosol or boiled cytosol, fortified with GSH, was incubated at 37°, a marked time-dependent decrease in NPS was seen in cytosol from mouse liver and rat kidney, a moderate decrease in rat testis cytosol, and no change in rat liver cytosol (Fig. 1) This suggests that the retention or depletion of GSH and other NPS groups in cytosolic fractions was dependent upon heat-labile factors. This finding is consistent with known rates of GSH turnover in rat liver and kidney, but not mouse liver [39, 40] NPS levels also may be increased artifactually in cytosolic fractions due to blood GSH in poorly perfused tissue. Regardless of the source of cytosolic NPS, it could prove to be an important consideration in comparing EH activity in the different tissues.

Our previously described radiometric partition assay offers several advantages over other assays for cEH, mEH, and GST [14, 15]. The speed and sensitivity of the assays, the low volatility of the substrates and products, and the ability to measure three enzyme activities with geometric isomers, TSO and CSO, are three major attributes of these assays. While cEH and mEH are differentiated by substrate specificity, pH optima, and, in part, by tissue subfractionation, differentiation of EHs from GST depends upon preferential extraction of the diols and epoxides by hexanol [14] For EH assays the epoxides are extracted with isooctanol or dodecane Thus, as suggested previously [13–15], in cytosol containing high levels of NPS, the single solvent extraction failed to separate diols from conjugates which could interfere with the single partition assay (Fig 2) When cytosols from rat liver, kidney, and testis were incubated with radiolabeled TSO and CSO, product formation (i.e nonextracted radioactivity) in rat liver and testis, but not kidney, was decreased by preincubation of the cytosol with DEM or by dialysis of the cytosol. The effect of DEM was concentration dependent with maximal effect occuring at 0.5 mM (Fig. 2). The effect of preincubation with DEM or dialysis of the

Table 1 Relative tissue weight and nonprotein sulfhydryl (NPS) content in fresh homogenates and aged cytosol from tissues of control and clofibrate-fed rats

Tissue	Treatment	Relative tissue weight (g/100 g body wt)	NPS (mM) Fresh homogenate	NPS (mM) Aged cytosol
Liver	Control	4 20 ± 0 07	3 04 ± 0 15	4 63 ± 0 31
	Clofibrate	6 09 ± 0 12*	3 70 ± 0 32	6 81 ± 0 38*
Kidney	Control	0 86 ± 0 03	2 66 ± 0 11	0 20 ± 0 02
	Clofibrate	0 97 ± 0 04*	2 56 ± 0 04	0 34 ± 0 08
Testis	Control	1 04 ± 0 08	3 57 ± 0 08	1 52 ± 0 07
	Clofibrate	1 08 ± 0 06	2 94 ± 0 05*	0 83 ± 0 14*
Mouse liver	Control	6 11 ± 0 33	8 41 ± 0 12	0 50 ± 0 18

Tissues were removed from rats and mice as described in Methods Homogenates prepared within 60 min and cytosol fractions after storage for at least 3 days were used for NPS determination NPS content is presented as molarity in tissue studied Values are mean ±S E of at least four samples

* Significantly different from control, $P < 0\ 05$

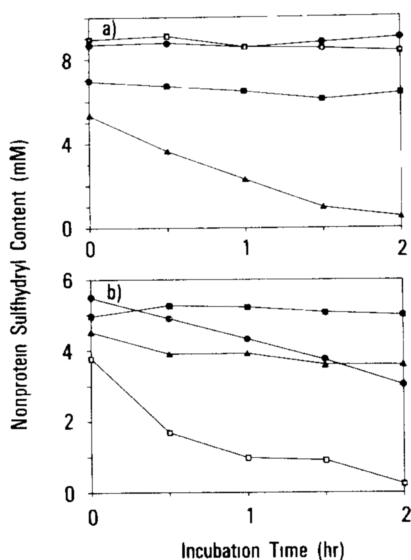

Fig 1 Variations in NPS content in cytosolic fractions during icubation at 37° Sufficient GSH was added to fresh and boiled cytosols to approximate the content in fresh tissue Fresh and boiled cytosols were then incubated at 37°, and aliquots were removed and mixed 1 1 with 10% trichloroacetic acid for NPS determination at the times indicated Key (a) rat fresh (●) and boiled (□) and mouse fresh (△) and boiled (■) liver cytosol, (b) rat kidney fresh (□) and boiled (●) and testis fresh (△) and boiled (■) cytosol Values are the means of two separate cytosols each assayed in duplicate

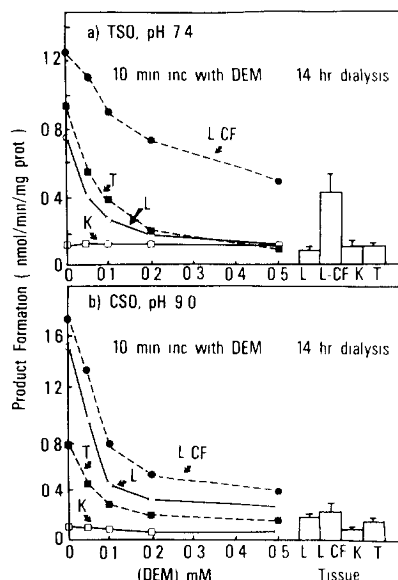

Fig 2 Effect of DEM or overnight dialysis on the apparent EH activity in rat tissue cytosol *Left* cytosols were diluted with the appropriate buffer and then preincubated at room temperature for 10 min with DEM at the final concentration noted *Right* cytosols were dialyzed overnight against 76 mM sodium phosphate (pH 7 4) and then diluted with the appropriate buffer Reactions were started by addition of radiolabeled (a) TSO to cytosols at pH 7 4 or (b) CSO to cytosols at pH 9 0 and incubated for 20 min at 37° The reactions were stopped by addition of 2 vol of isooctane followed by vortexing, with resultant extraction of all the epoxide After phase separation, aliquots of the aqueous phase were then taken for scintillation counting Key control rat liver cytosol (L, ○—○), clofibrate-fed rat liver cytosol (L–CF, ●--●), control rat kidney cytosol (k, □—□), and control rat testis cytosol (T, ■--■) Values are the mean of two separate determinations each performed in triplicate

cytosol correlates well with the level of NPS found in the respective cytosols These results suggest that NPS conjugates were being formed in cytosol from rat liver and testis These conjugates would remain in the aqueous phase along with the diols formed from hydrolysis of the TSO and CSO The above experiments do not exclude the possibility that DEM and dialysis may inhibit epoxide hydrolysis in the cytosol.

To resolve these questions, radiolabeled TSO and CSO were incubated with cytosol from rat liver, kidney, testis and mouse liver, and products and substrates were separated by TLC The GSH conjugates remained at the origin in this separation system while the diols and epoxides were separated as previously described for *trans-β*-ethylstyrene oxide [13]. Under these conditions, it was evident that neither preincubation with 0 5 mM DEM nor overnight dialysis of the cytosols inhibited diol formation from TSO or CSO in any of the tissues studied as previously shown for TSO hydrolysis in mouse liver cytosol [16]. The presence of GSH had no effect on TSO hydrolysis in any of the tissues studied as previously shown for *trans-β*-ethylstyrene oxide in mouse liver cytosol [13] Inclusion of 5 mM GSH, however, stimulated and inhibited the hydrolysis of CSO in rat liver and kidney respectively (Fig 3) Furthermore, conjugate formation correlated with cytosol content of NPS groups with the greatest amounts formed in rat liver followed by rat testis, rat kidney, and mouse liver for both TSO and CSO (Fig 3) These results confirm that the increased aqueous radioactivity produced from incubates of rat liver and testis cytosol with the two epoxides arose from inclusion of conjugates with NPS groups

As seen with CSO hydrolysis, competition of GSH for hydrolysis may be substrate, tissue, or species specific. Therefore, even with assays that separate products, the cytosolic NPS content must be considered. Both 10-min preincubation of the diluted cytosol with DEM and overnight dialysis of cytosol have now been shown to be effective modifications of the radiometric partition assay for EH in tissues containing significant amounts of NPS groups

With suitable one-step assays now available to measure EH in the rat, cEH, mEH, and GST activities were compared in cytosol and microsomes from liver, kidney, and testis of control and clofibrate-fed rats Treatment resulted in increased liver and kidney weight with no effect on testicular weight (Table 1) No significant changes were noted in cytosolic or microsomal protein contents (data not shown) These findings are compatible with previous studies on the effects of clofibrate on rat tissue [25, 26, 41] and provide the necessary data for approximation of total organ changes in the enzymatic activities to be described

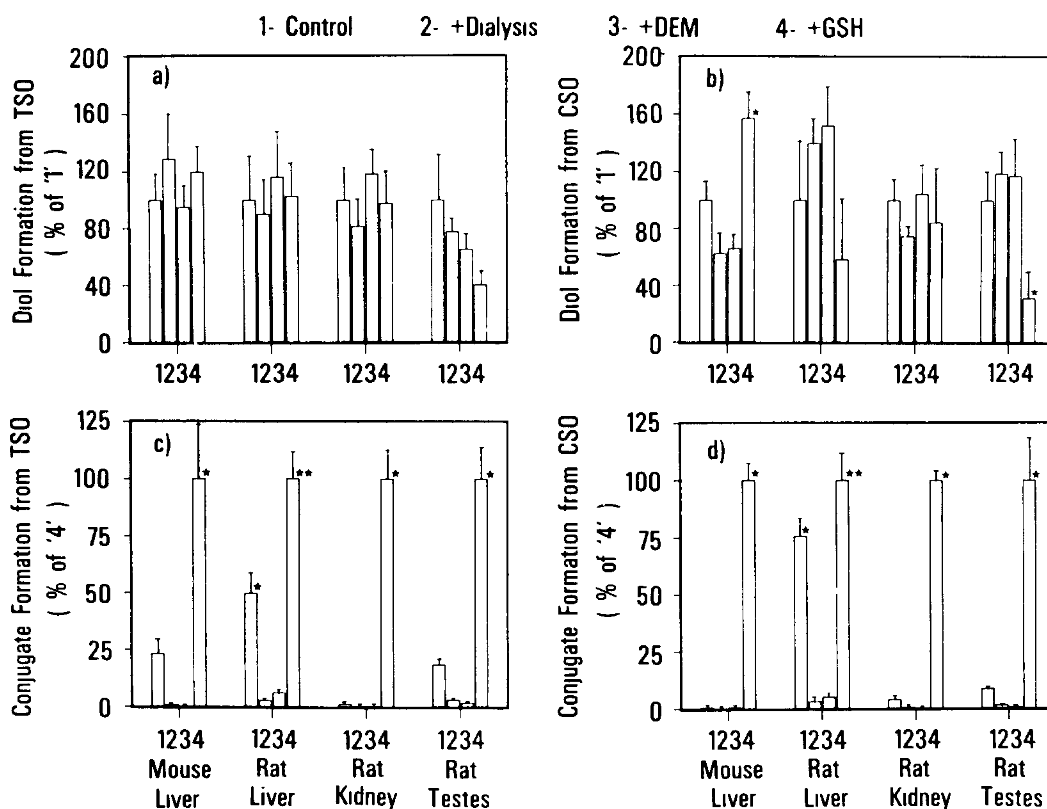

Fig 3 Effect of dialysis, DEM and GSH on product formation Cytosols were incubated as described in Fig. 2 with (1) no additions, (2) overnight dialysis, (3) 10-min preincubation with 0 5 mM DEM, and (4) with addition of 5 mM GSH. Reactions were stopped by addition of 0 5 vol of methanol containing cold diol (10 mg/ml), and aliquots were spotted on TLC plates and developed as described in Methods (a) Diol formation from TSO, (b) diol formation from CSO, (c) conjugate formation from TSO, and (d) conjugate formation from CSO. Values are the mean + S E of three separate determinations each performed in triplicate Values from the same tissue with a similar number of asterisks are not significantly different, $P < 0.05$

The hydrolysis of TSO, specific for cEH, was detectable in cytosol from all three tissues with the greatest amount of activity in the kidney followed by liver and then testis (Table 2). Treatment of rats with clofibrate resulted in significant increases in TSO hydrolysis in liver and kidney cytosol to 506 and 171% of controls respectively. As also shown for the mouse [18], the clofibrate-mediated increase in cEH activity shows a similar tissue distribution to peroxisome proliferation [41]

Activity comparable to cEH has also been detected in microsomes from mouse liver [42], kidney and testis [18] In the rat, TSO hydrolysis at pH 7.4 (i.e cEH-like activity) was detectable in liver, but not kidney or testis, microsomes (Table 2). Clofibrate treatment increased this activity to 126% of controls. While convincing evidence has been presented for cEH-like activity in the microsomes from mouse liver [42], the present data do not substantiate the presence of similar activity in the microsomes of rat tissue. First, TSO hydrolysis in rat microsomes is only 1–2% of CSO hydrolysis compared to 8–10% in mouse liver [18, 42]. Second, while TSO hydrolysis has been detected in four different mouse tissues [18], it was detectable only in liver microsomes of the rat. It should be noted that CSO hydrolysis was much higher in rat liver than kidney or testis microsomes Third, whereas both microsomal and cytosolic hydrolysis of TSO respond in similar fashion in mice treated with clofibrate [18, 19], in rat liver microsomes the increase was more similar to CSO hydrolysis Therefore, unlike the situation in mouse tissue, TSO hydrolysis in rat liver microsomes may arise from slow turnover by mEH

Conjugation of CSO to GSH was detectable in the cytosol from all three rat tissues (Table 2) with highest activity in the testis and lowest in the kidneys Similar testis to liver ratios have been reported for the cytosolic conjugation of 1-chloro-2,4-dinitrobenzene and *p*-nitrobenzylchloride, while conjugation of styrene-7,8-oxide is lower in testis than liver [43–45] The ratio of CSO conjugation in kidney to liver is much greater than for the above-mentioned substrates [43–45] Whether this tissue distribution of CSO conjugation is due to isozyme specificity [12] remains to be determined. GST activity towards CSO also was found in liver and kidney microsomes at approximately 20% of the level (per mg protein) in cytosol (Table 2) Previous studies have described the microsomal localization of GST in rat liver [46],

Table 2 Effect of dietary clofibrate on activity associated with cytosolic epoxide hydrolase (cEH) and glutathione S-transferase (GST)

Tissue	Treatment	cEH (TSO, pH 7 4) Dialyzed cytosol (pmoles/min/mg protein)	cEH (TSO, pH 7 4) Microsomes (pmoles/min/mg protein)	cGST (CSO + GSH pH 7 4) Dialyzed cytosol (pmoles/min/mg protein)	cGST (CSO + GSH pH 7 4) Microsomes (pmoles/min/mg protein)
Liver	Control	107 ± 9	354 ± 29	10 6 ± 0 6	1 8 ± 0 3
	Clofibrate	541 ± 89*	445 ± 10*	21 2 ± 5 3*	3 6 ± 0 6*
		(5 06)†	(1 26)	(2 00)	(2 03)
Kidney	Control	120 ± 22	ND‡	6 4 ± 0 6	1 3 ± 0 1
	Clofibrate	206 ± 26*	ND	11 1 ± 1 6*	2 7 ± 0 2*
		(1 71)		(1 73)	(2 02)
Testis	Control	102 ± 1	ND	13 7 ± 1 0	ND
	Clofibrate	98 ± 2	ND	13 0 ± 1 0	ND
		(0 96)		(0 95)	

Male rats were fed diets containing 0 5% (w/w) clofibrate in ground chow or ground chow only for 14 days Tissues were prepared and assays were conducted as described in Methods Values are the mean ±S E for four rats

* Significantly different from control, P < 0 05

† Values in parentheses represent ratio of treated to control

‡ ND, not detectable

and similar activity in extrahepatic microsomes has now been found Dietary clofibrate treatment resulted in an approximate doubling of activity in cytosol and microsomes from liver and kidney, but not testis (Table 2) A similar response in cytosolic CSO conjugation is also found in mouse cytosols [18, 19], but is substrate dependent as a decrease in 1,2-dichloro-4-nitrobenzene is also found [17] Zuccato *et al* [47] also found a substrate-dependent response of GST to another peroxisome proliferator, di-2-ethylhexylphthalate. These findings suggest that the response of GST to clofibrate and other peroxisome proliferators may be isozyme specific.

Hydrolysis of CSO and BPO is associated primarily with mEH [31, 32]. Hydrolysis of both epoxides was readily detectable in microsomes from all three tissues The micromal activities were tissue dependent, however, so that hydrolysis of CSO was fastest in liver with similar activities in kidney and testis microsomes, whereas hydrolysis of BPO was fastest in liver, intermediate in kidney, and slow in testis microsomes (Table 3) Substrate-dependent differences in the tissue distribution of mEH have also been noted when estroxide, androstene oxide [48], and styrene-7,8-oxide [44, 45] are used as substrates Treatment with clofibrate resulted in increases in liver mEH for CSO and BPO of 149 and 126% of controls respectively This is similar to the increase in hydrolytic rate of octene-1,2-oxide to 131% of controls reported by Levin *et al* [7] In addition, the rates of hydrolysis of CSO and BPO in kidney microsomes were 172 and 154% of controls, respectively, whereas no changes were seen in testis microsomes (Table 3) It is of interest that in con-

Table 3 Effect of dietary clofibrate on activities associated with microsomal epoxide hydrolase (mEH)

Tissue	Treatment	Microsomes CSO (nmoles/min/mg protein)	Microsomes BPO (nmoles/min/mg protein)	Dialyzed cytosol CSO (pmoles/min/mg protein)	Dialyzed cytosol BPO (pmoles/min/mg protein)
Liver	Control	23 9 ± 2 0	31 6 ± 1 5	104 ± 2	340 ± 27
	Clofibrate	35 6 ± 3 0*	39 8 ± 2 8	160 ± 5*	367 ± 17
		(1 49)†	(1 26)	(1 54)	(1 08)
Kidney	Control	2 1 ± 0 1	16 1 ± 0 4	81 ± 4	93 ± 13
	Clofibrate	3 6 ± 0 2*	24 8 ± 0 9*	100 ± 4*	187 ± 13*
		(1 72)	(1 54)	(1 24)	(2 00)
Testis	Control	3 5 ± 0 1	6 1 ± 0 4	282 ± 3	266 ± 42
	Clofibrate	3 4 ± 0 4	5 5 ± 0 1	258 ± 2*	333 ± 70
		(0 96)	(0 90)	(0 91)	(1 25)

Rats were treated and tissue was prepared as described under Table 2 mEH was assayed with both *cis*-stilbene oxide (CSO) and benzo[*a*]pyrene-4 5-oxide (BPO) as substrates at pH 9 0 Values are mean ±S E of four rats

* Significantly different from control, P < 0 05

† Values in parentheses are ratios of treated to control

trast to cEH and GST, the highest increase in mEH activity occurred in the kidney for both substrates

Hydrolysis of both substrates was also detectable in cytosol from rat liver, kidney and testis, as previously shown with mice [18]. In mice and rats, CSO (at pH 9.0) is slowly turned over by purified cEH at approximately 2–5% the rate of TSO hydrolysis at pH 7.4 ([49]; M. H. Silva and B. D. Hammock, unpublished data) In mouse, but not rat, cytosol, the ratio of TSO to CSO hydrolysis is so high that corrections are made for the slow turnover of CSO by cEH [18, 19]. Similar corrections in rat cytosols would not change the reported activities more than 5% and were therefore omitted from this study Hydrolysis of CSO was fastest in testicular cytosol with slower activities in liver and kidney cytosol, and it was increased in liver and kidney cytosol to 154 and 124% of controls after clofibrate treatment In contrast, the cytosolic hydrolysis of BPO was fastest in liver with successively slower activities in testis and then kidney BPO hydrolysis was increased by clofibrate treatment only in the kidney cytosol (Table 3) These results clearly indicate that differential hydrolysis of epoxides occurs in different rat tissue compartments

As a further test of this supposition, correlation between rates of hydrolysis of TSO, CSO and BPO in microsomes and cytosol was determined Rates of hydrolysis of a single substrate within cytosol (c) or microsomes (m) in all three tissues (N = 3) from control (N = 6) and clofibrate-fed (N = 6) rats were treated as a single group (N = 36), and the individual values for cTSO, cCSO, cBPO, mCSO and mBPO were matched to arrive at the correlation coefficients (Table 4). Data from our previous report on EH in tissue from control and clofibrate-fed mice [18] were treated in a similar fashion for comparative purposes. This correlation study required combination of activities from three different tissues of control and treated rats in order to study a suitable population size This procedure necessitates the assumption that similar enzymes are responsible for hydrolysis of a single epoxide in each tissue before and after clofibrate treatment. Little, or no, data are available at this time to refute or confirm this assumption The significance of these findings is therefore difficult to interpret at this time, but may be useful when the highest and lowest correlations are inspected The rates of hydrolysis of CSO and BPO in microsomes by a single (or closely related family) enzyme, mEH, are well established [1–5, 31, 32, 50], and the high correlation between hydrolysis of these two epoxides (0 83 in rats, 0.96 in mice) comes as no surprise The distinction of cEH from mEH and its specificity for TSO hydrolysis are also well established [4, 5, 14, 31, 32, 49, 50], yet intermediate coefficients were found between rates of hydrolysis of the substrates for these two enzymes The lowest correlation in both species was between hydrolysis of cTSO and cCSO (−0 04 in rats, 0 41 in mice) Previous papers have reported evidence for an mEH-like activity in the cytosol of several species [8–11, 18, 19] and, recently, TSO hydrolysis has been separated from CSO hydrolysis in mouse liver cytosol by means of an affinity-column specific for cEH [49] Further work is required to identify and characterize this putatively distinct EH in cytosol

The preceding studies have considered several questions concerning epoxide-metabolizing enzymes in rat tissue A technological difficulty arising from retention of NPS groups in rat liver and testis has been identified, and existing partition assays for EH have been modified to deal with this problem The tissue distribution of hydrolysis and GSH conjugation of previously unstudied epoxides has now been carried out in cytosol and microsomes of rat liver, kidney and testis which demonstrated that, while cEH in rat liver is lower than in mouse liver, comparable activities exist in testis and kidney, and GST, but not cEH-like, activity can also be found in kidney microsomes Additional evidence is presented that an mEH-like enzyme, distinct from cEH,

Table 4 Correlation between values for hydrolysis of epoxides in cytosol and microsomes from liver, kidney, and testis of control and clofibrate-fed rats and mice

Male rats		Male mice*	
Activities compared	Correlation coefficient	Activities compared	Correlation coefficient
mCSO vs mBPO	0 826†	mCSO vs mBPO	0 964†
cCSO vs mBPO	−0 529†	cTSO vs cBPO	0 816†
cTSO vs mBPO	0 526†	cCSO vs mCSO	0 734†
cCSO vs cBPO	0 518†	cBPO vs mBPO	0 721†
cBPO vs mCSO	0 500†	cBPO vs mCSO	0 703†
cTSO vs mCSO	0 418†	cCSO vs mBPO	0 679†
cCSO vs mCSO	−0 172	cCSO vs cBPO	0 518†
cBPO vs mBPO	0 121	cTSO vs mCSO	0 509†
cTSO vs cBPO	0 078	cTSO vs mBPO	0 476†
cTSO vs cCSO	−0 038	cTSO vs cCSO	0 410†

Activities for TSO, CSO, and BPO hydrolysis in cytosol (c) and CSO and BPO in microsomes (m) from liver, kidney, and testis of six control and six clofibrate-fed rats or mice were paired (N = 36) and the ten possible correlation coefficients were determined as previously described [21] Coefficients are listed in decreasing order from unity

* Data for mice tissue were described previously [11]

† Correlation coefficient is significantly different from zero, $P < 0.05$

can be detected in cytosol, and the responsiveness of these activities to clofibrate treatment has now been documented in the male rat The increase of cEH activity in response to peroxisome proliferators was found to be even greater in rat than in mouse cytosol. The relationship of the increase in cEH activity to peroxisome proliferation, a unique organelle response associated with hypolipidemia and carcinogenesis, is not yet resolved Due to the lack of knowledge concerning the mode of action of these chemicals and the potential of cEH substrates for toxic action, this question certainly merits further consideration

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