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ASSESSMENT OF RAT LIVER MICROSOMAL EPOXIDE HYDROLASE AS A MARKER OF HEPATOCARCINOGENESIS

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Abstract—The influence of eleven xenobiotics on the activity and amount of hepatic microsomal epoxide hydrolase was determined. Activity was assayed using three different substrates after rats were fed, throughout 3 weeks, diets containing one of six hepatocarcinogens, viz. 2-acetylaminofluorene, 3′-methyl-4-dimethylaminoazobenzene, 4′-fluoro-4-dimethylaminoazobenzene, thioacetamide, aflatoxin B_1 and ethionine. Five hepatocarcinogens induced activity 4- to 10-fold; ethionine was relatively ineffective as an inducer. Two non-carcinogenic analogues of hepatocarcinogens, viz. fluorene and *p*-aminoazobenzene, caused no appreciable increase in enzyme activity, but phenobarbital, barbital and 1-naphthylisothiocyanate induced activity 2- to 3-fold. All eleven xenobiotics increased the amount of microsomal epoxide hydrolase 2- to 9-fold when examined immunochemically using either a radial diffusion assay or an enzyme-linked immunosorbent assay (ELISA). Serum glutamic oxaloacetic acid transaminase activity was not appreciably elevated by feeding ten of the xenobiotics, suggesting that inductions were not owing to toxicity. Using ELISA, microsomal epoxide hydrolase was detected in post-microsomal (PM) supernatant fractions from control rat liver, thus confirming an earlier report by Gill *et al.* [*Carcinogenesis* **3**, 1307 (1982)]. The eleven xenobiotics induced the amount of ELISA-detectable antigen in PM supernatant fractions by 3- to 34-fold. Longer centrifugation of PM supernatant fractions yielded a pellet fraction that contained 92 ± 1.2% of the ELISA-detectable antigen irrespective of the xenobiotic regimen. Relationships between xenobiotic induction of microsomal epoxide hydrolase activity and amount and hepatocarcinogenesis are discussed.

Hyperplastic nodules, induced in rat liver by chronic feeding of 2-acetylaminofluorene (AAF) [1], are believed to be the cellular precursors of some hepatomas [1–7]. Compared to normal rat liver, nodules show enzyme deficiencies and excesses [8–12]. Among these enzymatic changes, Farber [12] recognized patterns that characterized several different focal lesions, viz. small foci which had low epoxide hydrolase activity but high γ-glutamyl transpeptidase activity [13] and larger nodules which had high activities for both enzymes but were either persistent or non persistent. Non-persistent nodules remodeled with appropriate, concomitant reversals of enzyme activities [13–15], while persistent nodules proliferated, became basophilic, and appeared to progress to hepatocellular carcinoma [15]. Farber [12] believed it "very likely that . . . most changes induced in target cells by 'carcinogens' have no direct relevance to cancer." Although perturbation of epoxide hydrolase activity was prominent in the schema of AAF-induced hepatic cancer, i.e. observed following 7 days of chronic feeding [16] and throughout nodule and hepatoma development [13–15], the relevance of this change is open to question. Reid [17] postulated that changes induced in target tissues by carcinogens but not by non-carcinogenic analogues might prove to be "key changes" in carcinogenesis. Accordingly, we tested whether several hepatocarcinogenic and non-hepatocarcinogenic xenobiotics induced hepatic microsomal epoxide hydrolase after short-term chronic feeding in a manner similar to that seen with AAF [16].

Farber and associates [7, 18, 19] and Okita *et al.* [20] detected by immunodiffusion against back-absorbed anti-nodule rabbit serum an antigen in microsomes from hyperplastic nodules and primary hepatomas that did not diffuse from normal microsomes; they called it preneoplastic (PN) antigen. An important feature of PN antigen lay with the observation that some proteins in microsomes from hyperplastic nodules and hepatomas dissociated more readily from the lipid bilayer than similar proteins in microsomes from normal rat liver [7, 18–20]. Among proteins that readily dissociated was PN antigen [21, 22], later identified as the microsomal enzyme epoxide hydrolase [16]. Recently, Gill *et al.* [23], using an enzyme-linked immunosorbent assay (ELISA), observed low levels of microsomal epoxide hydrolase in post-microsomal (PM) supernatant fractions from normal rat liver. We confirm their finding

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and report further that hepatocarcinogens markedly induce the level of ELISA-detectable microsomal epoxide hydrolase antigen in PM supernatant fractions.

MATERIALS AND METHODS

Male Holtzman rats were housed from birth in rooms with 12-hr light cycles beginning at 7:00 a.m. Until used for experiments, they were fed Purina Rat Chow. Rats on experiments were fed a semi-synthetic diet [24] alone or supplemented with 0.0012% aflatoxin B_1 (Afl); 0.05% AAF; 0.06% 3'-methyl-4-dimethylaminoazobenzene (3M), 4'-fluoro-4-dimethylaminoazobenzene (4F), *p*-aminoazobenzene (AB), fluorene (Fl); 0.07% thioacetamide (TA); 0.08% 1-naphthylisothiocyanate (NIT); or 0.3% ethionine (Eth). Barbital (Bar) and phenobarbital (PB) were added to the drinking water at 0.05%.

Rats were killed by cervical dislocation. Livers were quickly removed, perfused, and homogenized as previously described [21]. Microsomes were sedimented from post-mitochondrial supernatant fraction by centrifugation [20]. Cytosols were carefully aspirated to the top of the pellets; tubes were inverted to drain. Pellets were resuspended and washed once with 50 mM Tris–HCl, 24 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose, pH 7.5 (TKMS) buffer [22]. Washed pellets were stored in this buffer at −70°. In several experiments, PM supernatant fractions were centrifuged an additional 5.0×10^7 *g*-min (50 Ti Rotor, Beckman Instruments, Inc., Irving, CA) to yield a pellet which we called the low-density particulate (LDP) fraction.

Arene oxide hydrolase activity was assayed using [G-^{3}H]phenanthrene 9,10-oxide (7.4 mCi/mmole) or [11, 12-^{3}H]-benzo[*a*]pyrene 11,12-oxide (5.8 mCi/mmole) as substrates [25]. Styrene oxide hydrolase activity was determined by the procedure of Oesch *et al.* [26] using [7-^{3}H]styrene oxide (0.3 mCi/mmole) as substrate.

Epoxide hydrolase was purified by a modification of the procedure described by Guengerich *et al.* [27]. Microsomes were solubilized with Luberol PX, and were chromatographed on diethylaminoethyl cellulose (DE-52) and carboxymethyl cellulose (CM-52); both chromatography steps were performed in glycerol (20%). Final purification was achieved by chromatofocusing in 5% glycerol on PBE-94 using a pH gradient from 9 to 6; the enzyme eluted between pH 8.4 and 8.2. This preparation showed a single band on sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and analytical isoelectric focusing after staining the gels with Coomassie Blue or silver nitrate. The amino acid composition of this preparation was similar to that reported earlier [27, 28] and had a specific activity of 870 nmoles styrene glycol formed/min/mg protein.

Antibody to purified epoxide hydrolase was developed in a New Zealand rabbit by twice-monthly injections with 100 μg of purified enzyme emulsified in Freund's complete adjuvant. The rabbit antiserum was specific, e.g. it precipitated a single 50K band as analyzed by SDS-PAGE radioautography after *in vitro* translation using radiolabeled amino acids [29].

Radial immunodiffusion analysis was performed by the method of Thomas *et al.* [30]. ELISA assays were performed according to the procedure supplied by Kirkegaard and Perry Laboratories (Gaithersburg, MD) using 1/50,000 diluted anti-epoxide hydrolase rabbit serum as the primary antibody and goat anti-rabbit immunoglobulin serum conjugated with horseradish peroxidase conjugate as the second antibody. Quantitative readings of color intensity from 2,2-azinobis[3-ethyl-benzthiazoline-6-sulfonate] substrate were obtained by use of the Microelisa Autoreader (Dynatech, Alexandria, VA), and the primary antibody was inhibited by standard pure protein or unknown mixtures.

Lipids were extracted from microsomes and from the LDP fraction using the procedure of Folch *et al.* [31]. The phosphate content of these extracts was determined by the method of King [32]. Protein was determined by the method of Lowry *et al.* [33] using purified bovine serum albumin as a standard. Radioactivity was determined in a liquid scintillation system [34, 35]. Serum glutamic oxaloacetic acid transaminase (SGOT) activity was determined using a Sigma kit (St. Louis, MO) based on the method of Karmen [36]. Activity was expressed in International Units/ml (25°).

The carcinogens 2-AAF, Afl, Eth and TA were purchased from the Aldrich Chemical Co. (Milwaukee, WI), the Calbiochem-Behring Corp. (La Jolla, CA), the Sigma Chemical Co. (St. Louis, MO), and the J. T. Baker Chemical Co. (Phillipsburg, NJ) respectively. NIT was obtained from the Eastman Kodak Co. (Rochester, NY), while AB and Fl were obtained from ICN Pharmaceuticals, Inc. (Cleveland, OH). The two azo dyes, 3M and 4F, were synthesized according to the procedure of Giese *et al.* [37]. Labeled styrene oxide was synthesized from bromo-acetophenone by reduction with sodium borohydride in the presence of ^3H_2O and cyclization in 1 N NaOH. The reaction mixture was applied to a Florisil column in hexane, and [7-^{3}H]styrene oxide was eluted with 75% hexane: 25% diethyl ether. Florisil was purchased from the J. T. Baker Chemical Co. Chromatofocusing gel PBE-94 and polybuffers 96 and 74 were purchased from the Pharmacia Co. (Uppsala, Sweden). Goat anti-rabbit immunoglobulin horseradish peroxide conjugate was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN).

RESULTS

Influence of xenobiotics on microsomal epoxide hydrolase activity. Since it was shown previously that 7 days of AAF-feeding caused a marked increase in hepatic microsomal epoxide hydrolase activity [16], we did a preliminary study to determine the time-course of enzyme induction over a 3-week period. The activity was induced nearly 2-fold at 3 days, viz. a specific activity of 22 for AAF-fed versus a specific activity of 13 for basal-fed, while at 7 and 14 days, specific activities were 3-fold higher. Induction appeared to be maximum at 3 weeks, so this time was chosen for chronic feeding of xenobiotics.

The influence of chronic feeding of the eleven xenobiotics on microsomal epoxide hydrolase

Table 1. Influence of xenobiotics on the activity of microsomal epoxide hydrolase using three different substrates

Xenobiotic*	Enzyme activity (nmoles/min/mg protein) SO†	Ratio‡	PO	Ratio	BPO (Act) (10)§	Ratio	Average ratio‖
None	12 ± 1**		21 ± 2		2 ± 0.2		
3M	93 ± 16	8	241 ± 12	12	20 ± 0.2	10	10 ± 1.2
AAF	60 ± 5	5	211 ± 10	10	17 ± 0.2	9	8 ± 1.5
4F	43 ± 2	4	164 ± 8	8	16 ± 0.2	8	7 ± 1.3
TA	40 ± 5	3	111 ± 3	5	10 ± 0.2	5	4 ± 0.7
Afl	30 ± 3	2	96 ± 11	5	8 ± 0.2	4	4 ± 0.9
Eth	17 ± 3	1	33 ± 5	2	3 ± 0.5	1	1 ± 0.3
PB	38 ± 3	3	††		††		3
Bar	27 ± 4	2	70 ± 8	3	6 ± 0.2	3	3 ± 0.3
Fl	26 ± 9	2	27 ± 5	1	2 ± 0.3	1	1 ± 0.3
NIT	16 ± 2	1	51 ± 1	2	5 ± 0.3	3	2 ± 0.6
AB	14 ± 2	1	21 ± 1	1	3 ± 0.2	2	1 ± 0.3

* Xenobiotics were added to a semi-synthetic diet [24] and fed continuously throughout 21 days. Abbreviations are identified in Materials and Methods.

† SO, PO and BPO refer to [7-^{3}H]styrene oxide, [G-^{3}H]phenanthrene 9,10-oxide and [11,12-^{3}H]benzo[*a*]pyrene 11,12-oxide respectively.

‡ Ratios were $\bar{x}$-value for a particular xenobiotic divided by $\bar{x}$-value for None.

§ Each value was multiplied by 10 for convenience in tabulation.

‖ Values are $\bar{x} \pm$ S.E. for ratios with SO, PO and BPO as substrates.

** Values are $\bar{x} \pm$ S.E. for determinations made on five male rats.

†† Not determined.

activity when assayed using styrene oxide (SO), phenanthrene 9,10-oxide (PO), or benzo[*a*]pyrene 11,12-oxide (BPO) as substrate is recorded in Table 1. Five of six hepatocarcinogens induced enzyme activity an average of 4- to 10-fold; ethionine was a less efficient inducer. Three of five non-hepatocarcinogenic xenobiotics, viz. PB, Bar and NIT, induced enzyme activity an average of 2- to 3-fold; Fl and AB were generally less efficient inducers. Activities determined using PO and BPO as substrates yielded xenobiotic/none ratios that were similar in most instances. Activities determined using SO as substrate yielded ratios that compared reasonably well with PO- and BPO-ratios in several instances, viz. 3M, TA, Eth, Bar, Fl, and AB, but in other instances, SO-ratios were about half PO- and BPO-ratios, viz. AAF, 4F, Afl, and NIT. Substrate-dependent differences in enzyme activity following xenobiotic administration were observed previously [27] and were interpreted as evidence for multiple forms of microsomal epoxide hydrolase [27, 38].

Cutler [39] demonstrated correlations between liver damage visible histologically and increases in SGOT activity. Recently, it was reported that rats given AAF in choline-devoid diets [40] or those given acute injections of *N*-hydroxy-AAF [41] showed appreciable elevations in SGOT activity. To determine whether hepatotoxicity developed during the feeding regimes, we assayed SGOT activities among rats after feeding ten of eleven xenobiotics (phenobarbital was not tested) throughout 21 days. Only two of the xenobiotics caused significant changes in SGOT activity, viz. 3M caused significant elevation in activity, while Bar caused a small decrease. Since the increase caused by 3M was modest, i.e. 1.5-fold compared to a 20-fold increase seen with a toxic dose of CCl_4 (1 ml/kg body wt), it appears that hepatotoxicities associated with xenobiotic feeding had not developed sufficiently to cause appreciable enzyme leakage and/or hepatocyte lysis.

Influence of xenobiotics on enzyme amount. Two immunoassay methods were used to determine the amount of epoxide hydrolase antigen in hepatic microsomes after 3 weeks of xenobiotic feeding, a radial diffusion [30] and an ELISA method. The data are listed in Table 2. Hepatocarcinogens increased the amount of epoxide hydrolase an average of 4- to 9-fold; non-hepatocarcinogenic xenobiotics increased the amount of enzyme-antigen an average of 2- to 6-fold. In six instances, the two immunoassay methods yielded estimates of fold-increases that were in reasonable agreement; in five instances, however, the radial diffusion assay yielded appreciably lower estimates of fold-increases, viz. 3M, Afl, Eth, Fl and AB. These differences suggested that xenobiotic–hepatic tissue interactions influenced antigen–antibody complex formation and stability. Earlier Guengerich *et al.* [42], using immunodiffusion, observed antigen heterogeneity among microsomal epoxide hydrolase purified from livers of normal or xenobiotic-treated rats. This result also was interpreted as evidence for multiple forms of the enzyme [27, 42].

A statistical comparison of fold-increases in enzyme activity versus fold-increases in enzymes amount (deleting PB owing to insufficient activity data) showed that five of the xenobiotics influenced activity and amount to comparable extents; a similar conclusion was reached in two earlier reports wherein xenobiotics were injected [30, 43]. Among the five others, four xenobiotics increased amount to a significantly greater extent than activity, viz. Eth, Fl, NIT, and AB, while 4F was a better inducer of enzyme activity than enzyme amount.

Table 2. Influence of xenobiotics on the amount of microsomal epoxide hydrolase detectable by immunoassay

Xenobiotic*	Radial assay†	Ratio	ELISA‡	Ratio	Average ratio§
None	12.5 ± 0.8‖		8.8 ± 1.7‖		
AAF	99.0 ± 14.1	8	59.4 ± 9.2	7	8 ± 0.5
3M	85.9 ± 11.8	7	96.7 ± 13.7	11	9 ± 2
TA	59.1 ± 8.7	5	34.7 ± 10.4	4	4 ± 0.5
Afl	56.2 ± 7.5	5	73.3 ± 27.2	8	6 ± 1.5
4F	51.1 ± 9.5	4	44.7 ± 8.6	5	4 ± 0.5
Eth	24.4 ± 4.1	2	42.4 ± 12.0	5	4 ± 1.5
PB	50.5 ± 10.7	4	52.7 ± 5.2	6	5 ± 1
NIT	38.6 ± 5.0	3	37.9 ± 8.2	4	4 ± 0.5
Bar	33.2 ± 5.8	3	17.7 ± 4.3	2	2 ± 0.5
Fl	33.1 ± 6.7	3	47.5 ± 6.5	5	4 ± 1
AB	29.4 ± 1.8	2	45.2 ± 4.8	5	4 ± 1.5

* Xenobiotics were administered exactly as described in Table 1.
† Values listed are Δ diameter^2/μg protein
‡ Values listed are μg epoxide hydrolase/mg protein.
§ Values are $\bar{x}$ ± S.E for ratios determined using radial assay and ELISA.
‖ Values are $\bar{x}$ ± S.E. for determinations made on five male rats.

Influence of xenobiotics on detection of epoxide hydrolase antigen in PM supernatant fractions. Oesch *et al.* [44] reported that PM supernatant fractions from hyperplastic nodules were devoid of "material immunologically cross-reactive with microsomal epoxide hydrolase." Gill *et al.* [23], using ELISA, reported that PM supernatant fractions from normal rat liver contained low levels of microsomal epoxide hydrolase or "an immunologically related protein." Since ELISA yielded data on microsomal epoxide hydrolase levels that were comparable with radial diffusion data on microsomes from six of eleven xenobiotic-treated animal groups (Table 2), perhaps ELISA could be used validly to determine levels of epoxide hydrolase antigen in PM supernatant fractions from rats fed the eleven xenobiotics throughout 3 weeks; the data are recorded in Table 3. In normal rat liver, we observed protein immunologically related to epoxide hydrolase at a level of 7 ± 4 ng/mg protein in PM supernatant fractions, thus lending confirmation to the earlier report by Gill *et al.* [23]. The six hepatocarcinogens increased the amount detected 3- to 34-fold; among non-hepatocarcinogens, the increase ranged from 4- to 22-fold.

Table 3. Influence of xenobiotics on the detection by ELISA of epoxide hydrolase antigen in post-microsomal supernatant fractions of rat liver

Xenobiotic	Amount*	Ratio
None	7 ± 4†	
3M	239 ± 83	34
TA	183 ± 49	26
Eth	160 ± 55	23
AAF	146 ± 19	21
4F	64 ± 22	9
Afl	24 ± 13	3
NIT	157 ± 39	22
Fl	87 ± 22	12
PB	38 ± 17	5
AB	29 ± 8	4
Bar	26 ± 6	4

* Amount is expressed as ng epoxide hydrolase/mg of post-microsomal protein.
† Values are $\bar{x}$ ± S.E. for determinations made on five male rats.

Long-term centrifugation of PM supernatant fractions from all twelve feeding regimens allowed recovery of a pellet fraction that contained 92 ± 1.2% of the epoxide hydrolase antigen detectable by ELISA. Electrophoresis of pellet fractions through agarose containing antibody to purified epoxide hydrolase allowed observation of rocket-like precipitin lines, illustrating again immunochemical identity. Rocket height failed to show proportionality with antigen concentration, however, and quantitation was not possible. Pellet fractions had phospholipid–protein ratios that were 2.6-fold higher than similar ratios for microsomes from normal or AAF-fed rats. Pellet fractions prepared from PM supernatant fractions of AAF-fed rats catalyzed the hydration of styrene oxide at 11 ± 3.5 nmoles glycol/min/mg protein. These results suggest the presence of an enzyme in the cytosol fraction of rat liver homogenates that is immunologically and catalytically similar to microsomal epoxide hydrolase, and demonstrates that all the xenobiotics tested caused an increase in the detectable level of this enzyme.

DISCUSSION

Since microsomal epoxide hydrolase was shown to be associated with proteins that appeared to be markers for focal changes preceding development of malignant foci [7, 16, 18–20], it was important to test whether changes in activity and/or amount of this enzyme could be a "key change" [17] in carcinogenesis. As shown by average ratios in Table 1, five of six hepatocarcinogens appreciably induced microsomal epoxide hydrolase activity, while ethionine caused low induction; a similar lack of induction was noted previously [45, 46]. Furthermore, three of five non-hepatocarcinogenic xenobiotics induced

enzyme activity. This activity profile supports a conclusion that induction of microsomal epoxide hydrolase activity is not a "key change" leading to malignant transformation. On the other hand, the 2-fold disparity between activities with styrene oxide as substrate compared to activities with the two arene oxides as substrate, e.g. activities for rats fed AAF, 4F, TA, Afl, and NIT (Table 1), suggest that valid estimates of epoxide hydrolase activity after xenobiotic administration may not be possible with the technology employed in this study. Multiple forms of microsomal epoxide hydrolase may be differentially induced by xenobiotics [27, 42], but, in this study and perhaps in earlier studies, e.g. [45, 46], the different forms were not separately estimated.

As shown in Table 2, all of the xenobiotics significantly increased the amount of epoxide hydrolase antigen detected by either immunodiffusion assay or ELISA. Since both hepatocarcinogens and non-hepatocarcinogens were effective inducers of antigen, this biochemical change also would not appear to be a "key change". With six of the xenobiotics, estimates of antigen level by the two methods agreed reasonably well; with the other five, however, estimates with the diffusion method were appreciably lower than estimates by ELISA, viz. 3M, Afl, Eth, Fl and AB. Here, also, it would appear that the technology employed may have been insufficient to the task of accurately determining the level of microsomal epoxide hydrolase antigen following xenobiotic administration.

Thomas *et al.* [30, 47] and Pickett *et al.* [43] observed parallel increases in activity and amount of microsomal epoxide hydrolase following injection of several xenobiotics including AAF and PB. In our hands, five xenobiotics, which included AAF, caused fold-increases in activity and amount that were essentially similar, but among five others, four were better inducers of amount, while one was a better inducer of activity. In earlier studies [48, 49], 3-methylcholanthrene (3MC) induced epoxide hydrolase activity against some substrates, but not against others. Recently, Kawabata *et al.* [50] detected different amounts of epoxide hydrolase antigen in centrilobular, midzonal and periportal regions of hepatocytes following induction with PB, 3MC or *trans*-stilbene oxide. It seems likely that these differences relate to the multiple forms of microsomal epoxide hydrolase demonstrated by Guengerich *et al.* [27, 42], but suggest additionally that induction of epoxide hydrolase activity and/or amount need not occur coordinately. Nevertheless, the foregoing observations suggest the existence of several parameters that are uncontrolled and/or poorly understood which markedly influence kinetics of substrate–enzyme complex formation in livers of xenobiotic-treated rats. A similar statement seems justified concerning parameters regulating the availability of epoxide hydrolase immunodeterminants and the formation of stable antigen–antibody complexes in various extracts from livers of xenobiotic-treated rats. Until these parameters are understood and controlled, definitive assignment of the role of epoxide hydrolase in carcinogenesis would seem premature.

Data in Table 3 confirm reports by Gill *et al.* [23] concerning detection of microsomal epoxide hydrolase antigen in PM supernatant fractions of rat liver, and extend this report by showing that eleven xenobiotics significantly induced the level of this antigen in PM supernatant fractions. Gill *et al.* [51] recently determined the level of microsomal epoxide hydrolase activity in PM supernatant fractions from normal and neoplastic human liver and normal Rhesus monkey liver. In this report we measured the activity of microsomal epoxide hydrolase in pellet fractions from PM supernatant fractions of AAF-fed rats. Thus, microsomal epoxide hydrolase as a cytosolic component has been verified by several laboratories, and it appears to have catalytic competence.

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